APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2005, p. 5501–5510 Vol. 71, No. 9
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.9.5501–5510.2005

Bifidobacterium longum Endogalactanase Liberates Galactotriose from Type I Galactans

Sandra W. A. Hinz, Marieke I. Pastink, Lambertus A. M. van den Broek, Jean-Paul Vincken, and Alphons G. J. Voragen*

Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Received 19 January 2005/Accepted 9 April 2005

A putative endogalactanase gene classified into glycoside hydrolase family 53 was revealed from the genome sequence of *Bifidobacterium longum* strain NCC2705 (Schell et al., Proc. Natl. Acad. Sci. USA 99:14422–14427, 2002). Since only a few endo-acting enzymes from bifidobacteria have been described, we have cloned this gene and characterized the enzyme in detail. The deduced amino acid sequence suggested that this enzyme was located extracellularly and anchored to the cell membrane. *galA* was cloned without the transmembrane domain into the pBluescript SK(−) vector and expressed in *Escherichia coli*. The enzyme was purified from the cell extract by anion-exchange and size exclusion chromatography. The purified enzyme had a native molecular mass of 329 kDa, and the subunits had a molecular mass of 94 kDa, which indicated that the enzyme occurred as a tetramer. The optimal pH of endogalactanase activity was 5.0, and the optimal temperature was 37°C, using azurine-cross-linked galactan (AZCL-galactan) as a substrate. The K_m and V_{max} for AZCL-galactan were 1.62 mM and 99 U/mg, respectively. The enzyme was able to liberate galactotrisaccharides from (β1→4)galactans and (β1→4)galactooligosaccharides, probably by a processive mechanism, moving toward the reducing end of the galactan chain after an initial midchain cleavage. GalA's mode of action was found to be different from that of an endogalactanase from *Aspergillus aculeatus*. The enzyme seemed to be able to cleave (β1→3) linkages. Arabinosyl side chains in, for example, potato galactan hindered GalA.

Bifidobacteria play an important role in carbohydrate fermentation in the colon. They contain a large number of carbohydrate-modifying enzymes. Since the genome sequence of *Bifidobacterium longum* recently became available (36), more information about *Bifidobacterium* glycolytic enzymes has been uncovered. The genome reveals that approximately 5% of all annotated genes are involved in the modification of carbohydrates.

Several studies on in vitro fermentations of (β1→4)-linked (arabino)galactans with different bifidobacteria show that mainly *B. longum* strains were able to grow on these arabinogalactans (9, 11). This is consistent with the genome sequence of *B. longum* because it reveals the presence of many different putative enzymes potentially able to degrade arabinogalactans. Most of these enzymes are probably located intracellularly, and their sequence suggests that they can degrade the side chains of galactans (arabinofuranosidases and arabinosidases) or galactooligosaccharides (β-galactosidases) (36). Interestingly, the genome sequence also suggests that *B. longum* contains an endogalactanase (annotated as YvfO and further referred to as GalA in this study), which is predicted to be extracellular. This is rather exceptional, because only few endo-acting enzymes have been described in bifidobacteria so far (2, 24).

Most endogalactanases described to date are able to degrade the (β1→4)-linked galactosyl backbone of type I arabinogalactans (6, 20, 21, 28, 39). Neither the substrate specificity of GalA is clear nor if it truly acts with an endomechanism. It is possible that this enzyme is an essential link in galactan utilization, together with β-galactosidases, arabinofuranosidases, and transporters of galactooligosaccharides. To get more insight into galactan utilization by *B. longum*, we have cloned the *galA* gene, characterized this enzyme in detail, and compared its mode of action with an endogalactanase from *Aspergillus aculeatus*.

* Corresponding author. Mailing address: Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands. Phone: 31 317 483209. Fax: 31 317 484893. E-mail: Fons.Voragen@WUR.NL.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bifidobacterium longum* strain NCC490 was kindly provided by the Nestlé Research Centre (Lausanne, Switzerland). The strain was grown anaerobically using MRS medium, pH 6.0 (Becton Dickinson, Franklin Lakes, New Jersey) supplemented with 0.5 g/liter cysteine at 37°C. DNA cloning was performed using the *Escherichia coli* strain XL1-Blue MRF′ (Promega, Madison, Wisconsin). The *E. coli* strain was grown in Luria-Bertani (LB) broth or solidified LB medium (15 g agar/l) supplemented with 100 μg/ml ampicillin, when appropriate.

The *E. coli* cells containing the *galA* gene were grown in LB broth or solidified LB medium, supplemented with 100 μg/ml ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).

Chemicals, substrates, and enzymes. Chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. Potato arabinogalactan was obtained as described by van de Vis (40). Azurine-cross-linked galactan (AZCL-galactan) was purchased from Megazyme (Bray, Ireland). Endogalactanase from *Aspergillus aculeatus* was purified as described by van de Vis et al. (41). Arabinofuranosidase from *Aspergillus niger* was purchased from Megazyme. Restriction enzymes and other enzymes used for DNA manipulation were obtained from MBI Fermentas (St. Leon Rot, Germany) and were used according to the instructions of the manufacturer. The mixture of transgalactooligosaccharides (TOS) was kindly provided by Borculo Domo Ingredients (Zwolle, The Netherlands) and was fractionated as described by Van Laere et al. (43). [β-D-Gal*p*-(1→4)]$_m$-D-Gal*p* and [β-D-Gal*p*-(1→4)]$_n$-β-D-Gal*p*-(1→3)-D-Gal*p* oligosaccharides, with m = 1 to 3 and *n* = 1 or 2, were obtained as described by Hinz et al. (16).

Cloning of the endogalactanase gene. Genomic DNA of *B. longum* was isolated using a modified Marmur procedure as described by Johnson (18). A PCR was carried out on the genomic DNA with Easy A polymerase (Stratagene, La

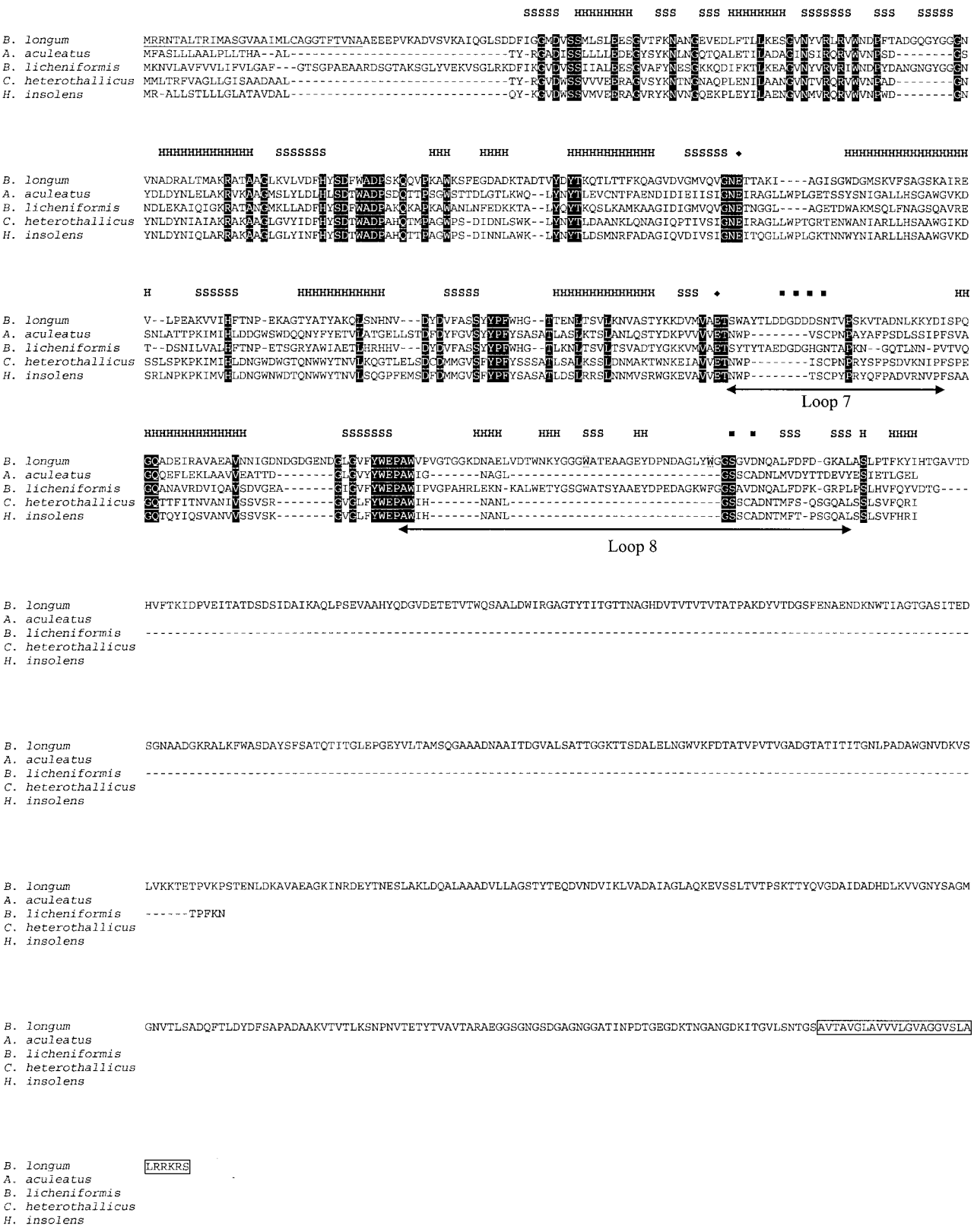

FIG. 1. ClustalW comparison of the amino acid sequence of endogalactanase from *Bifidobacterium longum* (GalA) and endogalactanases from *Aspergillus aculeatus* (accession no. ASNGAL1A), *Bacillus licheniformis* (AAO31370), *Corynascus heterothallicus* (AAN99814), and *Humicola insolens* (AAN99815). Amino acid identity is indicated by vertical black shading, and the catalytic residues E_{192} and E_{297} are indicated by diamonds. The secondary structure elements are indicated by "H" for helices and "S" for strands. The signal peptide of GalA is underlined, whereas its transmembrane domain is boxed. The residues involved in calcium binding are indicated by squares. The Trp residues representing subsites −2, −3, and −4 are shaded in grey.

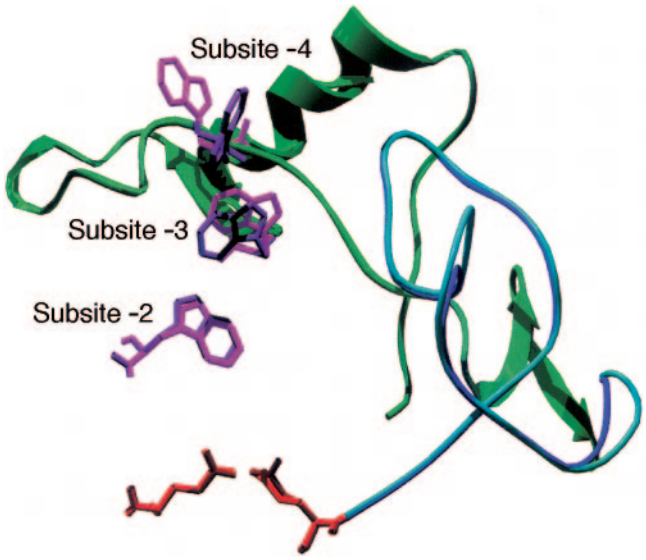

FIG. 2. Overlay of the catalytic residues, Trp residues involved in substrate binding, and loop 7/8 region of the 3D structures of GalA (lighter shade) and endogalactanase from *Bacillus licheniformis* (darker shade). The catalytic residues are indicated in red, the Trp residues (representing subsites −2, −3, and −4) in pink, loop 7 in blue, and loop 8 in green.

Jolla, California), using two primers for amplification of the *galA* gene without the transmembrane domain (XbaI restriction sites were introduced at the beginning and at the end of the gene for cloning purposes). Primers used were (with the XbaI site underlined) 257F (5′-CCCCCCCTCTAGACAAAGGAGAAAA AGCATGCG), and 257R (5′-CCCCCCCTCTAGATCAGCTACCGGTATTG CTCAG). The forthcoming DNA fragments were ligated into a *p*GEM T-easy vector (Promega) and transformed into *E. coli* XL1-Blue MRF′ cells. The cells were grown for 16 h at 37°C on solid S-Gal (3,4-cyclohexenoesculetin-β-D-galactopyranoside)/LB agar plates (Sigma), supplemented with 100 μg/ml ampicillin. Colonies containing a PCR fragment were identified as white colonies. Plasmid DNA was prepared by following the QIAGEN plasmid purification method (QIAGEN, Hilden, Germany). The *galA* genes were cut out from the plasmids with XbaI and purified and cloned into an XbaI-digested pBluescript vector (Stratagene). This vector was transformed into *E. coli* XL1-Blue MRF′ cells.

Isolation and characterization of endogalactanase. *E. coli* cells containing the *galA* gene were grown overnight at 37°C (1 liter) and harvested by centrifugation (15 min, 8,000 × *g*, 4°C). The supernatant was used for activity measurement, and the cells were suspended in 90 ml 50 mM sodium acetate buffer, pH 5, and disrupted by sonic treatment (10 min; amplitude, 30%; duty cycle, 0.3 s on and 0.7 s off; Digital Sonifier; Branson, Danbury, CT) on ice. Subsequently, the suspension was centrifuged (15 min, 8,000 × *g*, 4°C), the cell extract was collected, the pellet was suspended in 40 ml 50 mM sodium acetate buffer (pH 5), and a second sonic treatment was performed. This step was repeated twice. The cell extracts were pooled and applied onto a Q-Sepharose (Amersham, Little Chalfont, United Kingdom) anion-exchange column, using a BioPilot pump system (Amersham). Elution was done with a linear gradient of 0 to 0.5 M NaCl in 50 mM sodium acetate, pH 5, at a flow rate of 57 cm/h. Fractions with the highest endogalactanase activity were pooled and further purified on a Superdex 200 PG (Amersham) size exclusion column. Elution was performed with 0.15 M NaCl in 20 mM potassium phosphate buffer, pH 6.0, at a flow rate of 76 cm/h. Fractions with the highest endogalactanase activity were pooled and concentrated with anion-exchange chromatography under the same conditions as described above.

TABLE 1. Physicochemical properties of endogalactanase from *Bifidobacterium longum*

Parameter (units)	Endogalactanase value
K_m (mg/ml)[a]	1.62
V_{max} (U/mg)[a]	99
Deduced molecular mass (Da)	91,288
Molecular mass (kDa) (SDS-PAGE)	94
Native molecular mass (kDa)	329
Proposed molecular structure	Tetramer
Optimum pH	5.0
Optimum temperature (°C)	37

[a] AZCL-galactan as substrate.

Protein concentration was determined by the method of Bradford (5) using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the Pharmacia Phastsystem according to the instructions of the supplier (Amersham). Coomassie brilliant blue staining was used for the detection of proteins on PhastGel 10 to15% gradient gels (Amersham). The native molecular mass was estimated by size exclusion chromatography using the Akta Purifier equipped with a Superdex 200 PG column (Amersham), as described above. The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa).

Enzyme assays. Endogalactanase activity was measured by determining the hydrolysis of AZCL-galactan (according to instructions of the supplier) at 37°C after 15 min of incubation. The reaction mixture (250 μl) consisted of 1.85 μg of enzyme, 16 mM potassium phosphate buffer (pH 6.0), and 2.5 mg AZCL-galactan. The reaction was stopped by adding an equal volume of 0.5 M glycine-NaOH buffer (pH 9.0) containing 2 mM EDTA. After centrifugation (15 min, 10,000 × *g*), the absorbance of the supernatant was measured at 595 nm (A_{595}). The absorbance at 595 nm is a measure for the activity of the enzyme. In a parallel experiment, the A_{595} of a number of representative incubations with AZCL-galactan was related to the amount of reducing end groups formed. From this, a conversion factor was calculated, which enabled the conversion of the A_{595} readings to enzyme activity units. One unit of activity was defined as 1 μmol of reducing sugars liberated per min under the specified conditions. This assay was also used to measure the enzyme's optimum temperature and pH and for kinetic experiments. The optimum temperature was determined between 4 and 70°C. For determination of the optimum pH, McIlvain buffers (0.1 M citric acid, 0.2 M disodium phosphate) in the range of pH 2.6 to 7.6 were used. For kinetic experiments, the AZCL-galactan concentration was varied in the range of 0.08 to 16.0 mg/ml.

The mode of action of GalA was determined by high-performance anion-exchange chromatography (HPAEC) and high-performance size exclusion chromatography (HPSEC). The incubations were performed in triplicate with 5 mg/ml potato galactan in 5 mM sodium acetate buffer, pH 5.0, and 3.3 μU/ml enzyme at 37°C for different time intervals. The incubations were stopped by heating the incubation mixtures for 10 min at 100°C. After centrifugation (10

TABLE 2. Sugar composition of potato arabinogalactan and tetramer fraction obtained after partial degradation of galactan as described by Hinz et al. (16)

Fraction	Monosaccharide[a] composition (mol%)						Carbohydrate content[b] (%)	Gal/Ara ratio
	Fuc	Rha	Ara	Gal	Glc	GalA		
Potato galactan	1.2 ± 0.2	6.5 ± 0.3	13.9 ± 0.2	65.8 ± 0.3	1.2 ± 0.0	11.3 ± 0.3	76.0 ± 2.6	4.7
Tetramer			5.9 ± 0.1	94.1 ± 0.1			97.6 ± 3.6	16

[a] Fuc, fucose; Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; GalA, galacturonic acid.
[b] Expressed as wt/vol.

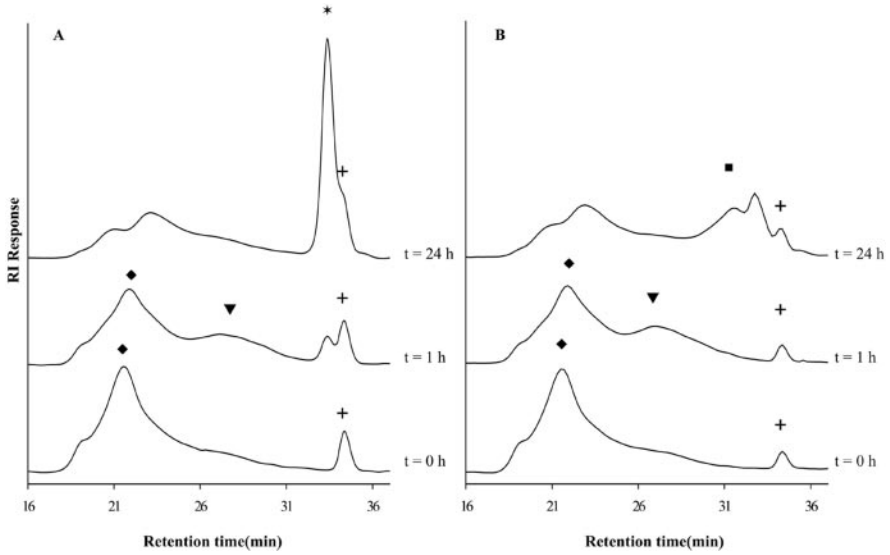

FIG. 3. HPSEC profiles of potato arabiogalactan degradation after 0, 1, and 24 h of incubation by GalA (A) and endogalactanase from *A. aculeatus* (B). Symbols: ◆, polymeric potato arabinogalactan; ▼, degradation products of potato arabinogalactan with an average molecular mass of 11 kDa; ✶, low-molecular-mass oligosaccharides (approximately 370 Da); ✚, acetate buffer; ■, higher-molecular-mass oligosaccharides (approximately 500 to 2,400 Da). RI, refractive index.

min, 10,000 × *g*), the supernatant was analyzed by HPAEC, HPSEC, and the Nelson-Somogyi method.

The substrate specificity was also measured by HPAEC in triplicate. The incubations were performed with 1 mg/ml substrate in 45 mM sodium acetate buffer, pH 5.0, and 0.067 U/ml enzyme at 37°C for 30 and 120 min. The incubations were stopped and analyzed as described above.

The influence of arabinosyl side chains on the hydrolytic activity of GalA was measured by HPAEC. The incubations were performed with 5 mg/ml potato galactan in 5 mM sodium acetate buffer, pH 5.0, and 13.6 U/ml arabinofuranosidase at 30°C for 24 h. The incubations were stopped by heating the incubation mixtures for 10 min at 100°C. After cooling to 37°C, 3.3 μU/ml GalA was added and the samples were incubated at 37°C for 48 h. The incubations were stopped and analyzed as described above. All reactions were carried out in triplicate.

Analytical methods. HPAEC was performed using a Thermo-Quest high-performance liquid chromatography system equipped with a Dionex CarboPac PA-1 (4 mm ID by 250 mm) column in combination with a CarboPac PA guard column (3 mm ID by 25 mm) and a Dionex ED40 PAD detector (Dionex Co., Sunnyvale). A flow rate of 1 ml/min was used with the following gradient of sodium acetate in 0.1 M NaOH: 0 to 40 min, 0 to 400 mM; 40 to 41 min, 400 to 1,000 mM. Each elution was followed by a washing step for 5 min with 1,000 mM sodium acetate in 0.1 M NaOH and an equilibration step of 15 min 0.1 M NaOH.

HPSEC was performed on three TSKgel columns (7.8 mm ID by 30 cm per column) in series (G4000 PWXL, G3000 PWXL, G2500 PWXL; Tosohaas, Stuttgart, Germany), in combination with a PWX-guard column (Tosohaas). Elution took place at 30°C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored by refractive index detection using a Shodex RI-72 detector (Kawasaki, Japan). Calibration was performed using dextrans (Amersham).

For matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), an Ultraflex workstation (Bruker Daltronics GmbH, Germany) was used. The mass spectrometer was calibrated with a mixture of maltodextrins. The samples were mixed with a matrix solution (1 μl each). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile and water (3:7). The prepared sample and matrix solutions were put on a gold plate and dried under a stream of warm air.

The concentration of reducing sugars was determined according to the Nelson-Somogyi method (29), using galactose as standard.

DNA sequencing and sequence analysis. An automated model 373 DNA sequencer (Applied Biosystems) was used to determine the nucleotide sequence of the gene. The DNA sequence data are available at the GenBank nucleotide databases under the accession number NC_004307. The BLAST2 program (1; available at http://www.ncbi.nlm.nih.gov/) was used for detecting sequence homologies. The SWISS-MODEL version 36.0003 program (14, 31, 37) (available from http://www.expasy.org/swissmod/SWISS-MODEL.html) was used for homology modeling.

RESULTS

Characterization of *galA* from *Bifidobacterium longum*. The *galA* gene consisted of an open reading frame of 2.6 kb. Approximately 10 nucleotides upstream of the start codon, a putative ribosomal binding site, AGGAG, was found. Analysis of the open reading frame predicted a molecular mass of 91,366 Da and an isoelectric point of 4.19 of the translation product.

GalA was classified in the glycoside hydrolase family 53 (GH53) according to the method of Henrissat et al. (15) at http://afmb.cnrs-mrs.fr/CAZY/index.html. A ClustalW comparison of four endogalactanases of which the three-dimensional (3D) structure is known (*Aspergillus aculeatus* GenBank accession no. ASNGAL1A [7, 35], *Bacillus licheniformis* strain AAO31370 [4, 34], *Corynascus heterothallicus* strain AAN99814 [23], *Humicola insolens* strain AAN99815 [19, 23]) and GalA from *B. longum* was performed (Fig. 1). Based on the 3D structures, the catalytic residues of GalA were suggested to be E_{192} and E_{297}. As expected, these 2 amino acid residues are conserved in all GH53 members (Fig. 1). The alignment showed that the enzyme was twice as large as the other endogalactanases. Analysis of the domain structure, using Prodom (8) (http://protein.toulouse.inra.fr/prodom/2003.1/html/form.php), showed that the catalytic domain is located between amino acids 55 and 429; the domain structure of the C-terminal extension was not further specified by the program.

Signal peptide and transmembrane domain prediction using SignalP (30) (http://www.cbs.dtu.dk/services/SignalP/), PSORT (27) (http://psort.nibb.ac.jp/), and SOSUI (17) (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) showed that GalA had a signal peptide and a transmembrane domain (Fig. 1).

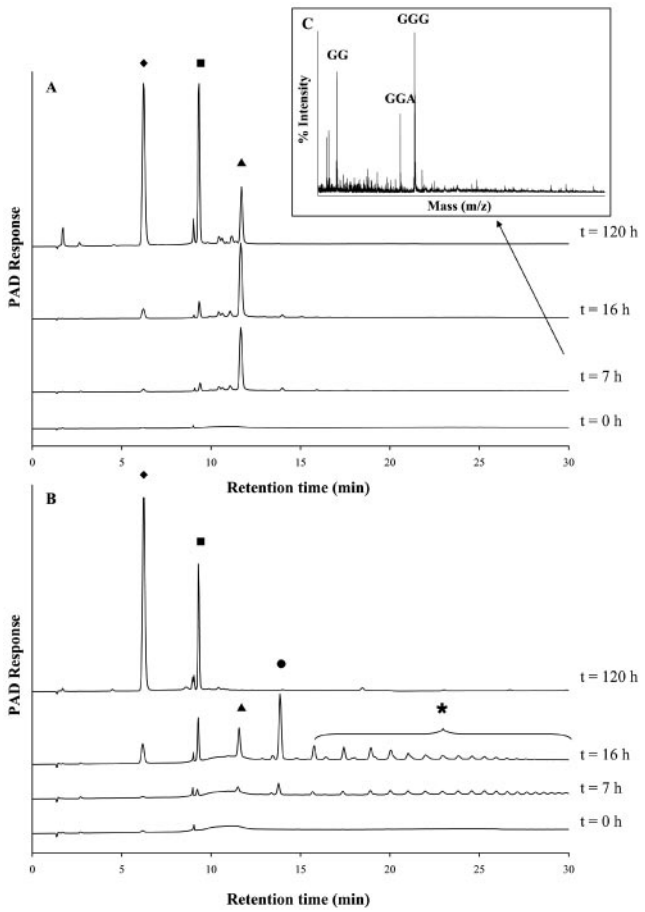

FIG. 4. HPAEC profiles of potato arabinogalactan degradation after 0, 7, 16, and 120 h of incubation by GalA (A) and endogalactanase from *A. aculeatus* (B) and a MALDI-TOF MS profile of the potato arabinogalactan digest after 7 h of incubation with endogalactanase from *B. longum* (C). Symbols: ◆, galactose; ■, galactodisaccharide [β-D-Gal*p*(1→4)β-D-Gal]; ▲, galactotrisaccharide [β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*]; ●, galactotetrasaccharides [β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*]; ★, galactooligosaccharides with a degree of polymerization of ≥5; GG, galactobiose; GGA, arabinosyl-galactobiose; GGG, galactotriose. PAD, pulsed amperometric detection.

These results suggested that GalA is an extracellular enzyme which is anchored to the cell membrane. The *galA* gene was cloned without the transmembrane domain.

A putative 3D structure of GalA was obtained by homology modeling, using the structure of the endogalactanase from *B. licheniformis* (4, 34) as a template. Of the four enzymes with a known 3D structure, the one from *B. licheniformis* had the highest identity (54%) with GalA. Amino acids 39 to 440 were incorporated in the model. For the *B. licheniformis* endogalactanase, it has been reported that loops 7 and 8 are longer than those in fungal endogalactanses (34). These loops contained residues involved in calcium binding. Further, the *B. licheniformis* endogalactanase showed the presence of three Trp residues (two of which were part of loop 8) representing subsites −2, −3, and −4 in the substrate-binding groove of the enzyme. The presence of subsites −3 and −4 was consistent with the enzyme mainly liberating galactotetrasaccharides from galactan. The putative 3D structure of GalA also showed the presence of the extended loops 7 and 8, the residues involved in calcium binding, and the two Trp residues in loop 8 (Fig. 1). Interestingly, an overlay of the structures of GalA and the *B. licheniformis* endogalactanase (Fig. 2) showed a difference in orientation of these aromatic amino acid residues between the two enzymes, particularly of the Trp (Trp405) residue of subsite −4. It seems as if the subsite −4 Trp of GalA points away from the substrate-binding groove, which might indicate that this enzyme releases shorter products.

Purification and physicochemical properties. The total endogalactanase activity of the 1-liter cell culture was 271 U in the cell extract and 209 U in the cell culture supernatant. The cell extract was used for the purification of GalA by anion-exchange chromatography and size exclusion chromatography because it contained the most activity. The enzyme was pure after these chromatographic steps, as judged by SDS-PAGE and Coomassie brilliant blue staining. The physicochemical properties of GalA are given in Table 1. The native molecular mass of the enzyme was determined by size exclusion chromatography, whereas the molecular mass of the subunits was determined by SDS-PAGE with Coomassie brilliant blue staining. GalA had a native molecular mass of 329 kDa, and the subunits (SDS-PAGE) showed a molecular mass of 94 kDa, which corresponds well with the molecular mass calculated from the deduced amino acid sequence (91 kDa). This indicated that GalA occurs as a tetramer.

Mode of action. The mode of action of GalA toward arabinogalactan was determined by the degradation of potato galactan. The monosaccharide composition of potato arabinogalactan is shown in Table 2, suggesting that this arabinogalactan preparation also contained some remnants of pectic material. The Gal/Ara ratio was 4.7 (16). The galactan degradation was followed by HPSEC (Fig. 3) and HPAEC (Fig. 4). A shift in the molecular mass of galactan occurred after 1 h of incubation, together with the appearance of a peak in the low-molecular-mass region (Fig. 3A). After prolonged incubation, the peak in the low-molecular-mass region increased and the galactan was reduced further in molecular size. The mode of action of GalA was compared with an endogalactanase from *A. aculeatus* which is also a GH53 family member. Initially, this latter enzyme had a similar shift in molecular mass of the polymeric galactan after 1 h (Fig. 3B), but prolonged incubation showed the formation of oligomers larger than the ones observed after incubation with GalA. HPSEC analysis revealed that 20 and 22% of the starting material was degraded after 1 h, whereas at the end point degradation (data not shown), 67 and 60% was degraded by GalA and the endogalactanase from *A. aculeatus*, respectively. HPAEC was used to determine the nature of the oligomers. The HPAEC profiles of the digests obtained with endogalactanase degradation (Fig. 4A) showed that GalA had a preference to liberate galactotrisaccharides. After prolonged incubation, galactose and galactobiose were also formed. Besides these products, some minor oligosaccharides were released which probably correspond to galactobiose with an arabinosyl residue as determined by MALDI-TOF MS (Fig. 4C). The degradation pattern of galactan by endogalactanase from *A. aculeatus* appeared to follow a different pattern (Fig. 4B). After an incubation time of 16 h, a range of oligosaccharides with a different degree of polymerization was

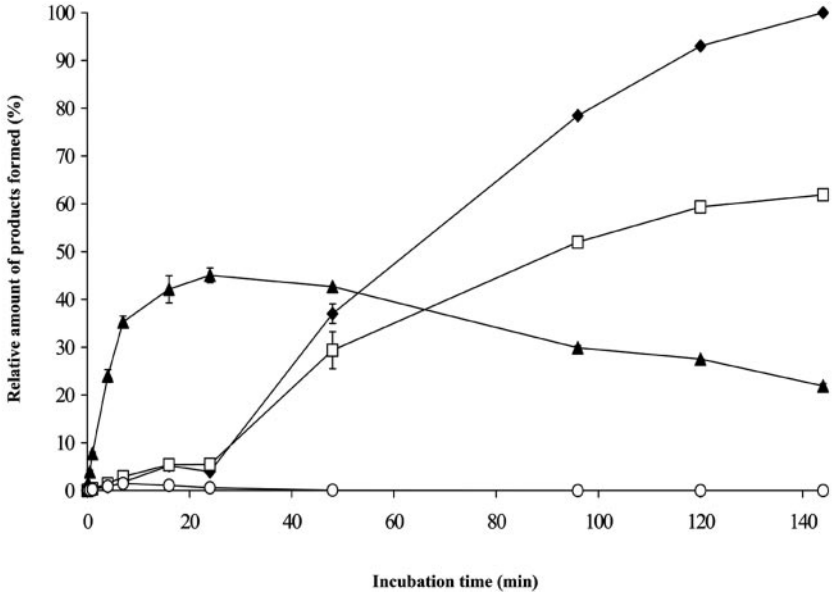

FIG. 5. The formation of galactotrisaccharide (▲), galactodisaccharide (□), galactose (◆), and galactotetrasaccharide (○) during the degradation of potato arabinogalactan by GalA over time. The amount of products formed is relative to the HPAEC peak area of the maximal amount of galactose formed. The standard deviation is indicated by error bars.

formed, of which the galactotetraose peak was the most predominant one. Exhaustive incubation showed degradation of all oligomers to galactose and galactobiose.

The degradation of potato galactan with GalA was monitored in time (Fig. 5). The results from this experiment showed clearly that the enzyme initially liberated galactotrisaccharides from the polymeric galactan. After an incubation of 24 h, the enzyme started to degrade the trisaccharide to galactose and galactobiose. A very small amount of galactotetrasaccharide was found during the first 20 h of incubation.

To compare the mode of action of GalA and *A. aculeatus* endogalactanase, the molecular weight of the galactan was plotted versus the amount of reducing sugars formed upon incubation for different periods of time (Fig. 6). An exo-acting enzyme will form a large amount of reducing sugars with a slow collapse of the molecular weight. On the other hand, an endo-acting enzyme will show a much lower formation of reducing sugars, with a large decrease in molecular weight. The curves clearly showed that the mode of action of GalA was different from that of *A. aculeatus*. This latter enzyme was characterized as an endo-acting enzyme (41). GalA showed behavior toward the potato galactan, which was clearly different from an endo-acting enzyme.

As mentioned before, GalA has a putative calcium-binding

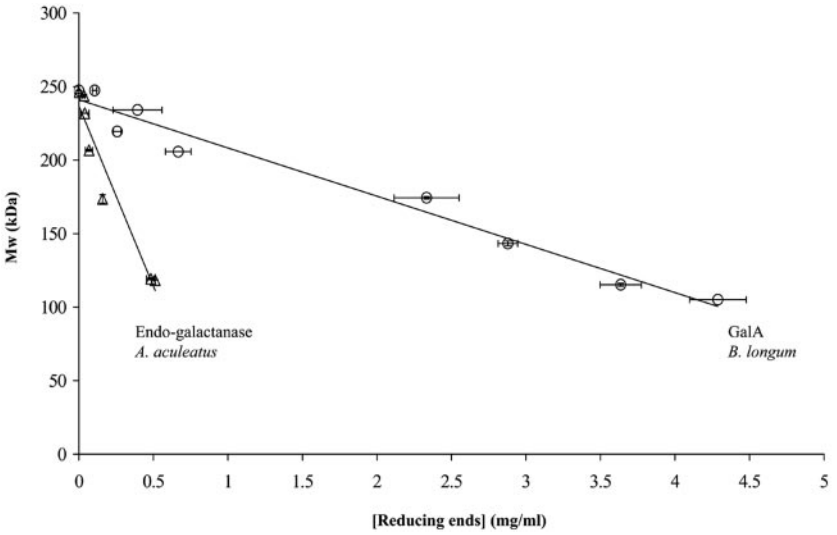

FIG. 6. The amount of reducing sugars formed by the action of GalA (○) and endogalactanase from *A. aculeatus* (△) versus the reduction of molecular weight of the polymeric galactan. The standard deviation is indicated by error bars.

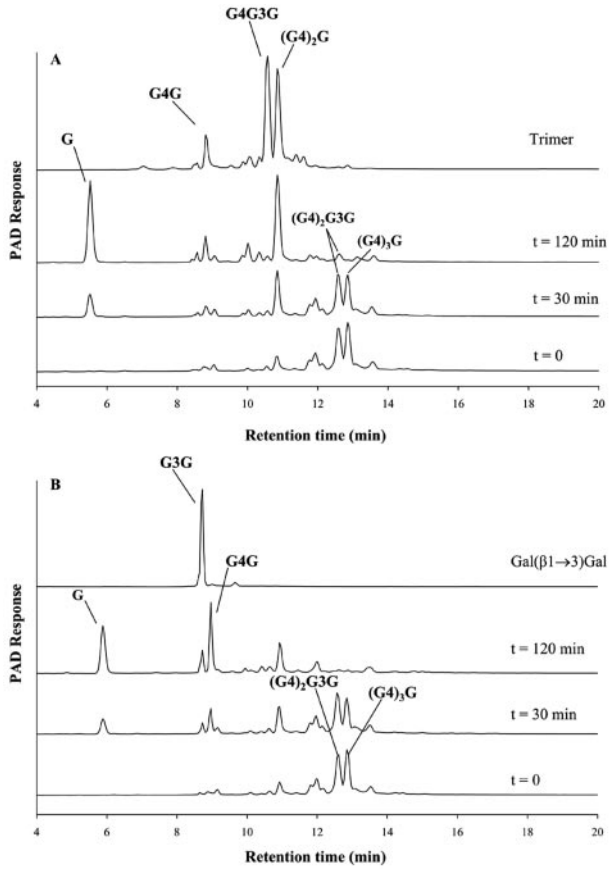

FIG. 7. HPAEC diagrams of the degradation of the tetramer fraction (purified after partial digestion of potato galactan with endogalactanase from *A. niger* [16]) with GalA (A) and endogalactanase from *A. aculeatus* (B). G, galactose; G4G, β-D-Gal*p*(1→4)β-D-Gal*p*; G3G, β-D-Gal*p*(1→3)β-D-Gal*p*; $(G4)_2G$, β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*; G4G3G, β-D-Gal*p*(1→4)β-D-Gal*p*(1→3)β-D-Gal*p*; $(G4)_3G$, β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)β-D-Gal*p*; $(G4)_2G3G$, β-D-Gal*p*(1→4)β-D-Gal*p*(1→4)-β-D-Gal*p*(1→3)β-D-Gal*p*. PAD, pulsed amperometric detection.

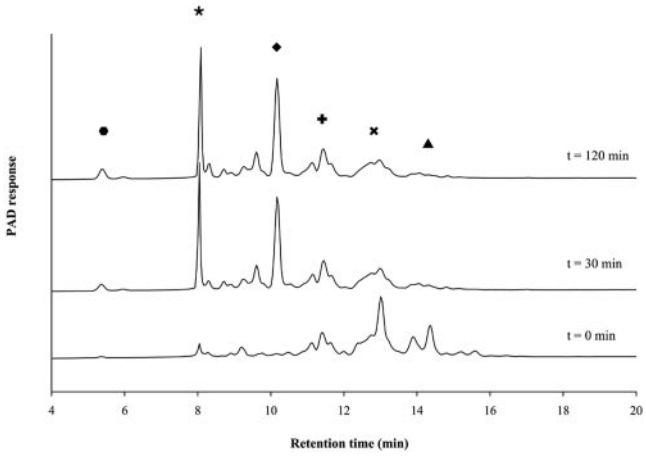

FIG. 8. HPAEC profiles of TOS degradation with GalA. The identity of several peaks in the TOS HPAEC profile are unknown, but oligosaccharides present include $[\beta\text{-D-Gal}p\text{-}(1\rightarrow4)]_n\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p$, $[\beta\text{-D-Gal}p\text{-}(1\rightarrow4)]_n\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow6)\text{-}\beta\text{-D-Glc}p$, $\alpha\text{-D-Glc}p\text{-}(1\leftrightarrow1)\text{-}\beta\text{-D-Gal}p$, $\beta\text{-D-Gal}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Glc}p\text{-}(1\leftrightarrow1)\text{-}\beta\text{-D-Gal}p$, $[\beta\text{-D-Gal}p\text{-}(1\rightarrow4)]_n\text{-}\alpha\text{-D-Glc}p\text{-}(1\leftrightarrow1)\text{-}\beta\text{-D-Gal}p$, $\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\leftrightarrow1)\text{-}\beta\text{-D-Gal}p\text{-}[(1\rightarrow4)\text{-}\beta\text{-D-Gal}p]_n$, with $n = 1$ to 4 (12). ●, galactose and/or glucose; ★, galactosyl dimers; ◆, galactosyl trimers; ✚, galactosyl tetramers; ✕, galactosyl pentamers; ▲, galactosyl hexamers. PAD, pulsed amperometric detection.

site. To examine whether GalA required calcium ions for activity, it was incubated with potato galactan in the presence of 1 mM EDTA or 1 mM $CaCl_2$. In both cases, GalA released a similar amount of galactotriose as without these additions, indicating that calcium is not necessary for the enzyme's activity. It is possible that calcium plays a role in the stability of the enzyme, but this was not further investigated.

Substrate specificity. As described previously, potato galactan contained a small amount of (β1→3) linkages in the backbone (16). However, in none of the performed experiments with GalA galactotriose with a (β1→3) linkage was this found. This might suggest that the (β1→3) linkages were degraded by the enzyme. Therefore, a galactooligosaccharide mixture containing β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p* and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p* was incubated with GalA. Table 2 shows the monosaccharide composition of this tetramer fraction. Only the trisaccharide Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p* was found when both galactotetrasaccharides were completely degraded (Fig. 7A). From this result, it was concluded that GalA is able to cleave the (β1→3) linkage in the tetramer. Since a small amount of β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p* is still available after an incubation of 120 min, it is suggested that the (β1→4) linkage may be degraded faster than the (β1→3) linkage. GalA is not capable to degrade type II galactan from gum arabic (data not shown), in which every galactosyl residue of the backbone is substituted with arabinosyl-galactosyl side chains (41). It is not clear whether the enzyme is hindered by the larger amount of side chains or whether it cannot degrade contiguous (β1→3)-linked galactosyl residues. The tetramer fraction was also treated with the endogalactanase from *A. aculeatus* (Fig. 7B). This endogalactanase liberated mainly galactobiose and galactose from both the galactotetrasaccharides. This enzyme was less active toward the (β1→3) linkage, since more disaccharide Gal*p*-(1→3)-β-D-Gal*p* accumulated in contrast with the incubation with GalA.

TOS is a mixture of different types of β-galactooligosaccharides derived after the transglycosylation of lactose with a β-galactosidase and can be used as a prebiotic. TOS was incubated with GalA. The enzyme liberated galactotrisaccharides from the substrate which were mainly pentasaccharides, resulting in an increased concentration of di- and trisaccharides (Fig. 8). However, the enzyme was unable to hydrolyze all the different types of oligosaccharides in the mixture of TOS, since some peaks in the chromatogram remained unaltered.

Potato galactan contains arabinosyl side chains in a Gal/Ara ratio of 4.7 (Table 2). In Fig. 4A and C, the presence of trisaccharides containing arabinosyl residues was already indicated. The influence of the arabinosyl residues on the activity of GalA was examined by preincubation of potato galactan with an arabinofuranosidase from *A. niger*. This enzyme was able to remove the arabinosyl residues from the galactan. After the pretreatment, the galactan was subjected to degradation

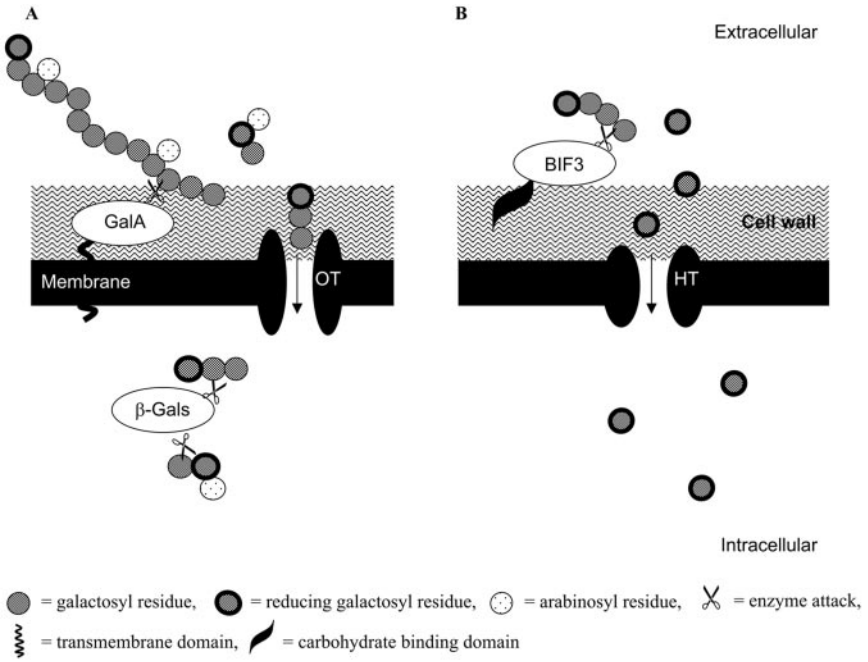

FIG. 9. Schematic representation of the putative degradation of galactans and internalization of galactooligosaccharides in *Bifidobacterium longum* (A) and *Bifidobacterium bifidum* (B). GalA, endogalactanase GalA from *B. longum*; BIF3, β-galactosidase BIF3 from *B. bifidum*; β-Gals, different β-galactosidases; OT, oligosaccharide transporter; HT, hexose transporter.

with GalA. The amount of trimer liberated from the substrate increased by 13% compared to the degradation of nonpretreated potato galactan when a pretreatment was performed (data not shown), which suggested that the enzyme is hindered by the arabinosyl side chains.

DISCUSSION

Several fermentation studies showed that *B. longum* was able to use polymeric arabinogalactan as a substrate (9, 11, 42). This paper provides the first description of a polysaccharide-degrading enzyme from *B. longum*, further supporting the view that galactans can be fermented by bifidobacteria. This enzyme, GalA, was able to degrade galactooligosaccharides with both (β1→4) and (β1→3) linkages; it was hindered by arabinosyl side chains of the type I arabinogalactan, and it was not active toward type II galactans. Interestingly, GalA liberated galactotrisaccharides from type I arabinogalactan. This is consistent with the GalA structure modeled in Fig. 2, which suggested that two Trp residues (subsites −2 and −3), and not three as with *Bacillus licheniformis* endogalactanase, are lining the substrate-binding groove. It thus seems that Trp405 of GalA is not positioned correctly for substrate binding, as a result of which galactotriose, and not galactotetraose, is the main product of the enzyme.

Several galactanases are found in bacteria (6, 20, 21, 39) and *Aspergillus* spp. (7, 10, 22, 41). Most endogalactanases described in the literature initially produce a range of oligosaccharides with different degrees of polymerization, and after prolonged incubations, only mono- and disaccharides remain. In this paper, we showed that GalA acts with a different mode of action. The HPSEC profiles of the galactan degradation showed that GalA initially seemed to act with an endomechanism (Fig. 3). However, the results presented in Fig. 6 suggested an exomechanism. Combining these results, we propose that GalA acts with a processive mechanism, i.e., after an initial midchain (or endo-) cleavage, the enzyme remains attached to the galactan and liberates galactotrisaccharides in an exofashion (3, 32). Because the enzyme liberated trisaccharides from the galactotetrasaccharides and the (β1→3) linkage was at the reducing end of the oligosaccharides, it was concluded that GalA is moving toward the reducing end of a galactan chain.

The processive mode of action of GalA might be related to its C-terminal extension. Most endogalactanases have a molecular mass of 30 to 50 kDa, whereas GalA was approximately twice as large (Fig. 1). A separate BLAST search with this C-terminal part revealed homology to bacterial endogalactanases (*Enterococcus faecium* strain ZP_00286096, *Thermotoga maritima* strain NP_229006, *Streptomyces avermitilis* strain NP_822499) and dextranase (*Paenibacillus* sp. strain AAQ91294). Because homology was found only with the noncatalytic parts of the mentioned enzymes, it is still unclear what the function of this C-terminal extension is. It might be involved in protein-protein interactions leading to the formation of the tetramer, since the GH53 galactanases with known 3D structure seem to occur as monomers (23, 34, 35). Another explanation might be that the extension is folded over the catalytic cleft in such a way that it is more difficult for the galactan to leave the catalytic site (and therewith enforcing processivity). For cellobiohydrolase Cel7D from *Trichoderma reesei* (44), it has been found that its processivity is related to a protein loop, which covers the groove with the catalytic residues, giving the enzyme a tunnel-like appearance. It should be noted that this loop is only 32

amino acids long, whereas the C-terminal extension of GalA is approximately 430 amino acids. Truncation of the *galA* gene can help to unravel the nature of this C-terminal part.

As mentioned before, GalA is probably anchored at the extracellular side of the cell membrane. There, the processive mechanism of the enzyme may ensure that a galactan chain does not escape to the environment and remains attached to the enzyme until it is completely degraded. In this way, the enzyme can secure substrate for itself, when the galactotrisaccharides formed are directly transported into the bacterial cell (Fig. 9A), where they can be further degraded by β-galactosidases (33, 38). The *B. longum* genome sequence did not reveal any extracellular β-galactosidase. Furthermore, the genome sequence showed the presence of several putative oligosaccharide transporters (NP_695366, NP_695367, NP_696791, NP_696790, NP_696339, NP_696339, NP_695267), which await further characterization. A transport mechanism for galactooligosaccharides has been suggested for *Bifidobacterium lactis* (13), since this bacterium had seemed to grow better on galactotrisaccharides and galactotetrasaccharides than on mono- and disaccharides. It is unclear whether arabinosylated galactooligosaccharides (formed upon arabinogalactan degradation) can be taken up by *B. longum*.

The presence of oligosaccharide transporters is not necessarily common in bifidobacteria. It is known that *Bifidobacterium bifidum* contains a β-galactosidase (BIF3) which is expected to be located extracellularly (25) and contains a putative galactose-binding domain. Possibly, this binding domain mediates attachment of the enzyme to galactosyl residues of the constituent polysaccharides of the *Bifidobacterium* cell wall (26). In this case, galactooligosaccharides might be degraded extracellularly to monomers, which can be internalized through the more common hexose transporters, and no special oligosaccharide transporter will be needed (Fig. 9B). From the considerations given above, it is evident that bifidobacteria can have different toolboxes to utilize galactans or galactooligosaccharides. It will be necessary to conduct more research to further unravel the mechanisms by which bifidobacteria degrade and take up carbohydrates.

ACKNOWLEDGMENTS

The work described has been carried out with financial support from the Commission of the European Communities, specifically, RTD programme "Quality of Life and Management of Living Resources" QLK1-2000-30042 and "Nutritional enhancement of probiotics and prebiotics: Technology aspects on microbial viability, stability, functionality and on prebiotic function (PROTECH)."

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bailey, R. W., and A. M. Roberton.** 1962. Carbohydrases of a rumen strain of *Lactobacillus bifidus*. Biochem. J. **82:**272–277.
3. **Benen, J. A. E., H. C. M. Kester, and J. Visser.** 1999. Kinetic characterization of Aspergillus niger N400 endopolygalacturonases I, II and C. Eur. J. Biochem. **259:**577–585.
4. **Bjørnvad, M. E., I. G. Clausen, M. Schülein, L. Bech, P. R. Østergaard, and C. Sjøholm.** December 2001. Bacterial galactanases and use thereof. Denmark patent no. US 6,331,426 B1.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Braithwaite, K. L., T. Barna, T. D. Spurway, S. J. Charnock, G. W. Black, N. Hughes, J. H. Lakey, R. Virden, G. P. Hazlewood, B. Henrissat, and H. J. Gilbert.** 1997. Evidence that galactanase A from *Pseudomonas fluorescens* subspecies *cellulosa* is a retaining family 53 glycosyl hydrolase in which E161 and E270 are the catalytic residues. Biochemistry **36:**15489–15500.
7. **Christgau, S., T. Sandal, L. V. Kofod, and H. Dalbøge.** 1995. Expression cloning, purification and characterization of a β-1,4-galactanase from *Aspergillus aculeatus*. Curr. Genet. **27:**135–141.
8. **Corpet, F., F. Servent, J. Gouzy, and D. Kahn.** 2000. ProDom and ProDom-CG: tools for protein domain analysis and whole genome comparisons. Nucleic Acids Res. **28:**267–269.
9. **Crociani, F., A. Alessandrini, M. M. Mucci, and B. Biavati.** 1994. Degradation of complex carbohydrates by *Bifidobacterium* spp. Int. J. Food Microbiol. **24:**199–210.
10. **Degnan, B. A., and G. T. Macfarlane.** 1995. Arabinogalactan utilization in continuous cultures of *Bifidobacterium longum*: effect of co-culture with *Bacteroides thetaiotaomicron*. Anaerobe **1:**103–112.
11. **de Vries, R. P., L. Parenicova, S. W. A. Hinz, H. C. M. Kester, G. Beldman, J. A. E. Benen, and J. Visser.** 2002. The β-1,4-endogalactanase A gene from *Aspergillus niger* is specially induced on arabinose and galacturonic acid and plays an important role in the degradation of pectic hairy regions. Eur. J. Biochem. **269:**4985–4993.
12. **Fransen, C. T. M., K. M. J. Van Laere, A. A. C. van Wijk, L. P. Brüll, M. Dignum, J. E. Thomas-Oates, J. Haverkamp, H. A. Schols, A. G. J. Voragen, J. P. Kamerling, and J. F. G. Vliegenthart.** 1998. α-D-Glc*p*-(1,1)-β-D-gal*p*-containing oligosaccharides, novel products from lactose by the action of β-galactosidase. Carbohydr. Res. **314:**101–114.
13. **Gopal, P. K., P. A. Sullivan, and J. B. Smart.** 2001. Utilisation of galactooligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. Int. Dairy J. **11:**19–25.
14. **Guex, N., and M. C. Peitsch.** 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis **18:**2714–2723.
15. **Henrissat, B., I. Callebaut, S. Fabrega, P. Lehns, J. P. Mornon, and G. Davies.** 1995. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc. Natl. Acad. Sci. USA **92:**7090–7094.
16. **Hinz, S. W. A., R. Verhoef, H. A. Schols, J.-P. Vincken, and A. G. J. Voragen.** Type I arabinogalactan contains β-D-Gal*p*-(1-3)-β-D-Gal*p* structural elements. Carbohydr. Res., in press.
17. **Hirokawa, T., S. Boon-Chieng, and S. Mitaku.** 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14:**378–379.
18. **Johnson, J. L.** 1994. Similarity analysis of DNAs, p. 655–682. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
19. **Kofod, L. V., M. S. Kauppinen, L. N. Andersen, I. G. Claussen, and A. Müllertz.** June 2001. Enzyme with galactanase activity. Denmark patent no. US 6,242,237 B1.
20. **Kotake, T., S. Kaneko, A. Kubomoto, M. A. Haque, H. Kobayashi, and Y. Tsumuraya.** 2004. Molecular cloning and expression in *Escherichia coli* of a *Trichoderma viride* endo-β-(1→6)-galactanase gene. Biochem. J. **377:**749–755.
21. **Labavitch, J. M., L. E. Freeman, and P. Albersheim.** 1976. Structure of plant cell walls. Purification and characterization of a β-1,4-galactanase which degrades a structural component of the primary cell walls of dicots. J. Biol. Chem. **251:**5904–5910.
22. **Lahaye, M., J. Vigouroux, and J.-F. Thibault.** 1991. Endo-β-1,4-D-galactanase from *Aspergillus niger var. aculeatus*: purification and some properties. Carbohydr. Polym. **15:**431–444.
23. **Lee, S. K., Y. B. Kim, and G. E. Ji.** 1997. Note: purification of amylase secreted from *Bifidobacterium adolescentis*. J. Appl. Microbiol. **83:**267–272.
24. **Le Nours, J., C. Ryttersgaard, L. Lo Leggio, P. R. Østergaard, T. V. Borchert, L. L. H. Christensen, and S. Larsen.** 2003. Structure of two fungal β-1,4-galactanases: searching for the basis for temperature and pH optimum. Protein Sci. **12:**1195–1204.
25. **Møller, P. L., F. Jørgensen, O. C. Hansen, S. M. Madsen, and P. Stougaard.** 2001. Intra- and extracellular β-galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. Appl. Environ. Microbiol. **67:**2276–2283.
26. **Nagaoka, M., H. Shibata, I. Kimura, S. Hashimoto, K. Kimura, H. Sawada, and T. Yokokura.** 1995. Structural studies on a cell wall polysaccharide from *Bifidobacterium longum* YIT4028. Carbohydr. Res. **274:**245–249.
27. **Nakai, K., and M. Kanehisa.** 1991. Expert system for predicting protein localization sites in gram-negative bacteria. Proteins **11:**95–110.
28. **Nakano, H., S. Takenishi, S. Kitahata, H. Kinugasa, and Y. Watanabe.** 1990. Purification and characterization of an exo-1,4-β-galactanase from a strain of *Bacillus subtilis*. Eur. J. Biochem. **193:**61–67.
29. **Nelson, N.** 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. **153:**375–380.
30. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identifica-

tion of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
31. **Peitsch, M. C.** 1995. Protein modeling by e-mail. Bio/Technology **13:**658–660.
32. **Robyt, J. F., and D. French.** 1970. Multiple attack and polarity of action of porcine pancreatic α-amylase. Arch. Biochem. Biophys. **138:**662–670.
33. **Rossi, M., L. Altomare, A. Gonzàlez Vara y Rodriguez, P. Brigidi, and D. Matteuzzi.** 2000. Nucleotide sequence, expression and transcriptional analysis of the *Bifidobacterium longum* MB 219 *lacZ* gene. Arch. Microbiol. **174:**74–80.
34. **Ryttersgaard, C., J. Le Nours, L. Lo Leggio, C. T. Jørgensen, L. L. H. Christensen, M. Bjørnvad, and S. Larsen.** 2004. The structure of endo-β-1,4-galactanase from *Bacillus licheniformis* in complex with two oligosaccharide products. J. Mol. Biol. **341:**107–117.
35. **Ryttersgaard, C., L. Lo Leggio, P. M. Coutinho, B. Henrissat, and S. Larsen.** 2002 Aspergillus aculeatus β-1,4-galactanase: substrate recognition and relations to other glycoside hydrolases in clan GH-A. Biochemistry **41:**15135–15143.
36. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. USA **99:**14422–14427.
37. **Schwede, T., J. Kopp, N. Guex, and M. C. Peitsch.** 2003. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. **31:**3381–3385.
38. **Tochikura, T., K. Sakai, T. Fujiyoshi, T. Tachiki, and H. Kumagai.** 1986. *p*-Nitrophenyl glycoside-hydrolyzing activities in Bifidobacteria and characterization of β-D-galactosidase of *Bifidobacterium longum* 401. Agric. Biol. Chem. **50:**2279–2286.
39. **Tsumura, K., Y. Hashimoto, T. Akiba, and K. Horikoshi.** 1991. Purifications and properties of galactanases from alkalophilic *Bacillus* sp. S-2 and S-39. Agric. Biol. Chem. **55:**1265–1271.
40. **van de Vis, J. W.** 1994. Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans. Ph.D. thesis. Wageningen Agricultural University, Wageningen, Germany.
41. **van de Vis, J. W., M. J. F. Searle-van Leeuwen, H. A. Siliha, F. J. M. Kormelink, and A. G. J. Voragen.** 1991. Purification and characterization of endo-1,4-β-D-galactanase from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinanases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan. Carbohydr. Polym. **16:**167–187.
42. **Van Laere, K. M. J., R. Hartemink, M. Bosveld, H. A. Schols, and A. G. J. Voragen.** 2000. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. J. Agric. Food Chem. **48:**1644–1652.
43. **Van Laere, K. M. J., T. Abee, H. A. Schols, G. Beldman, and A. G. J. Voragen.** 2000. Characterization of a novel β-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. Appl. Environ. Microbiol. **66:**1379–1384.
44. **von Ossowski, I., J. Stahlberg, A. Koivula, K. Piens, D. Becker, H. Boer, R. Harle, M. Harris, C. Divne, and S. Mahdi.** 2003. Engineering the Exo-loop of *Trichoderma reesei* Cellobiohydrolase, Cel7A. A comparison with *Phanerochaete chrysosporium* Cel7D. J. Mol. Biol. **333:**817–829.