Molecular Cloning and Characterization of a \( \beta \)-L-Arabinobiosidase in *Bifidobacterium longum* That Belongs to a Novel Glycoside Hydrolase Family*\(^*\)

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Extensin is a glycoprotein that is rich in hydroxyprolines linked to \( \beta \)-l-arabinofuranosides. In this study, we cloned a *hypBA2* gene that encodes a novel \( \beta \)-l-arabinobiosidase from *Bifidobacterium longum* JCM 1217. This enzyme does not have any sequence similarity with other glycoside hydrolase families but has 38–98% identity to hypothetical proteins in *Bifidobacterium* and *Xanthomonas* strains. The recombinant enzyme liberated \( \beta \)-l-arabinofuranose (Araf)-\( \beta \)-l-arabinofuranose disaccharide from carrot extensin, potato lectin, and Araf-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose disaccharide (Ara3-Hyp but not Araf-\( \alpha \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose) (Ara3-Hyp) and Araf-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose (Ara3-Hyp), which indicates that it was specific for unmodified Ara3-Hyp substrate. The enzyme also transglycosylated 1-alkanols with retention of the anomeric configuration. This is the first report of an enzyme that hydrolyzes Hyp-linked \( \beta \)-l-arabinofuranosides, which defines a new family of glycoside hydrolases, glycoside hydrolase family 121.

Extensin, a hydroxyproline-rich glycoprotein (HRGP),\(^2\) is a structural protein that is found in plant cell walls (1, 2). Potato lectin is also a HRGP that contains an extensin-like domain and two chitin-binding domains (3). These proteins contain repetitive Ser-Hyp motifs with hydroxyproline (Hyp) residues that are O-glycosylated with 1-4 arabinofuranosyl (Araf) residues with \( \beta \)-l-arabinofuranosyl linkages. For example, the structures of Ara3-Hyp and Ara4-Hyp, which are the major constituents of Hyp-linked arabinofuranosides, are Araf-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose (Ara3-Hyp) and Araf-\( \alpha \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose-\( \beta \)-l-arabinofuranose (Ara3-Hyp), respectively (4–6).

Previously, we cloned an *engBF* gene that encodes a novel endo-\( \alpha \)-N-acetylglucosaminidase from *Bifidobacterium longum* JCM 1217 on the basis of the sequence of BL0464, a hypothetical protein from *B. longum* NCC2705 (7), which contains C-terminal FIVAR domains that are involved in association with the bacterial cell wall (Pfam 07554). Similar to BL0464, BL0421, another hypothetical protein in *B. longum*, has three FIVAR domains (KEGG). BL0420, the gene that flanks BL0421, encodes a protein that also contains C-terminal FIVAR domains and belongs to glycoside hydrolase (GH) family 43, which includes xylosidases and arabino-1,2-arabinobiosidases. Schell et al. (8) predicted that BL0420 and BL0421 may be involved in binding or degrading xylan or hemicellulose. We hypothesized that these proteins are localized on the cell surface and coordinately degrade arabinan or xylan. Because BL0421 is not homologous to any known glycoside hydrolases, we predicted that the hypothetical protein is a novel enzyme.

Glycosidases that recognize the sugar chains of glycoproteins are useful in elucidating the presence and function of glycans. For instance, endo-\( \beta \)-N-acetylglucosaminidase selectively cleaves N-linked glycans from glycoproteins. In this study, we cloned the gene of a BL0421 homolog from *B. longum* JCM 1217 and characterized the properties of the recombinant enzyme, which is a novel \( \beta \)-l-arabinobiosidase. This is the first report of an enzyme that catalyzes the degradation of Hyp-linked \( \beta \)-l-arabinofuranosides.

**EXPERIMENTAL PROCEDURES**

**Substrates**—p-Nitrophenyl (pNP) substrates were obtained from Sigma. Sugar beet arabinan, debranched arabinan, arabinoxylan, and rhamnogalacturonan were purchased from Megazyme and purified by ethanol precipitation. Potato lectin was purified from potato tubers as described previously (9), by using affinity chromatography with a Chitopearl Basic BL-01 (Fuji Spinning) chitin column. Extensin was prepared from carrot root as described previously (10). The extensin was used to prepare Hyp-linked \( \beta \)-l-arabinofuranosides as follows. First, a sample of extensin was hydrolyzed for 16 h at 105 °C with 0.22 M Ba(OH)\(_2\), and then the hydrolysate was neutralized with sulfuric acid and centrifuged at 15,000 \( \times \) g for 20 min. The supernatant was applied to a Dowex 50 W \( \times \) 4 resin (\( \mathrm{H}^+ \) form). After washing the resin with 10 mM acetic buffer (pH 2.5), the Hyp-linked \( \beta \)-l-arabinofuranosides were eluted with 1.5 M aqueous ammonia and then evaporated to dryness. Next, the dried residue was
dissolved in water and applied onto a BioGel P2 column with distilled water. Finally, the eluate fractions containing Araf-β1,2-Araf-β-Hyp (Ara2-Hyp), Ara3-Hyp, and Ara4-Hyp were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Hyp and AraA2-Hyp were dansylated as described by Gray (11). The artificial cis form of Hyp-linked O-glycans was isomerized from the natural trans form by using an alkaline hydrolysis procedure (12). The cis and trans conformers were separated by high performance liquid chromatography (HPLC) on a Cosmosil 5C18-AR-II (10 cm × 250 mm, Nacalai) column with a mobile phase of methanol and 10 mM sodium phosphate (pH 2.5) (60/40, v/v) and a constant flow rate (1.0 mL·min⁻¹) at 30 °C. The elution was monitored by a fluorescence detector (FP-202; JASCO) with excitation and emission wavelengths of 365 and 530 nm, respectively. Finally, the eluate was desalted using a Sep-Pak Plus tC18 cartridge.

Expression and Purification of Recombinant HypBA2—The genomic DNA of B. longum JCM 1217 was extracted by using a FastPure DNA kit (Takara) and then used to perform PCR amplification of the gene for the BL0421 homolog, HypBA2. The forward (5’-AGGAGATATACCATGGCCGATACGC-TGCTCGAGTGCGGCCGCCG-3’) and reverse (5’-TGCTCGAGTGCGGCCGCCG-GAAGATGAACC-3’) primers were designed from nucleotides 96–112 and 4345–4357, respectively, of BL0421 from B. longum NCC2705. The underlined nucleotides represent the Ncol and NotI sites, respectively. The PCR amplification of hypBA2-CA486, which encodes amino acids 33–1464, was designed to eliminate the N-terminal signal peptide and C-terminal cell surface binding domain (residues 1465–1943). Then, the amplicon was digested with Ncol and NotI and inserted into the corresponding sites of the pET-23d vector (Novagen) to generate a C-terminal His₆-tagged recombinant protein. The resulting pET23d-hypBA2-CA486 plasmid was transformed into Escherichia coli BL21 (DE3) cells and then grown at 20 °C by using the Overnight Express Autoinduction System (Novagen). Subsequently, the cell cultures were centrifuged, and then the pellet was resuspended in BugBuster protein extraction reagent (Novagen). The His-tagged proteins were purified on a TALON metal affinity resin (Clontech), desalted by dialysis with a cellulose membrane (Wako), and concentrated by using a 10-kDa ultrafiltration membrane (Millipore). In addition, the PCR product of the full-length hypBA2 gene was sequenced on an ABI 3100 DNA sequencer using a Big-Dye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems).

Analysis of HypBA2 Hydrolysis and Transglycosylation Products—Arabinan and debranched arabinan (1.6 mg) were incubated at 30 °C for 2 h or overnight with 1.0 mg of purified HypBA2 enzyme in 100 µl of 50 mM sodium acetate buffer (pH 6.0) with or without alcohol. The reaction was stopped by boiling for 3 min and then precipitated with 80% ethanol. The supernatant was dried in a centrifugal concentrator and dissolved in 10 µl of water and then analyzed by thin layer chromatography (TLC) on a Silica Gel 60 aluminum plate (Merck) using a 2:1:1 solvent mixture (v/v/v) of ethyl acetate/acetone/acid/water. The sugars were visualized by spraying an orcinol-sulfate reagent onto the plate (13).

For structural analysis of the transglycosylation product, 8 g of sugar beet arabinan was mixed with 100 µg of HypBA2 enzyme in 500 ml of 50 mM sodium acetate buffer (pH 6.0) with 30% methanol and then incubated at 30 °C overnight. Subsequently, the completed reaction was evaporated, redissolved in 200 ml of water, and precipitated with 80% ethanol. The supernatant was collected and evaporated to dryness. Afterward, the dried sample was dissolved in 1 ml of water and separated by HPLC on a Cosmosil Sugar-D (4.6 × 250 mm, Nacalai) column at 30 °C with a mobile phase of acetonitrile and water (75/25, v/v) and a constant flow rate (1.0 mL·min⁻¹). The elution was monitored by a refractive index detector (RI-8022; TOOSOH). Finally, the fraction that contained the transglycosylation product (~1 mg) was collected and used for NMR analysis.

Assay of β-1-Arabinobiosidase Activity—The hydrolytic activity of the HypBA2 enzyme was assayed by using dansylated cis-Ara₃-Hyp (Ara₃-Hyp-DNS) as a substrate. The 40-µl reaction mixture contained 100 mM sodium acetate buffer (pH 5.5), 35 µM substrate, and 0.44 milliunits·mol⁻¹ of the HypBA2 enzyme. One unit of enzyme activity was defined as the amount of enzyme that is needed to produce 1 µmol of cis-Ara₃-Hyp-DNS·min⁻¹. The amount of cis-Ara₃-Hyp-DNS was quantified by measuring the arabinose content by trifluoroacetic acid hydrolysis and high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis as described below. After incubating the reaction at 30 °C, the reaction was stopped by adding 10 µl of 10% trichloroacetic acid and then analyzed by HPLC. The sample was applied to a Cosmosil 5C18-AR-II (2.5 × 250 mm, Nacalai) column at 30 °C with a mobile phase of methanol and 20 mM sodium phosphate (pH 2.5) (60:40, v/v) and a constant flow rate (1.0 mL·min⁻¹). The elution was detected by fluorescence.

Transglycosylation of Ara₃-Hyp—The transglycosylation reactions were performed by incubating 10 nmol of Ara₃-Hyp with 3.5 milliunits·µl⁻¹ of the HypBA2 enzyme at 30 °C overnight in 100 µl of 50 mM sodium acetate buffer (pH 5.5) with 20% methanol, ethanol, or n-propyl alcohol as an acceptor. Subsequently, the reaction products were analyzed by TLC with a 2:1:1 solvent mixture of ethyl acetate/acetone/water (v/v/v). The sugars were visualized by spraying an orcinol-sulfate reagent onto the plate (13).

Assays for pH, Temperature, and Ca²⁺ Dependence—The pH dependence of enzyme activity was determined between pH 2.0 and 9.0 by using the following buffers: 50 mM sodium acetate (pH 3.5–6.0), 50 mM MES (pH 5.5–7.0), 50 mM sodium phosphate (pH 6.5–8.0), and 50 mM Tris-HCl (pH 7.5–9.0). The effect of temperature on enzyme activity was examined by using 50 mM sodium acetate buffer (pH 5.5) at 10–60 °C. The effect of Ca²⁺ on the enzyme activity was determined with 1 mM CaCl₂ or EDTA.

Sugar Composition Analysis—The enzyme reaction product was incubated with 2 mM trifluoroacetic acid for 1 h at 120 °C. Then the hydrolyzed sugar chains were analyzed by HPAECPAD with a CarboPac PA10 column (Dionex) at a flow rate of 0.7 ml·min⁻¹, as described previously (14).
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Oligosaccharide Analysis by HPAEC-PAD—The sugar chains were analyzed using HPAEC-PAD with a CarboPac PA1 column. The column was eluted at a flow rate of 1.0 ml \cdot min^{-1} by 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); 30–35 min, 100% eluent B.

MS and NMR Analysis—electrospray ionization-TOF MS analysis was performed on a Mariner Biospectrometry Work station (Applied Biosystems). MALDI-TOF MS was performed on a Voyager-DE mass spectrometer (Applied Biosystems) in the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. \(^{1}H\) and \(^{13}C\) NMR spectra were measured with a JMM-ECA600KS spectrometer (JEOL). Two-dimensional correlation spectroscopy, heteronuclear multiple bond correlation, and heteronuclear multiple quantum coherence spectra also were used to assign chemical shifts and anomeric configurations of enzyme products.

Deletion Mutagenesis—The KOD-Plus Mutagenesis kit (Toyobo) was used to create nine deletion mutants of HypBA2 by using the primers shown in supplemental Table S1. The deletion mutants were as follows: C\(^{1026}\) (aa 33–1316), C\(^{789}\) (aa 33–1154), C\(^{932}\) (aa 33–1051), C\(^{1012}\) (aa 33–918), C\(^{1014}\) (aa 33–904), C\(^{1064}\) (aa 33–877), C\(^{1080}\) (aa 33–861), N\(^{118}\) (aa 119–1457), and N\(^{1271}\) (aa 272–1457). These mutant enzymes were expressed and purified by using the same procedure as that for the HypBA2-C\(^{486}\) enzyme.

Assays of Bacterial Enzyme Activities—The Bifidobacterium strains used in this study were B. longum JCM 1217 and B. longum JCM 7054, B. adolescentis JCM 1275, B. bifidum JCM 1254, B. breve JCM 1192, B. longum subsp. infantis JCM 1222, and B. pseudolongum JCM 1205. The bacteria were grown in Gifu anaerobic medium broth (Nissui) for 4 days at 37 °C under anaerobic conditions. The cell cultures were centrifuged at 15,000 \cdot g for 20 min, and then the pellets were washed with 50 mM sodium acetate buffer (pH 6.0). Subsequently, they were resuspended in 100 mM sodium acetate buffer (pH 6.0) and incubated with 0.25 mM Ara\(_{2}\)-Hyp-DNS for 8 h at 37 °C. Finally, the reactions were analyzed by TLC. The spots on the plates were developed with a 3:1:1 mixture (v/v/v) of \(n\)-butyl alcohol/acetic acid/water and then visualized with ultraviolet (UV) light.

RESULTS

Molecular Cloning, Expression, and Purification of the HypBA2 Protein—HypBA2 consisted of 1,943 amino acid residues exhibiting 97.6% identity with that of BL0421. The recombinant HypBA2-C\(^{486}\) protein was expressed at 20 °C as a soluble protein. SDS-PAGE showed that the purified recombinant HypBA2 protein migrated as a single band with an apparent molecular mass of 157 kDa (supplemental Fig. S1), which was in agreement with its calculated molecular mass of 156,797 Da.

Identification of the Liberated Carbohydrate from Arabinian—To identify the substrate for HypBA2, we screened its hydrolysis of various synthetic pNP substrates and natural hemicelluloses. The enzyme did not have any activity with pNP-\(\alpha\)-\(\alpha\)-L-arabinofuranoside, pNP-\(\alpha\)-\(\alpha\)-L-arabinopyranoside, pNP-\(\beta\)-\(\beta\)-L-arabinobioside, arabinoxylane, or rhamnogalacturonan (data not shown). However, it released a carbohydrate from arabinian and debranched arabinian (supplemental Fig. S2A), which was composed of arabinose (supplemental Fig. S2B). Electrospray ionization MS analysis revealed two molecular ion peaks at \(m/z\) 305.12 and 321.09, which corresponded to the sodium and potassium adducts of arabinose (calculated \(m/z\) 305.08 and 321.06 for \(C_{10}H_{18}O_{9}Na\) [M + Na]+ and \(C_{10}H_{16}O_{9}K\) [M + K]+, respectively). Furthermore, transglycosylation products were detected in the presence of 20% methanol, ethanol, and \(n\)-propyl alcohol (supplemental Fig. S2C). HPLC analysis of the transglycosylation product in the presence of methanol is shown in supplemental Fig. S2D. Electrospray ionization MS analysis revealed a molecular ion peak at \(m/z\) 319.11, which is consistent with the sodium adduct of a transglycosylation product, namely, Ara\(_{2}\)-OMe (calculated \(m/z\) 319.10 for \(C_{11}H_{24}O_{6}Na\)). The structure of this product was determined by \(^{1}H\) and \(^{13}C\) NMR (Fig. 1 and supplemental Table S2). The observed multiplicity of the anomeric proton (doublet at 4.86 ppm) and its coupling constant (\(J_{1,2} = 4.4\) Hz) were consistent with the product being a methyl \(\beta\)-\(\beta\)-L-arabinofuranoside (15). In addition, two-dimen-
sional correlation spectroscopy, heteronuclear multiple bond correlation, and heteronuclear multiple quantum coherence spectra of the transglycosylation product specifically identified the product as Ara-β1,2-Arafβ-OMe.

Although a terminal β-Araf residue was detected in olive arabinan (16), the Ara-β1,2-Arafβ residue has never been found in arabinan. In this study, only small amounts of the liberated saccharides (about 0.02%) were produced from commercially available sugar beet arabinan. Because most of the arabinofuranosyl residues in arabinan are in the α-anomeric configuration, we assumed that the Ara-β1,2-Arafβ residue was a contaminant from plant tissues.

**Substrate Specificity of HypBA2**—A literature search for oligosaccharides that contain the Ara-β1,2-Arafβ moiety suggested that Hyp-linked β-1-arabinofuranosides from potato lectin and carrot extensin may be substrates for HypBA2. Therefore, we tested whether Ara2-Hyp, Ara3-Hyp, and Ara4-Hyp were substrates. HypBA2 liberated Ara-β1,2-Arafβ-arabinobiose from carrot extensin in the presence of 20% methanol and ethanol, enough for NMR analysis (17). HypBA2 transglycosylated methanol, and produce methyl glycosides that are stable glycoside hydrolases transglycosylate 1-alkanols, such as (data not shown) and potato lectin (Fig. 2). By addition, HypBA2 liberated arabinobiose from carrot extensin (data not shown) and potato lectin (Fig. 2, lane a, 3, and Cb); however, Ara2-Hyp and Ara3-Hyp were not hydrolyzed (Fig. 2A). In addition, HypBA2 liberated arabinobiose from carrot extensin (data not shown) and potato lectin (Fig. 2Cc), and SDS-PAGE showed the mobility shift of the protein band of potato lectin (Fig. 2B). However, the liberated arabinose was not detected (Fig. 2C). These results showed that HypBA2 is an exo-acting glycoside hydrolase that is specific for unmodified Ara-Hyp substrate and liberates β-1-arabinobiose (Fig. 3). However, because HypBA2 could not hydrolyze the Ara-β1,2-Arafβ-arabinobiose transglycosylation product (data not shown), HypBA2 might require the Ara-β1,2-Arafβ-arabinobiose structure for enzyme activity. Consequently, we classified the enzyme as a β-1-arabinobiosidase.

**Transglycosylation Activity of HypBA2**—Many retaining glycoside hydrolases transglycosylate 1-alkanols, such as methanol, and produce methyl glycosides that are stable enough for NMR analysis (17). HypBA2 transglycosylated with Ara-Hyp in the presence of 20% methanol and ethanol, but 20% n-propyl alcohol inhibited enzymatic activity (Fig. 4). The coupling constant (J1,2 = 4.4 Hz) in the 1H NMR spectrum of the transglycosylation product from the reaction in 30% methanol was consistent with Ara-β1,2-Arafβ-arabinobiose and a mechanism that retains the anomeric configuration.

**Effects of cis-trans Substrate Conformations, pH, Temperature, and Ca2+ on HypBA2 Activity**—HypBA2 liberated Ara-Hyp-DNS from both cis- and trans-Ara-Hyp-DNS (Fig. 5). The retention time (Rt) of trans-Ara-Hyp-DNS (Rt = 4.4 min) was longer than that of cis-Ara-Hyp-DNS (Rt = 4.0 min) (Fig. 5A). Similarly, the retention time of cis-Ara-Hyp-DNS (Rt = 5.8 min) was longer than that of cis-Ara-Hyp-DNS (Rt = 4.7 min) (Fig. 5B). Because the difference in retention times was larger for the cis form than that of the trans form, we used the cis form of Ara-Hyp-DNS to characterize the enzyme.

The optimal temperature and pH for HypBA2 activity were 30 °C and 5.5–6.0, respectively (supplemental Fig. S3). The Km and Kcat values were calculated as 10.7 μM and 2.7 s−1, respectively. Furthermore, 1 mM Ca2+ increased the enzymatic activity by 43.8%, but 1 mM EDTA decreased the activity to 1.4% (Table 1).

**Sequence Analysis of HypBA2**—HypBA2 consisted of 1,943 amino acids that included a putative signal peptide, 3 F5/8 type C, 2 Big4, and 3 FIVAR domains (Fig. 6A). The F5/8 type C domain (Pfam 00754) is referred to as carbohydrate-binding module (CBM) family 32 that is generally involved in galactose binding. The Big4 domain is a bacterial Ig-like domain found in bacterial surface proteins (Pfam 00732). The amino acid sequence of HypBA2 was not similar to any known GH...
families. However, it was 38–98% identical to other hypothet-
ical proteins from Bifidobacterium, Xanthomonas, and actino-
mycetes (Fig. 6B and supplemental Fig. S4). Interestingly, al-
most all hypBA2 homologs flank GH43 homologous genes
(Fig. 6B).

Activity of HypBA2 Deletion Mutants—Three deletion mu-
tants, HypBA2-CΔ629, -Δ789, and -CΔ893, were functional
enzymes; however, HypBA2-CΔ1026 and -CΔ1049 showed
only 0.1% and 8.5% of the activity of HypBA2-CΔ486, respec-
tively (Table 1). The enzyme activities of the other deletion
mutants, HypBA2-CΔ1064, -CΔ1080, -Δ118, and -Δ271, could not be measured because they were expressed in inclusion
bodies in E. coli. Finally, similar to the wild-type enzyme, Ca2+
also increased the activities of the deletion mu-
tants (Table 1).

Detection of β-L-Arabinobiosidase Activity in B. longum—
β-L-Arabinobiosidase activity was found in B. longum JCM1217
and B. longum JCM 7054 but not in B. adolescentis JCM1275,
B. breve JCM 1192, B. bifidum JCM 1254, B. pseudolon-
gum JCM 1205, or B. longum subsp. infantis JCM1222 (sup-
plemental Fig. S5). In addition, the arabinobiose product from

![FIGURE 3. Schematic drawing of the hydrolysis of Ara3-Hyp by HypBA2. The thin arrow indicates the proposed cleavage site for HypBA2.](image-url)

![FIGURE 4. Transglycosylation activity of HypBA2 with Ara3-Hyp as the substrate. HypBA2 was incubated with Ara3-Hyp either in the absence (lane 3) or presence of 20% methanol (lane 4), ethanol (lane 5), or n-propyl alcohol (lane 6) at 30 °C for 12 h. Lane 1, arabinose; lane 2, Ara3-Hyp. A2, Ara2; Me, methyl; Et, ethyl; Pr, propyl.](image-url)

![FIGURE 5. HPLC chromatograms of HypBA2 reactions with dansylated substrates. The trans (A) and cis (B) isomers of Ara3-Hyp-DNS were incubated either without (a) or with (b) HypBA2 at 30 °C for 30 min. c, Hyp-DNS isomers. HtD, trans-Hyp-DNS; HcD, cis-Hyp-DNS; A3HtD, trans-Ara3-Hyp-DNS; A1HtD, trans-Ara-Hyp-DNS; A3HcD, cis-Ara3-Hyp-DNS; A1HcD, cis-Ara-Hyp-DNS.](image-url)

**TABLE 1**
Effects of calcium ion and EDTA on the activity of deletion mutants

| Deletion mutant | Control | 1 mM Ca2+ | 1 mM EDTA |
|-----------------|---------|-----------|-----------|
| %               | %       | %         |           |
| CΔ486 (33–1457) | 100     | 143.8     | 1.4       |
| CΔ629 (33–1316) | 84.0    | 131.8     | 2.9       |
| CΔ789 (33–1154) | 99.7    | 162.6     | 1.8       |
| CΔ893 (33–1051) | 47.1    | 132.2     | 0.7       |
| CΔ1026 (33–918) | 0.1     | 4.6       | 0         |
| CΔ1049 (33–894) | 8.5     | 15.8      | 0.3       |
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Ara$_3$-Hyp was detected on HPAEC-PAD in *B. longum* JCM 1217 (data not shown). Homologs of *B. longum* hypBA2 also were found in the genomic sequences of *B. longum* subsp. *infantis* ATCC 55813, *B. pseudocatenulatum* DSM 20438, *B. catenulatum* DSM 16992, and *B. dentium* ATCC 27678, but not in *B. adolescentis* ATCC 15703 or *B. animalis* subsp. *lactis* DSM 10140 (Fig. 6). These results suggested that /H9252-L-arabinobiosidase exists in *B. longum* and some other bifidobacterial species.

**DISCUSSION**

Glycoside hydrolases involved in the degradation of α-1-arabinofuranoso-oligosaccharides were found in six GH families (GH3, GH43, GH51, GH54, GH62, and GH93) in the carbohydrate-active enzyme (CAZy) data base (18, 19). In addition, glycoside hydrolases in the GH42 and GH27 families possess α- and β-1-arabinopyranosidase activities, respectively (20, 21). In this study, we cloned and characterized a hypothetical protein, HypBA2, and identified it as a novel β-1-arabinobiosidase. This is the first report of an enzyme that hydrolyzes α-L-arabinofuranosides. HypBA2 is a retaining enzyme that has strict substrate specificity for Ara$_3$-Hyp. Deletion mutants of HypBA2 revealed that the N-terminal conserved region (aa 33–907) and the adjacent F5/8 type C domain (aa 908–1044) are critical for enzymatic activity. The homologous genes in *Bifidobacterium*, *Xanthomonas*, and actinomycetes also conserved this N-terminal region; however, it did not contain any known sequence motifs. Therefore, we propose that the enzyme be assigned to a new GH family 121.

**FIGURE 6. Structural analysis of HypBA2.** A, schematic representation of HypBA2 from *B. longum* JCM 1217. The lines indicate the lengths of the deletion mutants of HypBA2. B, phylogenetic relationships among HypBA2 orthologs. The black boxes indicate the conserved regions among the hypothetical ORFs. The lengths of the sequences are shown on the right side of each schematic sequence. The organisms and GenBank accession numbers (in parentheses) are as follows: XCV, *Xanthomonas campestris* pv. *vesicatoria* (CAJ24408); XFA, *X. fuscans* subsp. *aurantifolii* (CAJ24408); XAC, *X. campestris* pv. *campestris* (AAO47877); AM, *Actinosynnema mirum* (ACU36767); AO, *Amycolatopsis orientalis* (CBA16438); XA, *X. axonopodis* pv. *citri* (AAM37385); XOO, *X. oryzae* pv. *oryzae* (BAE69722); XCC, *X. campeslris* pv. *campestris* (AAO47877); SR, *Streptosporangium roseum* (ACZ85426); MA, *Micromonospora aurantiaca* (EFA34846); BL1, *B. longum* NCC2705 (AAN24258); BL2, *B. longum* JCM 1217 (AB562506); BI, *B. longum* subsp. *infantis* (EEQ54489); BP, *B. pseudocatenulatum* (EET71984); BC, *B. catenulatum* (EEB20620); and BD, *B. dentium* (EDT45268). The phylogenetic tree was constructed with the ClustalW program using the neighbor-joining method. Signal peptide of HypBA2 was predicted by using the SignalP 3.0 server.
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Bifidobacteria are strictly anaerobic lactic acid-producing bacteria that are common in the lower intestinal microflora of human and animals. Because simple digestive sugars are preferentially absorbed in the upper intestinal tract, these bacteria survive in the lower intestinal tract by using glycoside hydrodases to cleave complex oligosaccharides (22). For example, B. longum NCC2705 encodes 14 members of the GH43 and GH51 families including α-L-arabinofuranosidases (23), and monomeric carbohydrates, such as L-arabinose, induce α-L-arabinofuranosidase activity in B. longum NIZO B667 (24). Several reports indicate the ability of B. longum to grow on α-arabinose and α-L-arabinofuranosides (25–28). Furthermore, enzymes involved in α-arabinose metabolism to D-xylulose-5-phosphate are conserved in B. longum NCC2705: α-L-arabinosidase isomerase (BL0272), L-ribulose-5-phosphate 4-epimerase (BL0273), and putative L-ribulokinase (BL0274). As a result, arabinobiosidases play important roles in the metabolism of B. longum by producing arabinose that can be used as carbon and energy sources.

Previously, we cloned and characterized a Gal-β1,3-GalNAc-releasing enzyme, endo-α-N-acetylgalactosaminidase, from B. longum (7). Kitaoka et al. (29, 30) proposed that Gal-β1,3-GalNAc from core-1 mucin-type O-glycans and Gal-β1,3-GlcNAc from human milk oligosaccharides were metabolized by B. longum, which suggested that B. longum absorbs disaccharides. Because the C-terminal structure of HypBA2 was analogous to that of endo-α-N-acetylgalactosaminidase, it is likely that HypBA2 localizes to the cell wall of B. longum. Indeed, HRGPs that contain β-L-arabinofuranosides are widely distributed in land plants, mosses, ferns, and green algae (6). Therefore, β-L-arabinobiosidase probably liberates arabinobiose from HRGPs on the cell surface of B. longum, so that arabinobiose can be assimilated.

The cell walls of dicot plants contain 33–75% of Ara4-Hyp in the total Hyp residues (6), and potato lectin contains 47% of Ara4-Hyp in the total Hyp residues (6), and potato lectin contains 47% of Ara4-Hyp in the total Hyp residues (31). Ashford et al. (5) reported that fungus α-L-arabinofuranosidase hydrolyzed Ara4-Hyp to Ara3-Hyp. Because HypBA2 cannot hydrolyze Ara3-Hyp, an α-L-arabinofuranosidase will be needed for effective degradation of β-1-arabinofuranosides. The hypBA2 gene forms a cluster with a GH43 homologous gene, which is conserved in many HypBA2 homologs (Fig. 6B). Therefore, HypBA2 and the neighboring GH43 protein may coordinate action on β-1-arabinofuranosides. Interestingly, BL0420 indicates highly homologous with other neighboring GH43 proteins (35% identity with that of Xanthomonas albines). The gene cluster may be beneficial for the degradation of plant exten- sin, because many of Xanthomonas strains cause plant diseases. Bifidobacteria and Xanthomonas are phylogenetically distant enough. In addition, the gene cluster cannot be found in other gut bacteria. Hehemann et al. (32) reported that the human gut bacteria acquired genes for porphyran degradation from a marine bacterium. Therefore, a part of Bifidobacteria probably received the gene cluster by horizontal gene transfer from Xanthomonas or actinomycetes.

Many lectins and CBMs require calcium to bind carbohydrates (33). CBM32 members (referred to as F5/8 type C) also have a binding calcium (34, 35). All of the HypBA2 deletion mutants showed calcium-dependent activity (Table 1). In particular, calcium recovered the activity of HypBA2-CΔ893, but only partly recovered the activities of CΔ1026 and CΔ1049. Therefore, the F5/8 domain (aa 908–1044) is likely to be the calcium-binding domain and related to the substrate recognition.

Previously, Hyp-linked arabinofuranosides in plants have been analyzed by alkaline hydrolysis and column chromatography. Our finding that β-L-arabinobiosidase has strict substrate specificity for Ara3-Hyp can be used to develop a simpler technique to detect the presence and study the role of β-L-arabinofuranosides in plant glycoproteins.

Acknowledgments—We thank Dr. S. Fushinobu (The University of Tokyo, Tokyo, Japan) and Dr. M. Kitaoka (National Food Research Institute, Tsukuba, Japan) for helpful discussions.

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