Characterization of a Novel β-L-Arabinofuranosidase in Bifidobacterium longum

FUNCTIONAL ELUCIDATION OF A DUF1680 FAMILY MEMBER

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Background: β-L-Arabinofuranosyl linkages are found in many plant biopolymers, but the degradation enzyme has never been found.

Results: A novel β-L-arabinofuranosidase was found in Bifidobacterium longum.

Conclusion: β-L-Arabinofuranosidase plays a key role in Bifidobacterium longum for β-L-arabinooligosaccharides usage.

Significance: The members of DUF1680 family might be used for the degradation of plant biopolymers.

Pfam DUF1680 (PF07944) is an uncharacterized protein family conserved in many species of bacteria, actinomycetes, fungi, and plants. In a previous article, we cloned and characterized the hypBA1 gene as a β-L-arabinobiosidase in Bifidobacterium longum JCM 1217. In this study, we cloned a DUF1680 family member, the hypBA1 gene, which constitutes a gene cluster with hypBA2. HypBA1 is a novel β-L-arabinofuranosidase that liberates l-arabinose from the l-arabinofuranose (Ara1) β-L-arabinobioside (Ara2) disaccharide. HypBA1 also transglycosylates 1-alkanols with hypBA1-C486 was expressed and purified to homogeneity. The recombinant enzymeβ-L-arabinofuranosidase, the GH family 121.

Recently, we cloned a hypBA2 gene that encodes a novel β-L-arabinobiosidase from Bifidobacterium longum JCM 1217 on the basis of the sequence of hypBA1 from B. longum NCC2705, which belongs to the glycoside hydrolase (GH) family 121 (10). The enzyme is a novel β-L-arabinofuranosidase (Ara1-Ara2). Because the β-L-arabinofuranosidase is a single enzyme encoding a novel β-L-arabinofuranosidase, we investigated the gene cluster with Pfam DUF1680 (PF07944), which contains three glycoside hydrolases of unknown function.

PROCEDURES

Extensin, potato lectin, Hyp-linked β-L-araminofuranosidase, β-Ara1, and Araβ-L,2-Araβ-OMe (Araγ-Araδ) were prepared as described previously (10). Dansylated Hyp-linked β-L-arabinooligosaccharides were prepared as described by Gray (11). p-Nitrophenyl (pNP) substrates were obtained from Sigma. p-Arabinoside was obtained from Wako Chemicals. The chemical structures of substrates are shown in supplemental Fig. S1. HypBA2-CA486 was expressed and purified as described previously (10).

Expression and Purification of Recombinant HypBA1—The genomic DNA of B. longum JCM 1217 was extracted using a FastPure DNA kit (Takara) and then used for PCR amplification of the gene for the BL0422 ortholog, hypBA1. The forward (5′-AAGGAGATATACATATGAGCTACATCACTTCCC-3′) and reverse (5′-TGCTCGAGTGCGGCGCTCGACGCTGGAAGACA-3′) primers were designed from nucleotides 4–22 and 1959–1974, respectively, of BL0422 from B. longum NCC2705 to generate a C-terminal His6-tagged recombinant protein. The PCR amplification product of hypBA1 was cloned into the pET-23b vector (Novagen) with the In-Fusion Advantage PCR Cloning Kit (Clontech). The full-length hypBA1 gene was sequenced on an ABI 3100 DNA Sequencer.

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2 The abbreviations used are: Hyp, hydroxyproline; HRGP, hydroxyproline-rich glycoprotein; Ara1, ara-l-arabinofuranose; DNS, dansyl, 5-dimethylaminonaphthalene-1-sulfon; DUF, domain of unknown function; GH, glycoside hydrolase; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; RI, refractive index; GAM, Gifu anaerobic medium; pNP, p-nitrophenyl; PYF, peptone-yeast extract-Fildes.
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sequencer with a Big-Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems). The resulting pET23b-hypBA1 plasmid was transformed into Escherichia coli BL21 (ADE3) cells, which were then grown at 20 °C by using the Overnight Express Autoinduction System (Novagen). Subsequently, the cell cultures were centrifuged, and the resultant pellet was resuspended in BugBuster protein extraction reagent (Novagen). The Histagged proteins were purified on TALON metal affinity resin (Clontech), desalted by dialysis with a cellulose membrane (Wako), and concentrated using a 10-kDa ultrafiltration membrane (Millipore).

**Enzyme Assays**—The hydrolytic activity of the HypBA1 enzyme was assayed using dansylated cis-Ara2-Hyp2-Ara2-Hyp (cis-Ara2-Hyp-DNS) as a substrate. The 40-μl reaction mixture contained 50 mM sodium acetate buffer (pH 4.5), 25 μM substrate, 5 mM Tris(2-carboxyethyl)phosphine (TCEP), and 0.17 milliunits ml⁻¹ of the HypBA1 enzyme. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of cis-Ara-Hyp-DNS per minute. After incubating the reaction mixture at 37 °C, the reaction was stopped by adding 10 μl of 5% trichloroacetic acid, and then analyzed by HPLC. The sample was applied to a Cosmosil 5C18-AR-II 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 fluorescence detector (FP-202, JASCO) with excitation and emission wavelengths of 365 and 530 nm, respectively. The sugars were visualized by spraying an orcinol-sulfate reagent onto the plate (12). For structural analysis, the transglycosylation product from the reaction in 20% methanol was purified 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 (RI) detector (RI-8022, TOSOH), and the absorbance of the fraction containing the transglycosylation product was determined. The 13C NMR spectra were measured by a spectrometer (JEOL) in DMSO-d6.

**Expression and Purification of HypBA1**—The expression of HypBA1 was induced in Escherichia coli BL21 (DE3) by 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C, and then analyzed by HPAEC-PAD as described above, using an l-arabinose standard. In the case of cis-Ara-Hyp-DNS, liberated cis-Hyp-DNS were analyzed according to the same procedure used for cis-Ara2-Hyp-DNS.

**Transglycosylation of Ara2-Hyp**—The transglycosylation reactions were performed using Ara2-Hyp as a donor and 1-alkanols as acceptors. Thirty nanomoles of Ara2-Hyp were incubated at 37 °C for 3 h with 340 milliunits ml⁻¹ of HypBA1 in 100 μl of 50 mM sodium acetate buffer (pH 4.5) with 5 mM TCEP and 20% methanol, ethanol, or 1-propanol as an acceptor. Subsequently, the reaction products were analyzed by TLC with a 2:1:1 solvent mixture of ethyl acetate/acetic acid/water (v/v/v) and a constant flow rate (1.0 ml min⁻¹). The ACSN solution was added to the TLC plate, and the fractions were monitored using a UV detector (365 nm) (12). For structural analysis, the transglycosylation product from the reaction in 20% methanol was purified 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 (RI) detector (RI-8022, TOSOH), and the absorbance of the fraction containing the transglycosylation product was determined. The 13C NMR spectra were measured by a spectrometer (JEOL) in DMSO-d6.

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**RESULTS**

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BL0422, and coincided with that of BL11_0211 from B. longum JCM 1217, for which the complete genome sequence is available (15). The recombinant HypBA1 protein was expressed at 20 °C as a soluble protein. SDS-PAGE showed that the purified recombinant HypBA1 protein migrated as a single band with an apparent molecular mass of 74 kDa (supplemental Fig. S2), which was in agreement with its calculated molecular mass of 74,329 Da. The final yield of the purified enzyme was 140 mg/liter of culture.

Substrate Specificity and General Properties of HypBA1

The enzymatic activity for dansylated cis-Ara2-Hyp-DNS was determined at 35 °C-40 °C and 4.5, respectively (supplemental Fig. S4). The specific activity of the purified enzyme was 2.1 units/mg protein. The kinetic parameters for HypBA1 activity on different substrates are shown in Table 1. The kinetic parameters for HypBA1 were used as the acceptors, the transglycosylation products were used as the donors of dansylated substrates.

Substrates were incubated either without (lane a) or with (lane b) HypBA1 at 37 °C for 16 h. Ara-Hyp (lane 2), Ara2-Hyp (lane 3), Ara3-Hyp (lane 4), Ara2-Hyp (lane 5), pNP-α-L-arabinofuranoside (lane 7), pNP-α-L-arabinopyranoside (lane 8), pNP-β-L-arabinopyranoside (lane 9), and Ara2-Me (lane 10) were used as substrates. Lanes 1 and 6, L-arabinose standard.

Transglycosylation Activity of HypBA1—When 1-alkanols were used as the acceptors, the transglycosylation products were detected on TLC (Fig. 4A). The purified transglycosylation product (methyl L-arabinofuranoside) was hydrolyzed to L-arabinose by the HypBA1 treatment (Fig. 4B), which indicates that the methanol was linked by the β-anomeric form. The structure of this product was determined by 1H and 13C NMR (supplemental Fig. S6 and Table S3). The 1H NMR spectrum showed the anomeric proton as a doublet at 4.74 ppm with a coupling constant 1,2 = 4.8 Hz. Furthermore, the 13C NMR spectra revealed that the transglycosylation product was found to be consistent with a methyl β-1-arabinofuranoside (Ara-Me) (16). These data indicated that HypBA1 is a retaining enzyme.

Sequence Analysis of HypBA1—HypBA1 consisted of 658 amino acids that included DUF1680 without other sequence motifs (supplemental Fig. S7). HypBA1 was 38–98% identical...
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Critical Amino Acid Residues of HypBA1—The candidate acidic amino acid residues were selected for site-directed mutagenesis studies based on multiple alignments and the HMM logo of the DUF1680 family in the Pfam data base (17). Alanine substitutions were introduced at the positions of Glu-322, Glu-338, and Glu-366, which are highly conserved among the HypBA1 homologues (indicated as asterisks in supplemental Fig. S8). The mutant enzymes were purified for the determination of specific activities. The E322A and E366A mutant enzymes were recovered in insoluble fractions with BugBuster. The E338A enzyme exhibited a significant decrease in activity compared with the E322A mutant showed 1.5% of the wild-type enzyme activity (Table 2). The E322A enzyme was soluble, and only a small percentage (less than 1%) of the enzyme was rescued by sodium azide. The E338A enzyme was not rescued by sodium azide but was rescued by sodium azide more than in the E322A enzyme. The azide-rescued E338A enzyme showed the glycosyl azide product in HPLC and TLC (data not shown). The E366A mutant exhibited 25% of the wild-type enzyme activity (Table 2).

TABLE 2
The specific activities of HypBA1 mutants

| Mutant enzymes | Specific activity* | Percentage of specific activity |
|----------------|-------------------|-------------------------------|
| Wild type      | 2100              | 100%                          |
| E322A          | 32                | 1.5%                          |
| E338A          | 340               | 16%                           |
| E366A          | 0.028             | 0.0013                        |

* Enzymatic activities were determined using the cis isomer of Ara2-Hyp-DNS.

In Vitro Fermentability of Bifidobacterium—The candidate enzyme was assayed in the presence of 1-alkanols. A, HypBA1 was incubated with Ala2-Hyp-DNS in the absence (lane 1) or in the presence of 20% methanol (lane 2), ethanol (lane 3), or 1-propanol (lane 4) at 37 °C for 3 h. Lane 5, l-arabinose; lane 6, Ala2-Hyp. B, purified methyl L-arabinofuranoside was incubated without (lane 1) or with (lane 2) HypBA1 at 37 °C for 16 h. Lane 3, l-arabinose standard. Me, methyl; Et, ethyl; Pr, propyl.

to other DUF1680 members from bifidobacteria (supplemental Figs. S7 and S8). Duplicated DUF1680 members were found in the sequences of almost all Bifidobacterium species. HypBA1 (BLLJ_0211) constitutes a gene cluster with HypBA2 (BLLJ_0212) and a GH43 family member (BLLJ_0213) (supplemental Fig. S7). The gene cluster was conserved in B. longum NCC2705, B. longum subsp. infantis 157F, and B. pseudocatenulatum DSM 20438. In addition, the gene cluster with the GH43 family member was conserved in B. catenulatum DSM 16992 and B. dentium ATCC 27678.

DISCUSSION

The DUF1680 family has 597 members distributed among 315 species of enteric bacteria (i.e. Bifidobacterium, Bacteroides, Salmonella, Clostridium, and Escherichia), plant-pathogenic Xanthomonas, actinomycetes, fungi, and plants, as shown
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in the Pfam database. The members of this family are hypothetical proteins of unknown function and have no sequence similarity with other glycoside hydrolase families. In this study, we cloned the gene encoding a member of the DUF1680 family and characterized its product as a novel β-L-arabinofuranosidase. Therefore, we propose that the enzyme be assigned to a new family of glycoside hydrolases, the GH family 127.

β-Ara2 was a suitable substrate for HypBA1 as well as Ara1, Hyp and Ara2, Hyp, which contain the Araβ1,2-Araα structure at the nonreducing terminal. In extensins, β-L-arabinooligosaccharides are in close existence on repetitive Ser-Hyp motifs and contribute to protease resistance. It is thought that Hyp-linked β-L-arabinooligosaccharides do not occur naturally in the normal environment. Furthermore, HypBA1 did not directly release L-arabinose from extensin or potato lectin (supplemental Fig. S5). In addition, we showed that β-Ara2 was used as a carbohydrate source for B. longum, with enzymatic activity detected in the cell lysate (supplemental Table S4 and Fig. S6). Interestingly, the enzymatic activity was not detected in cells grown in the presence of L-arabinose or glucose. The amino acid sequence of HypBA1 lacks both a secretory signal and a transmembrane domain. Collectively, these results indicate that HypBA1 is an intracellular enzyme that degrades HypBA2-released β-Ara2, as schematically summarized in Fig. 7 and supplemental Fig. S9.

Previously, we characterized an endo-α-N-acetylglactosaminidase (BLLJ_0168) from B. longum JCM 1217, which releases Gal-β1,3-GalNαc (GNB) disaccharide from core-1 mucin-type O-glycans (19). Kitaoka et al. (19, 20) proposed a metabolic pathway for core-1 mucin-type O-glycans and Gal-β1,3-GalNαc disaccharides based on the characterization of the genes encoded in the GNB/LNB operon (BLLJ_1620-BLLJ_1626) of B. longum. B. longum contains the GNB/LNB-binding cassette (ABC)-type transporter (21, 22). Furthermore, there are 2 transmembrane subunits (BLLJ_0211 and BLLJ_0212) that have also been annotated as encoding subunits of the GNB/LNB-binding protein, and BLIJ_0208 and BLIJ_0209 are predicted to be a putative ABC-type sugar transport system in the GNB/LNB pathway. As shown in supplemental Fig. S9, the hypBA1 gene is located downstream of the GNB/LNB-binding subunits. BLIJ_0209 and BLIJ_0210 are predicted to share 28% identity with BLJ_0207 and BLJ_0208 and 30% identity with the GNB/LNB transport subunits BLJ_1624 and BLJ_1625. Furthermore, the gene (BLLJ_0207) is predicted to be a putative regulatory regulator. Thus, we expect that this gene will regulate the gene cluster containing the β-L-arabinofuranosidase degradation enzymes (BLLJ_0211-BLLJ_0213) by internalizing β-Ara2.

The β-L-arabinooligosaccharides metabolic pathway in B. longum is predicted as shown in supplemental Fig. S9. First, a GH43 family member (BLLJ_0213) releases L-arabinose from extensin (Ara1-Hyp to Ara2-Hyp), and then HypBA2 (BLLJ_0212) releases β-Ara2 (Ara3-Hyp to Ara-Hyp) on the bifidobacterial cell surface. Next, the released L-arabinose and β-Ara2 are internalized into the bifidobacterial cell by uncharacterized transport system and predicted β-Ara2 transport system (BLLJ_0208-BLLJ_0210), respectively. Then, HypBA1 (BLLJ_0211) degrades β-Ara2 to L-arabinose. Furthermore, the L-arabinose metabolic enzymes for the conversion to D-xylulose-5-phosphate, which have been characterized in Corynebacterium glutamicum ATCC 31831 (24), exhibit 50–59% identity with those of B. longum JCM 1217; L-arabinose isomerase (BLLJ_0342), L-ribulokinase (BLLJ_0340), and L-ribulose 5-phosphate 4-epimerase (BLLJ_0341). As a result, HypBA1 plays a key role in B. longum for β-L-arabinooligosaccharides usage as a carbohydrate and energy source.
Recently, Fukuda et al. (15) reported that *B. longum* has an advanced ability for fructose uptake and acetate production, with the released acetate improving the intestinal defense mediated by epithelial cells. In addition to fructose, L-arabinose is a naturally found common carbohydrate and is found as a component of biopolymers such as hemicellulose and pectin. *B. longum* JCM 1217 encodes a number of candidates for the \( \beta \)-L-arabinofuranosidase gene, 11 members of the GH43 gene family, and 4 members of the GH51 gene family. Several reports indicate that *B. longum* has the ability to grow on L-arabinose and \( \beta \)-L-arabinooligosaccharides (14, 23, 25–27). We showed that *B. longum* also uses \( \beta \)-Ara\(_2\) as a carbohydrate source (supplemental Table S4). Several \( \alpha \) - and \( \beta \)-L-arabinooligosaccharides degradation enzymes in *B. longum* might be involved in L-arabinose acquisition from plant polymers in the large intestine.

HypBA1 was identified as a retaining glycoside hydrolase, as described above. Hydrolysis by retaining glycoside hydrolases proceeds through a double-displacement mechanism with 2 catalytic residues. The catalytic residues typically utilized are either aspartate or glutamate residues. In the chemical rescue study, E366A mutant was rescued by the addition of azide, which suggests that Glu-366 is a catalytic residue for HypBA1. However, no glycosyl azide product was formed in the reaction mixture. A water molecule activated by azide ion might be reactivated E366A mutant without glycosyl azide production, as shown in GH43 \( \beta \)-xylosidase and GH14 \( \beta \)-amylase (28, 29).
B. longum JCM 1217 encodes 4 members of the DUF1680 family (BLLJ_0211, BLLJ_1826, BLLJ_1848, and BLLJ_0089), whereas B. longum NCC2705 encodes 2 members (BL0422 and BL0174) (supplemental Fig. S7). BL0422 constitutes a conserved gene cluster with the GH121 β-l-arabinofuranosidase gene and the GH43-encoding gene as well as BLLJ_0211. BL0174 (98.8% identity with BLLJ_1826) is flanked by a gene cluster with 5 GH43 members and 1 α-galactosidase (BL0176-BL0190), whereas BLLJ_1826 is flanked by a small gene cluster without GH43 members (BLLJ_1824-BLLJ_1820). Interestingly, BLLJ_1848 constitutes a gene cluster with 5 duplicated GH43 members (BLLJ_1850-BLLJ_1854), in which the cluster is replaced by insertion sequences in B. longum NCC2705.

HRGPs that contain β-l-arabinooligosaccharides are widely distributed in land plants, mosses, ferns, and green algae (30). Furthermore, terminal β-l-arabinofuranosidases are found in many plant biopolymers (3–8) and in yessotoxin from the dinoflagellate algae Protoceratium reticulatum (31, 32). Because DUF1680 family members are conserved in many species of bacteria, actinomycetes, fungi, and plants, they are thought to play a role in the effective degradation of plant biopolymers as well as HRGPs.

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