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Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083

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Abstract Arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* releases only C3-linked arabinose residues from double-substituted xylose residues. A genomic library of *B. adolescentis* DSM20083 was screened for the presence of the *axhD3* gene. Two plasmids were identified containing part of the *axhD3* gene. The nucleotide sequences were combined and three open reading frames (ORFs) were found. The first ORF showed high homology with xylanases belonging to family 8 of the glycoside hydrolases and this gene was designated *xylA*. The second ORF was the *axhD3* gene belonging to glycoside hydrolase family 43. The third (partial) ORF coded for a putative carboxylesterase. The *axhD3* gene was cloned and expressed in *Escherichia coli*. Several substrates were employed in the biochemical characterization of recombinant AXHd3. The enzyme showed the highest activity toward wheat arabinoxylan oligosaccharides. In addition, β-xylanase from *Trichoderma* sp. was able to degrade soluble wheat arabinoxylan polymer to a higher extent, after pretreatment with recombinant AXHd3. Arabinoxylan oligosaccharides incubated with a combination of recombinant AXHd3 and an α-L-arabinofuranosidase from *Aspergillus niger* did not result in a higher maximal release of arabinose than incubation with these enzymes separately.

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Introduction

Bifidobacteria can have several health-promoting effects in humans (Gibson and Roberfroid 1995; Gibson 1998). To increase the amount of bifidobacteria in the gastrointestinal tract, they can be added to the diet, which is an example of a probiotic. Another way to increase the amount of bifidobacteria is through the consumption of a prebiotic. The definition of a prebiotic is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health" (Gibson and Roberfroid 1995). Examples of prebiotic compounds are fructooligosaccharides, α-galactooligosaccharides, β-galactooligosaccharides and xylooligosaccharides (XOS) (Crittenden and Playne 1996). An in vivo study with XOS intake by humans showed the growth promotion of bifidobacteria (Okazaki et al. 1990).

The Japanese market started to pioneer the addition of XOS in food as a prebiotic; and the first synbiotic, a combination of a probiotic and prebiotic, containing bifidobacteria and XOS and some other components, was marketed in 1993 (Crittenden and Playne 1996). Nowadays, a whole range of products containing XOS is available (Vásquez et al. 2000). It is known that xylan is hardly fermented by bifidobacteria (Crociani et al. 1994), whereas XOS and/or arabinoxylan oligosaccharides (AXOS) can be easily fermented (Okazaki et al. 1990; Campbell et al. 1997; Jaskari et al. 1998; Van Laere et al. 2000; Crittenden et al. 2002). However, relatively little is known about the XOS/AXOS-degrading enzymes from bifidobacteria. Only a β-D-xylosidase purified from *Bifidobacterium breve* K-100 was shown to be able to release xylose from xylan (Shin et al. 2003a). The genome sequence of *B. longum* NC2705 revealed that most of the sugar-modifying enzymes were related to arabinan and xylan degradation. A total of 14 putative arabinoxylan-degrading enzymes were identified (Schell et al. 2002). So far, only an α-L-arabinofuranosidase from *B. longum* B667 has been cloned and characterized (Margolles and De Los Reyes-

Gavilán 2003). This enzyme showed exo-activity and could only release 25% of the arabinose residues from flour arabinoxylan upon prolonged incubation. Van Laere et al. (1997, 1999) isolated two different arabinofuranosidases from *B. adolescentis* able to release arabinose residues from arabinoxylan. These enzymes were named arabinofuranohydrolase-D3 (AXHd3; which hydrolyzed only C3-linked arabinose residues from double-substituted xylose residues) and AXHm23 (which released only arabinose residues that were C2 or C3 linked to a single-substituted xylose residue). These two enzymes, together with a β-xylosidase, were able to degrade AXOS completely (Van Laere et al. 1999).

Here, we report the identification of the *axhD3* gene and a xylanase gene (*xylA*) from *B. adolescentis* and the characterization of recombinant AXHd3. Also, their role in AXOS degradation is discussed.

Materials and methods

Enzyme purification and internal amino acid sequencing

The AXHd3 from *B. adolescentis* was purified as described by Van Laere et al. (1997, 1999). The protein was concentrated with a 10-kDa filter (Amicon) and the *N*-terminal sequence and internal amino acid sequences of the enzyme were determined (Eurosequence, Groningen, The Netherlands). The gel electrophoresis and blotting of the concentrated sample was as described by Van den Broek et al. (2003).

Genomic library and screening

A PCR reaction was performed with primers (Table 1) designed from obtained internal amino acid sequences, Supertaq DNA polymerase (Sphearo Q), and the genomic DNA of *B. adolescentis*, which was isolated as described by Van den Broek et al. (1999). The reaction conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 2 min, ending with 72°C for 10 min. PCR products obtained with the primers 1BFOR/2REV and 1BFOR/4REV were isolated from an agarose gel using the MBI DNA extraction kit (Fermentas). The purified product was cloned into pGEM-T Easy (Promega) for cloning purposes and nucleotide sequencing.

Primers 1BFOR and 4REV were used in PCR for digoxigenin (DIG)-labeling the *axhD3* fragment for colony hybridization. The genomic library of *B. adolescentis* DSM20083 was constructed as described by Van den Broek et al. (1999). Colony hybridization and detection was performed following the instruction of the manufacturer of the DIG-labeling kit (Boehringer Mannheim).

Table 1 Oligonucleotide primers used in the PCR experiments. Nucleotide codes: *N* A, T, C or G, *W* A or T, *S* C or G, *Y* C or T, *V* A, C or G, *R* A or G, *D* A, T or G

Primer	Sequence code (5′→3′)
AXHFORl	ATGATGATTACCTCAACTAATC
AXHREV	TCATTGCTCTCTTTCCTTCG
AXHPETFOR	CATATGATGATTACCTCAACTAATC
AXHPETREV	CTCGAGTTGCTCTCTTTCCTTCG
1AFOR	TAYGAYCTBGTNCAYTGGGA
1AREV	TCCCARTGNACVAGRTCRTA
1BFOR	GTNCAYTGGGARTTYATHG
1BREV	CDATRAAYTCCCARTGNAC
2FOR	ATCGTSCARGAYGAYCCNCA
2REV	CYTGNGGRTCRTCYTGSACGA
4FOR	ATGGGYCCBGTSACNGTNG
4REV	GCSACNGTSACVGGNCCCA

Sub-cloning of the *axhD3* gene

To facilitate the over-expression of recombinant AXHd3, the *axhD3* gene was sub-cloned from pGEM-T Easy into pET22b (Novogen). Using pGEM-T Easy plasmid DNA as the template, the gene sequence was amplified by PCR using KOD Hot Start DNA polymerase (Novogen), the primers AXHPETFOR and AXHPETREV (Table 1) and a thermal program comprising 25 cycles of 94°C for 1 min, 61°C for 1 min and 68°C for 1 min 45 s, following five initial cycles with an annealing temperature of 45.1°C. The 1.569 kb PCR product contained engineered *Nde*I and *Xho*I restriction endonuclease sites at the 5′ and 3′ termini, respectively, and the original stop codon was deleted to place *axhD3* in frame with the C-terminal poly-histidine purification peptide of the pET22b expression vector. The undigested PCR product was purified from an agarose gel using a QIAquick gel extraction kit (Qiagen), ligated into pCR-Blunt (Invitrogen) and sub-cloned into pET22b on a *Nde*I/*Xho*I restriction fragment, generating pRML2.

Over-expression and purification of recombinant AXHd3

Escherichia coli BL21(DE3), transformed with pRML2 was cultured on 20 g LB agar l^{-1} supplemented with 50 mg ampicillin l^{-1} for 16 h at 37°C. A single colony was used to inoculate 1 l LB liquid growth medium, supplemented with 50 mg ampicillin, that was incubated for 16 h at 37°C with shaking at 140 rpm. The starter culture, 10 ml, was used to inoculate a 2-l Erlenmeyer flask containing 1 l LB liquid growth medium, supplemented with 50 mg ampicillin, that was incubated at 37°C with shaking at 140 rpm. When the optical density at 600 nm (OD_{600}) reached 0.5, the temperature was reduced to 23°C and over-expression of recombinant AXHd3 was induced by supplementation of the growth medium with 1 mM isopropyl-thio-β-D-galactoside. After incubation for a further 5 h, the bacterial cells

were harvested by centrifugation and re-suspended in start buffer (10 mM HEPES buffer, pH 7.5, 10 mM imidazole, 500 mM NaCl). After sonication, cell debris was removed by centrifugation at 28,000 *g*, 4°C for 30 min and the supernatant was loaded onto a chelating sepharose column (15 cm long, 1.6 cm diam.; Amersham), charged with $NiSO_4$ and equilibrated with start buffer. Bound proteins, due to the presence of the C-terminal poly-histidine tail, were washed with 100 ml of start buffer, followed by 100 ml of start buffer containing 50 mM imidazole. Recombinant AXHd3 was eluted with a linear gradient of imidazole (50–500 mM). Purity was assessed by SDS-PAGE (Laemmli 1970) and fractions containing pure recombinant AXHd3 were pooled and precipitated by increasing the $(NH_4)_2SO_4$ concentration to 3.2 M. Precipitated recombinant AXHd3 was washed three times with 3.2 M $(NH_4)_2SO_4$.

The protein concentration was determined by the method of Bradford (1976) or the Folin/Lowry procedure (Lowry et al. 1951), using bovine serum albumin as the standard.

Enzyme assays

Substrates for α-L-arabinofuranosidase were *p*-nitrophenyl-α-L-arabinofuranoside (Sigma), medium-viscosity wheat arabinoxylan (Megazyme), sugar-beet arabinan (Megazyme), β-xylanase degraded wheat arabinoxylan (approx. 5% hydrolyzed) and Arabinazyme and Xylazyme AX test tablets (Megazyme). *p*-Nitrophenyl-α-L-arabinofuranoside was prepared at a concentration of 5 mM in 100 mM sodium phosphate buffer (pH 6.0). Arabinoxylan, sugar-beet arabinan and depolymerized arabinoxylan were used at a concentration of 10 g l^{-1} in 100 mM sodium phosphate buffer (pH 6.0).

Arabinofuranosidase was assayed as follows. To pre-equilibrated wheat arabinoxylan, depolymerized wheat arabinoxylan, or sugar-beet arabinan (0.5-ml sample, at 10 g l^{-1}) in 100 mM sodium phosphate buffer (pH 6.0), 0.2 ml recombinant AHXd3 was added and subsequently mixed and incubated at 40°C. The reaction was terminated after 3, 6, 9 and 12 min by placing the tube in boiling water for 3 min. A zero time value was obtained by incubating the enzyme in boiling water for 3 min before adding the substrate. To each tube, 2.5 ml Tris-HCl buffer (100 mM, pH 8.6) containing 2 mM EDTA was added, followed by 0.1 ml NAD^+ (10 g l^{-1}). The absorbance was measured at 340 nm (A_1) and then 10 µl galactose dehydrogenase (100 units ml^{-1}; Megazyme) were added. The absorbance was measured again (A_2), after incubation at ~25°C for 40 min (or at 40°C for 20 min). Free L-arabinose was calculated from $\Delta(A_2 - A_1)$ according to the instructions of the Lactose/Galactose kit (Megazyme).

pH activity was determined by incubating 0.2 ml recombinant AXHd3 with 0.5 ml depolymerized arabinoxylan (10 mg ml^{-1}) in 100 mM citrate/phosphate buffer at pH 4.0–7.0, otherwise as described above.

Action on *p*-nitrophenyl-α-L-arabinofuranoside was determined by incubating 0.2 ml suitably diluted enzyme with 0.2 ml substrate (5 mM) in 100 mM sodium phosphate buffer (pH 6.0).

Assays with Arabinazyme and Xylazyme AX tablets was performed according to the manufacture (Megazyme).

Hydrolysis of arabinoxylan

Depolymerized wheat arabinoxylan (5-ml sample, at 2 g l^{-1}) in either 100 mM sodium phosphate buffer (pH 6.0) or sodium acetate buffer (pH 4.5) was incubated with recombinant AXHd3 (7 units on wheat arabinoxylan, pH 6.0), *Aspergillus niger* arabinofuranosidase (500 units on *p*-nitrophenyl-α-L-arabinofuranoside, pH 4.5; Megazyme) or a mixture of both enzymes (pH 5.0) at 40°C. Aliquots (0.5 ml) were removed at various time-intervals and incubated at 100°C to inactivate enzyme activity. Distilled water (1.5 ml) was added to each sample and aliquots (0.2 ml) were removed for the determination of released arabinose, using the galactose dehydrogenase/NAD^+ assay. Total carbohydrate concentration was determined using the phenol sulfuric acid procedure (Dubois et al. 1956) with an arabinose/xylose (40:60) standard solution. The degree of hydrolysis was calculated from the arabinose released, as a percentage of total carbohydrate.

Wheat arabinoxylan (1 l sample, at 10 g l^{-1}) in 2 mM sodium phosphate buffer (pH 6.0) was incubated with 1,000 units recombinant AXHd3 at 40°C. Aliquots (1 ml) were removed at various time-intervals for the determination of free arabinose levels (i.e., extent of hydrolysis). After 2 h, the solution was incubated at 100°C for 20 min to inactivate recombinant AXHd3. To this solution, 10 ml sodium acetate buffer (1 M, pH 4.5) were added and the pH was adjusted to 4.5. β-Xylanase M1 (2,000 units, *Trichoderma* sp.; Megazyme) was added and the solution was incubated at 40°C for 60 min and then at 100°C to inactivate enzyme activity. In a parallel experiment, wheat arabinoxylan (1 l sample, at 10 g l^{-1}) in 10 mM sodium acetate buffer (pH 4.5) was incubated with 2,000 units β-xylanase M1 (*Trichoderma* sp.) at 40°C for 60 min and then at 100°C for 20 min. In both cases, the reaction solutions were concentrated to 100 ml, centrifuged at 12,000 *g* to remove insoluble protein material and 25 ml (approx. 2.5 g) applied to a column of Bio-Gel P-2 (95 cm long, 5 cm diam.; BioRad) and chromatographed at 60°C with elution by degassed distilled water. Fractions (20 ml) were collected and analyzed for carbohydrate using the phenol-sulfuric acid procedure.

Nucleotide sequence accession number

An automated DNA Sequencer 373 (Applied Biosystems) was used for determining the nucleotide sequence of the genes. The DNA sequence data (*axhD3*, *ces*, *xylA*) were submitted to the GenBank nucleotide databases under the accession number AY233379. The BLAST2 program (Altschul et al. 1997) was used for searching sequence homologies.

Results

Identification and cloning of the arabinofuranohydrolase-D3 gene and an endo-xylanase gene from *B. adolescentis*

The AXHd3 enzyme (Van Laere et al. 1997, 1999) was subjected to *N*-terminal and internal amino acid sequencing for cloning purposes. The *N*-terminus was blocked, but four internal amino acid sequences were obtained (SYDLVHWEFIAHALN, VIVQDDPQADLGYEG, TMG PVTVAEVTVDA, DADTPES). From these sequences, different primers were designed (Table 1) and different combinations were used in PCR reactions for the specific amplification of parts of the *axhD3* gene from *B. adolescentis*. The combinations 1AFOR/2REV, 1BFOR/2REV, 1AFOR/4REV and 1BFOR/4REV resulted, respectively, in fragments of approximately 400, 400, 1,200 and 1,200 bp, as estimated by agarose gel electrophoresis. Primers 1AFOR and 1BFOR were designed from the same internal fragment and therefore only the fragments obtained with the primer combinations 1BFOR/2REV and 1BFOR/4REV were sequenced. The combination 1BFOR/2REV resulted in a PCR fragment of 371 bp and 1BFOR/4REV gave a fragment of 1,284 bp. The primer combination 1BFOR/4REV was used for DIG-labeling of the PCR product which was used for colony hybridization. Two different types of plasmids hybridized with the labeled PCR fragment. The plasmids of these colonies were sequenced and both types of colony contained a part of the *axhD3* gene in their plasmid. The nucleotide sequence of both plasmids was combined and resulted in a DNA sequence of 3,674 bp. Two open reading frames (ORFs) and one partial ORF were identified. The first ORF was 1,140 bp, the second was 1,590 bp and the partial ORF contained 921 bp.

The deduced amino acid sequence of the first ORF contained 379 amino acids, encoding a protein of 43,785 Da. A database search revealed 52% identity with xylanase Y from *Bacillus halodurans* (NP_242971; AAN16076), 31% identity with an endoglucanase Y from *Cytophaga hutchinsonii* (ZP_00309326), 31% identity with an endo-1,4-β-xylanase precursor from an uncultured bacterium (AAS85781) and 27% identity with xylanase Y from *Bacillus* sp. KK-1 (AAC27700). The gene was designated *xylA*. The xylanases are members of family 8 of the glycoside hydrolases (GHs; Henrissat 1991) and therefore the putative *xylA* was classified in this family.

The deduced amino acid sequence of the second ORF contained 529 amino acids, encoding a protein of 59,403 Da. The deduced amino acid sequence contained all four internal amino acid sequences of the native AXHd3. Therefore, the gene was designated *axhD3*. The gene revealed 38% and 34% identity with a β-xylosidase from *C. hutchinsonii* (ZP_00310780) and an endo-1,4-β-xylanases from *Caldocellum saccharolyticum* (AAB87371), respectively. For a possible β-xylosidase from *Clostridium acetobytylicum* (AAK76859), belonging to family 43 of the GHs, 32% identity was found. As *axhD3* showed homology with members of GH family 43, it was classified in this family. Both GH families 8 and 43 have an inverting catalytic mechanism.

The deduced amino acid sequence of the partial ORF contained 307 amino acids. A database research revealed 44% identity with a putative esterase from *Salmonella enterica* subsp. *enterica* serovar *Thypi Str.* CT18 (NP_455874) and a putative carboxylesterase from *S. typhimurium* LT2 (NP_460582). This gene was designated *Ces* and showed highest homology with members of carbohydrate esterase (CE) family 10. Most members of this family are esterases acting on non-carbohydrate substrates.

Overview of arabinoxylan-degrading enzymes from *Bifidobacterium* spp

The amino acid sequence of *axhD3* was compared with the other known amino acid sequences of arabinoxylan-

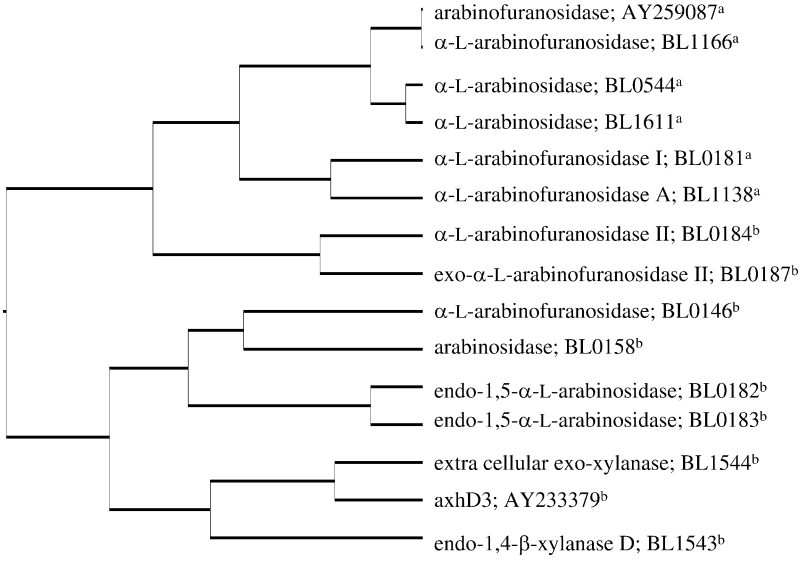

Fig. 1 Dendrogram based on amino acid sequences of arabinoxylan-degrading enzymes from *Bifidobacterium* spp. The dendrogram was constructed using the Clustal method. Superscript letters: *a* GH family 51, *b* GH family 43

degrading enzymes from *Bifidobacterium longum*. A dendrogram was constructed using the Clustal method; and two distinct groups were observed, one of which represented mainly members of GH family 51, while the other group contained exclusively members of GH family 43 (Fig. 1). The group containing the members of GH family 51 consisted of putative α-L-arabinosidases and α-L-arabinofuranosidases. The cloned α-L-arabinofuranosidase from *B. longum* B667 (AY259087; Margolles and De Los Reyes-Gavilán 2003) was similar to the α-L-arabinofuranosidase from *B. longum* NCC2705 (BL1166; Schell et al. 2002). The two putative related α-arabinofuranosidases II (BL0187, BL0184) belonging to GH family 43 (http://afmb.cnrs-mrs.fr/CAZY/index.html) were placed in the dendrogram in the group containing the members of GH family 51. AXHd3 showed the highest homology with a putative extracellular exo-xylanase (BL1544; *xynF*) from *B. longum* NCC2705.

Substrate specificity of recombinant AXHd3

A recombinant AXHd3 containing an additional histidine tag to facilitate enzyme purification was expressed in *E. coli*. The molecular mass was approximately 60 kDa as estimated by SDS-PAGE and is in agreement with the deduced amino acid sequence of *axhd3*, taking into account the poly-histidine tag at the C-terminus (1,083 Da). The pH optimum of recombinant AXHd3 was pH 6.0, which was similar to native AXHd3 (Van Laere et al. 1999). The enzyme was characterized using various substrates and showed the highest activity toward oligosaccharides obtained from wheat flour arabinoxylan digested by endo-β-xylanase (Table 2). Whereas native AXHd3 (Van Laere et al. 1999) apparently showed no detectable activity towards *p*-nitrophenyl-α-L-arabinofuranoside, recombinant AXHd3 was able to release *p*-nitrophenol from this substrate at a very low rate, as a large amount of the enzyme could now be employed. The recombinant AXHd3 showed no endo-activity against xylan and arabinan.

Soluble wheat arabinoxylan was incubated with an endo-β-xylanase from *Trichoderma* sp. (Fig. 2a) or with recombinant AXHd3 followed by incubation with endo-β-xylanase (Fig. 2b) to investigate any synergistic mechanism between the two enzymes. Size exclusion chromatography showed that wheat arabinoxylan was degraded to a higher extent by β-xylanase after pretreatment with recombinant AXHd3. The polymeric fraction was reduced and substantially higher amounts of mono-, tri-, tetra-, and hexamers were produced. The synergistic effect was also tested for the release of arabinose from wheat arabinoxylan preincubated with endo-β-xylanase by the recombinant AXHd3 and an α-L-arabinofuranosidase from *A. niger* (Fig. 3). High amounts of α-L-arabinofuranosidase were used to obtain maximal release of arabinose. Recombinant AXHd3 released approximately 10% of the arabinose residues from AXOS and the α-L-arabinofuranosidase from *A. niger* was able to release approximately 40% of the arabinose residues. However, no increase in the amount of maximal release of arabinose was observed after incubation with both enzymes together.

Table 2 Activity of recombinant AXHd3 from *B. adolescentis* DSM20083 on various substrates

Substrate	Specific activity (units mg^{-1} protein)
Wheat flour arabinoxylan	28.3
AXOS	90.4
Sugar-beet arabinan	4.0
p-Nitrophenyl-α-L-arabinofuranoside	0.095
Arabinazyme tablets (*endo*-1,5-α-L-arabinanase)	<0.001
Xylazyme tablets (*endo*-1,4-β-D-xylanase)	<0.001

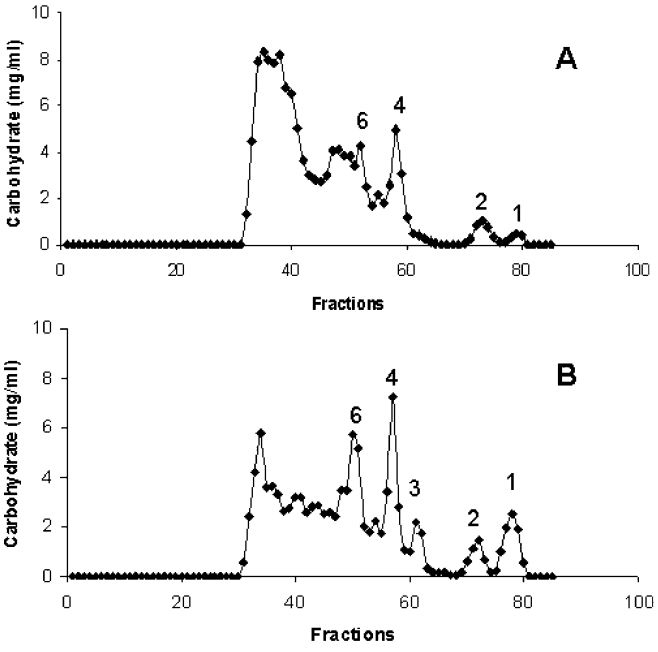

Fig. 2 Bio-Gel P2 gel permeation chromatography of the oligosaccharides produced by the hydrolysis of soluble wheat arabinoxylan by *Trichoderma* xylanase M1 (**a**) and recombinant AXHd3 from *B. adolescentis* followed by *Trichoderma* xylanase M1 (**b**). *Numbers* represent the degree of polymerization

Discussion

AXHd3 is a unique enzyme that is able to release only C3-linked arabinose residues from double-substituted xylose residues (Van Laere et al. 1997, 1999) and plays a role in the complete degradation of arabinoxylan and AXOS (Van Laere et al. 1997, 1999). The amino acid sequence revealed that the enzyme belongs to GH family 43, which has an inverting mechanism. This result supports the data obtained by Pitson et al. (1996), who classified AXHd3 as an inverting enzyme on the basis of its lack of glycosyl transfer to methanol.

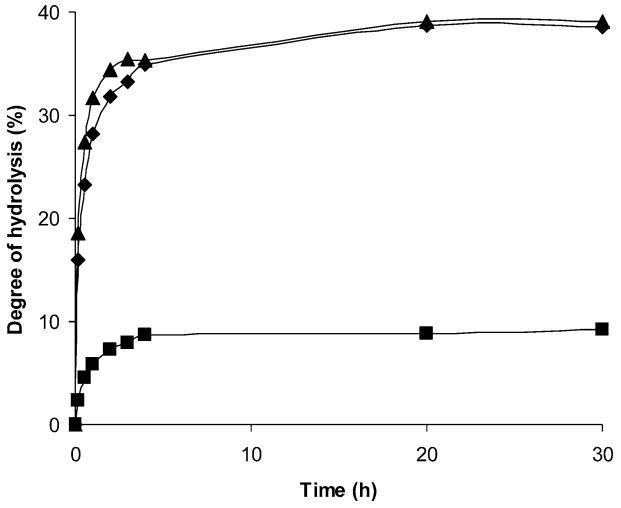

Fig. 3 Release of arabinose from wheat arabinoxylan oligosaccharides by recombinant AXHd3 from *B. adolescentis* (7 units; *squares*), *A. niger* α-L-arabinofuranosidase (500 units; *diamonds*) or a combination of both enzymes (*triangles*)

The highest homology for the deduced amino acid sequence of *axhD3* was found for putative β-xylosidases and β-xylanases. In addition, the highest homology for AXHd3 and the arabinoxylan-degrading enzymes of *B. longum* NCC2705 (Schell et al. 2002) was also found for a putative extracellular exo-xylanase (BL1544; *xynF*). We propose that, due to this high homology, it is possible for these enzymes to be assigned to arabinose-releasing activities instead of β-xylosidase or β-xylanase, moreover because these enzymes are not biochemically characterized yet. Homology for the deduced amino acid sequence of *xylA* was observed with putative xylanases but no homology was found with arabinoxylan-degrading enzymes from *B. longum* NCC2705 (Schell et al. 2002). It would be interesting if *xylA* codes for a true xylanase with an endo-activity, as most members of GH family 8 are glucanases. Intriguingly, the *xylA*-encoded gene product does not contain a signal peptide and it is thus assumed that the enzyme is intracellular. The cloning and biochemical characterization of this enzyme is now in progress.

To date, only a few arabinose-releasing enzymes have been characterized from *Bifidobacterium* spp, despite the presence of 11 genes coding for putative arabinose-releasing enzymes in the genome of *B. longum* NCC2705 (Schell et al. 2002). The only cloned enzyme is an α-L-arabinofuranosidase from *B. longum* B667 (*abfB*), which was active towards polymeric arabinoxylan and released ~25% of the total arabinose residues (Margolles and de Los Reyes-Gavilán 2003). It was concluded that the enzyme exhibited hydrolytic activity against α-1,3- and α-1,5-linked non-reducing terminal L-arabinose residues. However, activity against α-1,2 linkages could not be excluded. The enzyme was not tested against AXOS, but the activity toward arabinoxylan was rather low. The purified and characterized α-L-arabinopyranosidase and α-L-arabinofuranosidase from *B. breve* K110 were not tested for arabinoxylan-degrading activity. Arabinogalactan was used as substrate but no activity was observed for either enzyme (Shin et al. 2003b). AXHd3 from *B. adolescentis* was active towards arabinoxylan and oligomers derived from it. AXHm23 was only active towards AXOS (Van Laere et al. 1999). The recombinant AXHd3 showed the highest activity towards AXOS.

In general, it was found that AXOS and XOS are better substrates for growth/fermentation than their polymeric precursors or free monosaccharide compounds (Okazaki et al. 1990; Campbell 1997; Jaskari et al. 1998; Van Laere et al. 2000; Crittenden et al. 2002). Fermentation studies of *B. longum* co-cultured with *Bacteroides thetaiotaomicron* demonstrated that an arabinogalactan polymer was degraded by *Bact. thetaiotaomicron* to oligomers, which subsequently could be utilized by *Bifidobacterium longum* (Degnan and Macfarlane 1995). In another study, it was shown that arabinoxylan was degraded by the *Bacteroides fragilis* group and it was postulated that the produced oligosaccharides might provide a carbon source for bifidobacteria (Hopkins et al. 2003). These oligosaccharides might be imported into the cell before hydrolyzing them. In addition, the genome sequence of *Bifidobacterium longum* NCC2705 revealed the presence of eight high-affinity MalEFG-type oligosaccharide transporters (Schell et al. 2002), more than in any other published prokaryotic genome to date. It is speculated elsewhere that XOS can be transported into certain bacteria, where they are subsequently hydrolyzed and used as a carbon and energy source. For instance, members of the CE family 7 enzymes appear to lack signal peptides; and members of this family have been shown to be acetylated xylooligosaccharide esterases, exhibiting no detectable activity on acetylated xylan (Lorenz and Wiegel 1997; Vincent et al. 2003). The degradation of arabinoxylan is therefore assumed to be a co-operative process in the colon. The presence of specific enzymes like AXHd3 shows why *B. adolescentis* can utilize AXOS. Also, *B. longum* is able to grow on this substrate, an ability presumably enabled by the numerous genes coding for enzymes involved in arabinoxylan degradation (Schell et al. 2002).

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