

**Galactosyl hydrolases from *Bifidobacterium*
adolescentis and *Bifidobacterium longum***

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Galactosyl hydrolases from *Bifidobacterium adolescentis* and *Bifidobacterium longum*

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ABSTRACT

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Key words: galactan, galacto-oligosaccharides, β -galactosidase, α -galactosidase, endo-galactanase, substrate specificity, mode of action, bifidobacteria, transglycosylation, site directed mutagenesis.

Galactans and galacto-oligosaccharides are known for their prebiotic potential. Since not much is known about the enzymic machinery of bifidobacteria for galactan utilization, galactan-modifying enzymes of bifidobacteria were studied, which may assist in the development of prebiotics. Three galactan/galacto-oligosaccharide modifying enzymes were characterized; a $(\beta 1 \rightarrow 4)$ -specific β -galactosidase from *Bifidobacterium adolescentis* DSM20083, an extracellular endo-galactanase from *Bifidobacterium longum*, and a transglycosylating α -galactosidase from *B. adolescentis*.

(Arabino)galactans are plant polysaccharides. The structural variation within these galactans is large. A Gal $(\beta 1 \rightarrow 3)$ Gal structural element was found in the backbone of $(\beta 1 \rightarrow 4)$ -galactans (Type I). The $(\beta 1 \rightarrow 3)$ -galactosyl interruption appeared to be a common structural feature of Type I arabinogalactans with a frequency ranging from approximately 1 in 160 (potato, soy, citrus) to 1 in 250 (onion).

A β -galactosidase (β -Gal II) from *B. adolescentis* was cloned and expressed in *E. coli* cells. β -Gal II prefers $(\beta 1 \rightarrow 4)$ -galactosides over other galacto-oligosaccharides, like lactose. Only a low transglycosylation activity was found, which makes the enzyme less appropriate for tailoring prebiotics.

An endo-galactanase from *B. longum* was cloned and expressed in *E. coli* cells. It was suggested that the enzyme was located extracellular and anchored to the cell wall. The enzyme was able to liberate galacto-trisaccharides from $(\beta 1 \rightarrow 4)$ -galactans, probably by a processive mechanism, moving towards the reducing end of the galactan chain after an initial mid-chain cleavage. The enzyme seemed to be able to cleave $(\beta 1 \rightarrow 3)$ -linkages.

An α -galactosidase (AGA) from *B. adolescentis*, which has a high transferase activity, was cloned and expressed in *E. coli* cells. Site-directed mutagenesis was performed to increase the transglycosylation efficiency of the enzyme. A 16% increase in transglycosylation activity could be obtained with only a single mutation. Combining successful single mutations in double mutations did not result in an extra increase in transglycosylation efficiency.

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General introduction

Based on

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INTRODUCTION

Nowadays, there is an increasing interest in positively influencing the intestinal microflora through the diet. Carbohydrate digestion (in the upper part of the intestines) and carbohydrate fermentation (in the lower part) play an important role in the gastrointestinal tract (GIT) of humans and animals. Besides their direct physiological effect they also affect the gut ecosystem, which significantly contributes to the well being of humans (17). Bifidobacteria are one of the major groups in the GIT and it is claimed that they have several health-promoting effects (21-23) like preventing diarrhea (62), lowering cholesterol levels (3), immunostimulation (59), anticarcinogenicity (5, 57, 92), improved mineral absorption (71), and production of vitamins (15). In order to stimulate the growth of bifidobacteria in the GIT the concepts of probiotics and prebiotics have been developed. To positively influence the microbiota in the GIT probiotics and/or prebiotics can be applied in the diet as a functional ingredient. The definition of a probiotic is ‘a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the micro flora (by implantation or colonization) in a compartment of the host, and by that, exert beneficial health effects in this host’ (72). Bacteria used as probiotic are mainly from the genera *Bifidobacterium* or *Lactobacillus* (54, 82). A prebiotic can be defined as ‘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’ (22). Prebiotic ingredients used are often non-digestible oligosaccharides. The combination of probiotics and prebiotics is termed synbiotic. The definition is ‘a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT’ (22). More information on probiotics or an overview of the health benefits of pre- and probiotics is reviewed by several authors (10, 39, 41, 72, 77, 79).

Besides non-digestible oligosaccharides, the intake of dietary fibre also gives beneficial health effects to humans (75). Trowell et al. (84) proposed the generally accepted definition of dietary fibre as consisting of the remnants of edible plant cell wall polysaccharides, lignin, and associated substances resistant to (hydrolysis) digestion by the alimentary enzymes of humans. However, a great deal of debate has existed over this definition the last decades. A new definition/description was launched by the American Association of Cereal Chemists (55). Their criteria for dietary fibre were (I) it should be edible parts of plants or analogous

carbohydrates, (II) resistant to digestion and absorption in the small intestine, (III) completely or partially fermented in the large intestine, (IV) include polysaccharides, oligosaccharides, lignin, and associated plant substances, and (V) promote beneficial physiological effects. Two types of dietary fibre are distinguished; one precipitating in 80% (v/v) ethanol (insoluble dietary fibre), and the other remains in solution in 80% (v/v) ethanol (soluble dietary fibre; (49)). Non-digestible oligosaccharides (NDOs) belong to the soluble dietary fibre. Dietary fibres, like galactans and galacto-oligosaccharides are known for their prebiotic potential (1, 56, 61, 66), and commercial products containing these are available.

GALACTANS AND GALACTO-OLIGOSACCHARIDES

Galactans and arabinogalactans are plant polysaccharides, which can be divided in two groups, i.e. Type I and Type II. Type I arabinogalactan contains a ($\beta 1 \rightarrow 4$)-linked galactan backbone, which can be substituted with arabinosyl residues (6, 78), whereas type II arabinogalactans contain a backbone of predominantly ($\beta 1 \rightarrow 3$)-linked galactosyl residues. Type II arabinogalactans are most frequently branched, for example with galactosyl, arabinosyl, and rhamnosyl residues (78). A schematic overview of these polysaccharides is shown in Figure 1.1.

Besides galactans, also naturally occurring galacto-oligosaccharides are found. Many of these non-digestible oligosaccharides are found in plants, honey and milk. A group of galacto-oligosaccharides in legumes and sugar beet are the α -galacto-oligosaccharides raffinose and stachyose. A schematic representation of these oligosaccharides is shown in Figure 1.1. The trisaccharide raffinose consists of a galactosyl residue, which is ($\alpha 1 \rightarrow 6$)-linked with sucrose. Stachyose is a tetrasaccharide, which consist of the trisaccharide raffinose with a ($\alpha 1 \rightarrow 6$)-linked galactosyl residue. Also longer α -galacto-oligosaccharides are found, which, together with stachyose, are synthesized by stachyose synthases. Another naturally occurring α -galacto-oligosaccharide is melibiose, which was found in soy beans, grains, honey and grapes. This is a disaccharide in which a galactosyl residue is ($\alpha 1 \rightarrow 6$)-linked to a reducing glucosyl residue.

Another group of galacto-oligosaccharides are the enzymatically synthesized β -galacto-oligosaccharides, which are available as prebiotic food ingredients. A commercial preparation of galacto-oligosaccharides is TOS (trans-galacto-oligosaccharides), which is derived from

lactose as start substrate by a transglycosylating β -galactosidase. The mixture of TOS contains different types of galacto-oligosaccharides, like $[\beta\text{-D-Galp}-(1\rightarrow4)]_n\text{-}\beta\text{-D-Galp}-(1\rightarrow4)\text{-D-Glcp}$, $[\beta\text{-D-Galp}-(1\rightarrow4)]_n\text{-}\beta\text{-D-Galp}-(1\rightarrow6)\text{-D-Glcp}$, $\alpha\text{-D-Glcp}-(1\leftrightarrow1)\text{-}\beta\text{-D-Galp}$, $\beta\text{-D-Galp}-(1\rightarrow2)\text{-}\alpha\text{-D-Glcp}-(1\leftrightarrow1)\text{-}\beta\text{-D-Galp}$, $[\beta\text{-D-Galp}-(1\rightarrow4)]_n\text{-}\alpha\text{-D-Glcp}-(1\leftrightarrow1)\text{-}\beta\text{-D-Galp}$, $\beta\text{-D-Galp}-(1\rightarrow4)\text{-}\alpha\text{-D-Glcp}-(1\leftrightarrow1)\text{-}\beta\text{-D-Galp}-[(1\rightarrow4)\text{-}\beta\text{-D-Galp}]_n$, with $n=1\text{-}4$ (19). Lactose is naturally occurring in milk. It consists of a galactosyl residue that is $(\beta 1\rightarrow4)$ -linked to a reducing glucosyl residue.

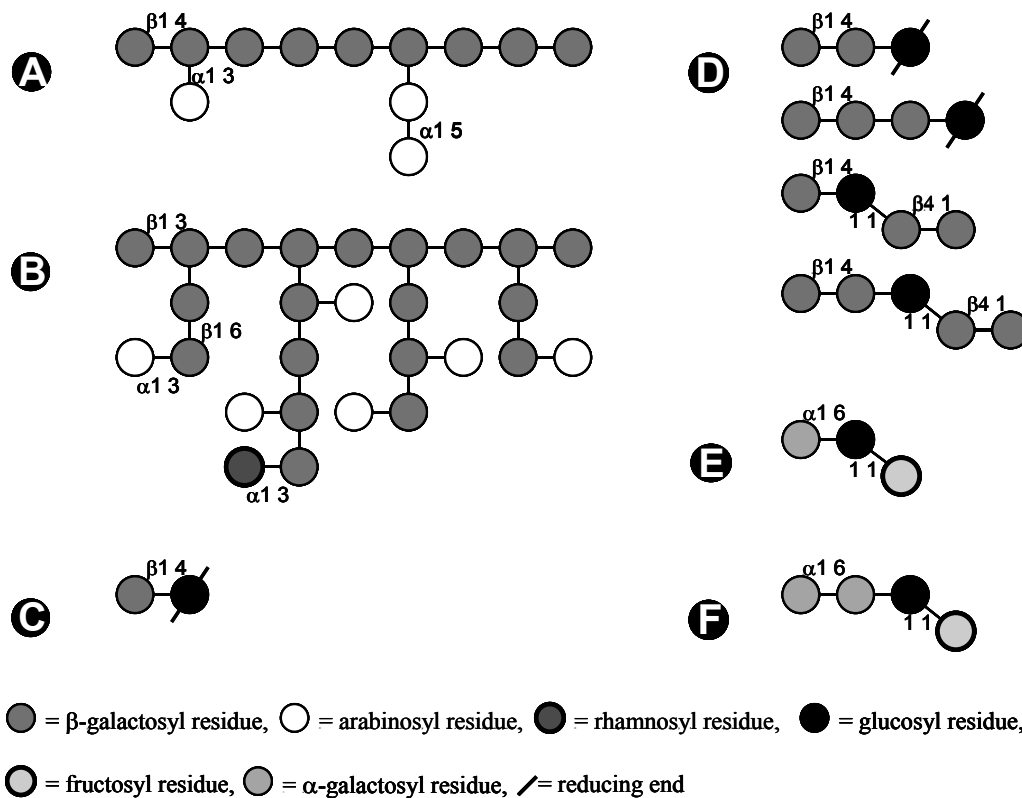


Figure 1.1: Schematic representation of galactans and galacto-oligosaccharides. A: Type I arabinogalactan, B: Type II arabinogalactan, C: lactose, D: some oligosaccharides from the TOS preparation, E: raffinose, F: stachyose.

BIFIDOBACTERIA

So far, not much is known about the galactan utilization of the gut micro-flora. Since bifidobacteria are one of the major bacterial groups in the GIT, and it is claimed that they have several health-promoting effects, the galactan utilization of bifidobacteria was of interest

to us. Bifidobacteria are gram-positive, anaerobic, non-spore-forming, and non-motile bacteria. The rods are often Y- or V-shaped (68). Tissier (80) reported in 1900 the isolation of the first bifidobacterium from the intestine of a child, and named it *Bacillus bifidus communis*. The genus *Bifidobacterium* was already recognized by Orla Jensen as a separate taxon in 1924, but it took 50 years before the genus *Bifidobacterium* was for the first time classified in the Bergey's Manual of Determinative Bacteriology (58). Presently, 34 species (including some different biotypes and subspecies) have been described (4, 32, 47, 65, 73, 94). The major habitat is considered to be the intestine of man and animals (4) and twelve species have been associated with humans as host. Bifidobacteria rapidly colonize the digestive tract of newly born infants and become the major bacteria in the colon. The number of these known *Bifidobacterium* spp. gradually decreases with age and also the composition of the *Bifidobacterium* spp. changes in time (31, 50). Bifidobacteria represent 1-3% of the total fecal bacterial community in adults (18, 45).

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Oligo- and polysaccharides will be degraded to monosaccharides and these will be converted to intermediates of the hexose fermentation pathway also called fructose-6-phosphate shunt or bifid shunt. Subsequently, they will be converted to short chain fatty acids and other organic compounds (11, 69). In general, gut bacteria degrade polymeric carbohydrates to low molecular weight oligosaccharides, which can subsequently be degraded to monosaccharides by the use of a wide range of depolymerizing enzymes. These glycosidases are found extracellularly, associated to the bacterial cell, or intracellularly.

The microbial enzyme activity can be induced by non-digestible oligosaccharides or dietary components. For example: the activity of β -galactosidase and α -arabinopyranosidase of *B. longum* is increased, when grown on arabinogalactan (8, 14, 67). Growth of *B. adolescentis* on xylose and arabinoxylan-derived oligosaccharides induced the production of two arabinofuranohydrolases (87). Also *B. longum* produced arabinofuranosidases during growth on arabinoxylan (7). Trindade and coworkers (83) found that sucrase activity of *B. lactis* was induced in the presence of sucrose, raffinose, and in small amounts by oligofructose.

The induction of enzymes, which are involved in the degradation of carbohydrates, can be repressed by the presence of glucose (12, 83). This repression of enzyme synthesis is a way of the bacteria to control the oligo- and polysaccharide metabolism. When a preferred carbon source is present, there will be no unnecessary production of large amounts of enzyme (14).

BIFIDOBACTERIUM CARBOHYDRASES

Japanese researchers have performed most of the early work on isolation and characterization of bifidobacteria enzymes (36, 37, 63, 64, 81). All the enzymes were isolated from *Bifidobacterium* species present in humans except for *B. pseudolongum*. Most of the data about heterologously produced enzymes from *Bifidobacterium* spp. has been published in the last five years. All enzymes are from bifidobacteria species present in humans, with the exception of those from *B. lactis*, which is commonly found in fermented milk. Most of the carbohydrases are α -galactosidases (GH family 36), β -galactosidases (GH family 2 or 42), and enzymes active towards gluco-oligosaccharides like α -glucosidases and sucrose phosphorylases (GH family 13).

Since the genome of *B. longum* NCC2705 has been published (70), more information about carbohydrate modifying enzymes of this organism became available. Five percent of the annotated genes of *B. longum* are carbohydrate modifying enzymes (like glycoside hydrolases and glycosyl esterases) and carbohydrate binding modules. For example in *L. lactis* 2.5% of annotated genes encode carbohydrate modifying enzymes, in *Clostridium perfringens* 3.1%, in *E. coli* K12 1.4%, in *Bacillus subtilis* 1.7%, and in *Mycobacterium tuberculosis* 1.1% (28). Only in *Bacteroides thetaiotaomicron*, also a large number of the annotated genes encode carbohydrate modifying enzymes (93). In Table 1.1 an overview is given of the annotated and predicted (arabino)galactan and galacto-oligosaccharide modifying enzymes of *B. longum* NCC2705. This table shows also arabinosidases and arabinofuranosidases. It is expected that not all these enzymes will be involved in the degradation of arabinogalactan; some of them will be involved in the degradation of arabinoxylans, because the genome sequence also reveals the presence of putative endo-xylanases (70). Since the exact substrate specificity of the arabinosidases and arabinofuranosidases is unknown, all enzymes annotated as such have been included in the table. For each enzyme the presence of transmembrane domains (TmD) and/or a signal peptide (SignP) has been indicated, which was based on sequence analysis using SignalP (53) (<http://www.cbs.dtu.dk/services/SignalP-2.0/>), PSORT (52) (<http://psort.nibb.ac.jp/>), and SOSUI (30) (http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html).

Remarkably, only a few enzymes contained a signal peptide, and it is therefore suggested that most (arabino)galactan and galacto-oligosaccharide degrading enzymes are located intracellularly. Only three enzymes were predicted to have an “endo” working mechanism.

This indicates that *B. longum* has probably a preference for oligosaccharides, which are transported into the cell for further utilization. This is confirmed by the presence of several putative oligosaccharide transporters annotated in the genome of *B. longum* (70). Also preliminary results of *B. breve* UCC2003 revealed a variety of oligosaccharide transporters (90). Examples of carbohydrate import by *Bifidobacterium* described in some detail include that of glucose and arabinose in *B. breve* (13), lactose, glucose, and galactose in *B. bifidum* (43, 44), lactose and glucose in *B. longum* (40), and of galacto-oligosaccharides (24) and sucrose in *B. lactis* (83). This indicates that bifidobacteria are very well adapted for the utilization of especially oligosaccharides in the colon.

Table 1.1: Overview of (arabino)galactan and galacto-oligosaccharide modifying enzymes from *Bifidobacterium longum* NCC2705

Sugar modifying enzyme	BL-number	GH Family	R/I ^a	TmD & Position TmD (aa)	SignP & Position SignP (aa)
Endo- β -galactosidase or galactanase	257	53	R	Yes, 871-892	Yes, 1-30
Endo- α -L-arabinosidase	182	43	I	No	No
Endo- α -L-arabinosidase	183	43	I	No	Yes, 1-31
α -Arabinofuranosidase	181	51	R	No	No
α -L-Arabinosidase	1611	51	R	No	No
α -L-Arabinosidase	544	51	R	No	No
α -L-Arabinofuranosidase	1166	51	R	No	No
Exo- α -L-arabinosidase	187	43	I	No	No
α -Galactosidase	1518	36	R	No	Yes/ No ^b , 1-37
α -Galactosidase	177	27	R	No	No
β -Galactosidase	259	42	R	No	No
β -Galactosidase	978	2	R	No	No
β -Galactosidase	1168	42	R	No	No

^a Catalytic mechanism: R is retaining, I is inverting

^b With SOSUI a signal peptide was found from amino acid 1 to 37. With SignalP and PSORT no signal peptide was found.

CLASSIFICATION OF CARBOHYDRASES

Enzymatic hydrolysis of glycosidic bonds in oligo- and polysaccharides is carried out with one of two stereo chemical outcomes: net retention or net inversion of the anomeric configuration. Glycosidases are classified as either retaining or inverting as first proposed by Koshland (42). Retaining glycoside hydrolases have a double displacement (S_N1) mechanism (74) involving a glycosyl-enzyme intermediate. The retaining enzyme has two carboxylic acids in the catalytic center. One carboxylic acid acts first as an acid catalyst and protonates the glycosidic oxygen, while the other carboxylic acid acts as nucleophile and assists departure of the leaving group (Figure 1.2A). This is the glycosylation step and a glycosyl-enzyme intermediate is formed. Subsequently, the first carboxylic acid will behave as a base catalyst and activate the incoming nucleophile (water), resulting in the hydrolysis of the glycosyl-enzyme intermediate (deglycosylation step). The product formed has the same stereochemistry as the substrate. Generally, the distance of the carboxylic acids is approximately 5.5 Å in retaining enzymes (9, 48, 91). Inverting glycoside hydrolases have a single displacement (S_N2) mechanism (74) and have different carboxyl acids acting as acid and base (Figure 1.2B). In this case the protonation of the glycosidic oxygen and departure of the leaving group are accompanied by a concomitant attack of a water molecule, which is activated by the carboxylic base catalyst. The product has the opposite stereochemistry as the substrate (9, 48, 91). The distance of the carboxylic acids is approximately 6.5-9.5 Å in inverting enzymes (48).

For retaining enzymes the incoming nucleophile can be a sugar molecule instead of water. This can lead to the formation of oligosaccharides with a higher degree of polymerization or containing a new linkage type. Such reactions are called transglycosylation (Figure 1.3). Inverting enzymes do not have the capability to synthesize oligosaccharides. An example of a transglycosylating enzyme, derived from bifidobacteria, was described by Dumortier et al. (16). They showed that one of the β -D-galactosidases from *B. bifidum* with a high transgalactosylation activity was able to synthesize transgalacto-oligosaccharides (TOS), whereas other purified β -D-galactosidases were not able to produce TOS under the same conditions.

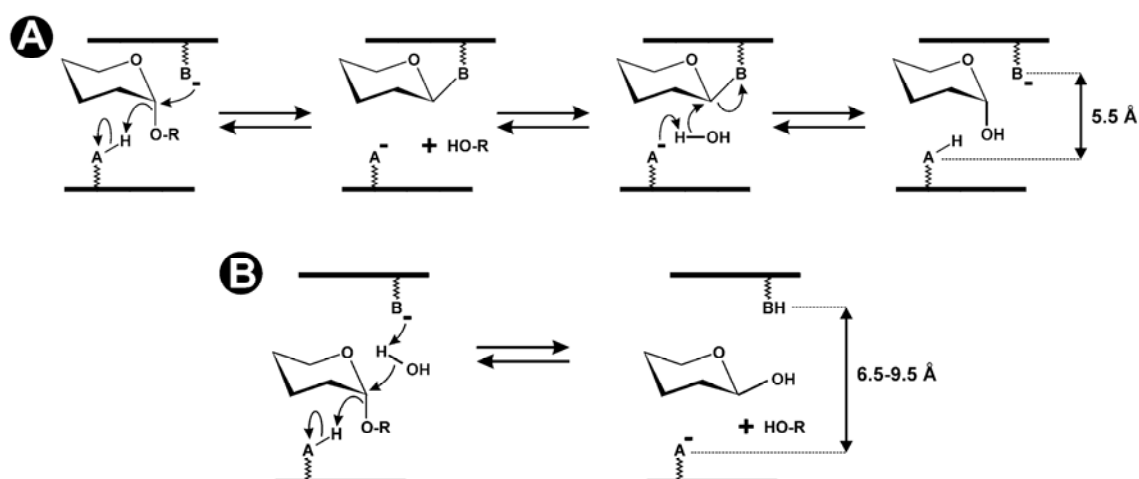


Figure 1.2: The two mechanisms of enzymatic glycosidic bond hydrolysis. **(A)** The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and assistance to the leaving group is provided by the nucleophile (B^-). The resulting glycosyl-enzyme intermediate is hydrolyzed by a water molecule. **(B)** The inverting mechanism, in which protonation of the glycosidic oxygen and departure of the leaving group are accompanied by a concomitant attack of a water molecule that is activated by the base residue (B^-).

In 1991 Henrissat (25) introduced a classification of glycoside hydrolases based on their amino acid sequence similarities. Glycoside hydrolases with a high degree of sequence homology were assigned to the same glycoside hydrolase family (GH family). This classification has predictive value with respect to the structural features (fold) of the enzymes, the evolutionary relationship between the enzymes, and catalytic mechanism (25, 26). This classification system is complementary to the International Union of Biochemistry enzyme nomenclature (EC numbers), which is based on substrate specificity of the enzyme (IUB 1984). The database is regularly updated (26, 27) and available at internet (<http://afmb.cnrs-mrs.fr/CAZY/index.html>). Currently, more than 88 GH families are known.

Enzymes in one GH family can have a different substrate specificity but also a different mode of action (endo or exo). However, it appeared that within a GH family the catalytic mechanism, retaining or inverting, was conserved (20). The catalytic residues are also conserved within a GH family (27) as well as the protein fold (9, 29). Although only limited information is available about the 3D structures of enzymes from bifidobacteria (38, 76), the 3D structure of more than 51 GH families is known. Some of the GH families are grouped into clans and they have a common ancestry and significant similarities in their tertiary

structure. Within a clan the catalytic residues and the catalytic mechanism are conserved (27). The information in the database can be used to compare the deduced amino acid sequence of genes or for sequenced genomes for searching homologies with similar carbohydrases.

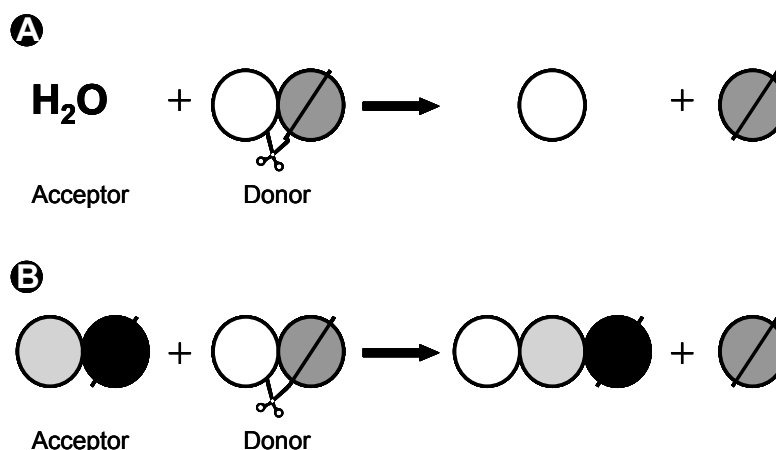


Figure 1.3: Schematic representation of hydrolysis (A) and transglycosylation (B).

AIM AND OUTLINE OF THE THESIS

Galacto-oligosaccharides are known for their prebiotic potential, and commercial products containing these are available. So far, not much is known about the enzymic machinery of bifidobacteria for galactan utilization. For this, the galactan-modifying enzymes of bifidobacteria are of interest to us. Understanding the mechanism of galactan and galacto-oligosaccharide degradation may assist in the development of better prebiotics. The aim of this research was to investigate this galactan utilization mechanism. Three galactan/galacto-oligosaccharide modifying enzymes were characterized to unravel this mechanism. The first enzyme, a β -galactosidase from *B. adolescentis*, was hardly active towards lactose. The second enzyme, an endo-galactanase from *B. longum*, was located extracellular and anchored to the cell membrane. The third galacto-oligosaccharide modifying enzyme, α -galactosidase from *B. adolescentis*, possessed a high transglycosylation activity. This latter enzyme was used for the synthesis of oligosaccharides. Subsequently, the enzyme was optimized for oligosaccharide synthesis by site directed mutagenesis.

As described before, galactans and arabinogalactans are plant polysaccharides, which can be divided in two groups, i.e. Type I and Type II. The structure of these arabinogalactans seem rather well established (2, 6, 78, 85). However, occasionally new structural elements are found in these polysaccharides (33). This suggests that the structural variation within the

arabinogalactans is larger than expected. In chapter 2, we add an example to arabinogalactan complexity, and report a new structural element to type I arabinogalactan.

For the degradation of galacto-oligosaccharides, bifidobacteria contain several β -galactosidases, which all have different properties (35, 51, 89). Van Laere et al. (89) found two β -galactosidases from *B. adolescentis*. One of these enzymes was not able to hydrolyze lactose, in contrast to other known β -galactosidases from bifidobacteria (16, 34, 51, 60). It may be expected that the various β -galactosidases provide bifidobacteria with flexibility in utilizing different types of galactans or galacto-oligosaccharides. In chapter 3 the β -galactosidase from *B. adolescentis*, as described by Van Laere et al. (89), was cloned and further characterized with respect to substrate specificity.

From fermentation studies it is known that *B. longum* can utilize arabinogalactan (8, 14, 67). The genome sequence of *B. longum* indicates that this organism is equipped with a membrane-bound, extracellular ‘endo- β -galactosidase or galactanase’ (Table 1.1), besides several β -galactosidases. The exact mechanism of action of this enzyme remains to be established, since the prefix “endo” suggests that the enzyme acts randomly on galactans, whereas the ending “osidase” suggests an exo mode of action. The fact that the enzyme is classified in GH family 53 does not give information about its mode of action. This ‘endo- β -galactosidase or galactanase’ was cloned and characterized for its mode of action and substrate specificity, as described in chapter 4.

Several glycoside hydrolases from bifidobacteria are found to possess transglycosylation activity (16, 51, 86). An α -galactosidase from *B. adolescentis* with transglycosylation activity was isolated, cloned and expressed in *Escherichia coli* cells (46, 86, 88). The enzyme seemed to have potential for elongating non-digestible oligosaccharides (86, 88). Using enzymes from Bifidobacteria itself for generating non-digestible oligosaccharides may ensure that these carbohydrates are indeed degraded in the colon (56). Besides transglycosylation activity, α -galactosidase from *B. adolescentis* also has hydrolysis activity, which interferes with the synthesis activity. In chapter 5, the transglycosylation activity of this enzyme is characterized, and with site-directed mutagenesis amino acids close to the catalytic site of the enzyme are modified to obtain a higher transglycosylation activity, so that it will become more suitable for preparing prebiotics.

Finally, in chapter 6, the main findings are summarized and discussed in the context of the aims, literature and future perspectives.

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Type I arabinogalactan contains β -D-Galp-(1 \rightarrow 3)- β -D-Galp structural elements

Arabinogalactan type I from potato was partially degraded by endo-galactanase from *Aspergillus niger*. High-performance anion-exchange chromatography revealed that several of the oligomeric degradation products eluted as double peaks. To investigate the nature of these products, the digest was fractionated by Bio-Gel P2 chromatography. The pool that contained tetramers was treated with a (β 1 \rightarrow 4) specific galactosidase from *Bifidobacterium adolescentis* to obtain a dimer with deviating linkage type, which was further purified by BioGel P2 chromatography. By obtaining all ^1H and ^{13}C chemical shifts and the presence of intra residual scalar coupling (HMBC) it could be concluded that the dimer contained a (β 1 \rightarrow 3) linkage instead of the expected (β 1 \rightarrow 4)-linkage. Using the same NMR techniques as for the dimer, it was found that the pool of tetramers consisted of the following 2 galactose tetramers: β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) α / β -Galp-OH and β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 3) α / β -Galp-OH. The fact that the deviating (β 1 \rightarrow 3)-linked galactose was found at the reducing end of the tetramer showed that this deviating linkage is present within the backbone. The (β 1 \rightarrow 3)-galactosyl interruption appeared to be a common structural feature of type I arabinogalactans with a frequency ranging from approximately 1 in 160 (potato, soy, citrus) to 1 in 250 (onion).

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INTRODUCTION

Galactans and arabinogalactans are plant polysaccharides (7, 8, 10, 18), that are covalently bound to rhamnogalacturonan type I (11) or non-covalently bound as hemicelluloses (13). They can also occur as a side chain of protein (arabinogalactan-protein, AGP) (10), or as a polysaccharide present in exudate gums, not linked to other constituents (7, 18). Generally, two types of arabinogalactans are distinguished. Type I arabinogalactan contains a (β 1 \rightarrow 4)-linked galactan backbone, which can be substituted with L-Araf at the O3 position (7, 18). Type I arabinogalactans are found linked to rhamnogalacturonan I regions of pectic molecules (13, 17), for example, in potato. Linkage analysis by permethylation showed that 64% of the linkages in potato arabinogalactan are (β 1 \rightarrow 4)-Galp-linkages (19).

In comparison to type I arabinogalactans, type II arabinogalactans are more widespread in plants (7). The structural basis of this type of arabinogalactans is a backbone of β -galactopyranose residues that are predominantly (1 \rightarrow 3)-linked. Type II arabinogalactans are most frequently branched with D-Galp, L-Araf, L-Rhap, D-GlcpA, and D-GalpA, although other side chains such as D-Manp, D-Xylp, and D-Glcp are also possible (18). Only minor amounts of (β 1 \rightarrow 3)(β 1 \rightarrow 6)-linked galactan are found within potato galactans (16).

Although the structure of arabinogalactans seems rather well established (2, 7, 18, 19), occasionally new structural elements are found in these polysaccharides. Huisman et al. (15) showed the presence of (1 \rightarrow 5)-Araf residues in the backbone, and terminal Arap residues at the galactosyl side chains, of type I arabinogalactans from soy. This suggests that the structural variation within the arabinogalactans is larger than expected. In this paper, we add another example to arabinogalactan complexity and report a new structural element to type I arabinogalactan.

MATERIAL AND METHODS

Materials

Potato, soybean, onion, and citrus arabinogalactan were obtained as described by Van de Vis (19). The endo-galactanase from *Aspergillus niger* was purified by Van de Vis et al. (20). The β -galactosidase from *Bifidobacterium adolescentis* DSM 20083 was cloned and purified by

Hinz et al. (14). A (β 1→4)-galacto-disaccharide standard was purchased from Megazyme International Ireland Ltd. (Bray, Ireland).

Enzymatic degradation of potato arabinogalactan

A solution of 100 mg/ml potato arabinogalactan in 10 mM NaOAc buffer pH 5 was partially digested with an endo-galactanase from *Aspergillus niger* (26.4 μ g, 26 U) for 384 hours at 30°C, while continuously mixed. The incubation was stopped by heating for 10 min at 100°C and insoluble material was removed by centrifugation (5 min at 10,000 x g). The degradation was followed by high-performance anion-exchange chromatography (HPAEC) and high-performance size-exclusion chromatography (HPSEC).

Fractionation of arabinogalacto-oligosaccharides

The arabinogalacto-oligosaccharides were separated from the polymeric material by fractionation on a Bio-Rad Bio-Gel P2 (200-400 mesh, Richmond, USA) size-exclusion column (100 x 2.6 cm), using an Amersham Biotech Hiload system (Little Chalfont, UK). Components were eluted with millipore water at 60°C (flow rate was 0.5 ml/min) and monitored by refractive index detection using a Shodex RI-72 detector (Kawasaki, Japan). Fractions (5 ml) were collected and appropriate fractions were pooled as indicated in Figure 2.2.

Analysis of sugar composition

The sugar composition of the various Bio-Gel P2 pools was determined using methanolysis according to de Ruiter et al (9). For this, the different pools were treated with 2N HCl in dry methanol for 16 h at 80°C, followed by 1 h of 2N CF₃CO₂H (TFA) at 121°C. The released sugars were quantified using HPAEC.

Enzymatic degradation of arabinogalacto-oligosaccharides

Bio-Gel P2 pools III, IV, and V were incubated with 0.015 U of the β -galactosidase β -Gal II from *B. adolescentis*. The incubations were carried out in 20 mM phosphate buffer, pH 6 at 37°C for 60 min, and stopped by heating for 10 min at 100°C. Insoluble material was removed by centrifuging (10 min at 10,000 x g). The reactions were analyzed by HPAEC.

End-point degradation of arabinogalactans

A solution of 1 mg/ml arabinogalactan in 50 mM NaOAc buffer pH 5 was incubated with the endo-galactanase from *A. niger* (0.14 µg, 0.26 U) for 24 hours at 30°C, under continuous stirring. The total volume of the incubation was 100 µl. The incubation was stopped by heating for 10 min at 100°C, and insoluble material was removed by centrifugation (10 min at 10,000 x g). The incubation was monitored by HPAEC.

Estimation of the (β1→3)/(β1→4) ratio in arabinogalactan from different sources

50 µl of the end-point incubations were incubated with the β-galactosidase β-Gal II from *B. adolescentis* (0.15 µg, 0.012 U). The volume of the reaction mixtures was adjusted to 100 µl with 20 mM phosphate buffer pH 6, and incubated at 37°C for 60 min. The incubations were stopped by heating for 10 min at 100°C, and insoluble material was removed by centrifuging (10 min at 10,000 x g). Reaction products were determined by HPAEC analysis.

Analytical methods

High-performance size-exclusion chromatography was performed on three TSKgel columns (7.8 mm ID x 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. Calibration was performed using dextrans.

High-performance anion-exchange chromatography was performed on a Thermo-Quest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column in combination with a Dionex CarboPac PA guard column (3 mm ID x 25 mm) and a Dionex ED40 PAD-detector (Dionex, Sunnyvale, USA). A flow rate of 1 ml/min was used.

Arabinogalacto-oligosaccharides were determined using a gradient of sodium acetate in 100 mM NaOH: 0-40 min, 0-400 mM NaOAc in 100 mM NaOH; 40-41 min, 400-1,000 mM NaOAc in 100 mM NaOH; 41-46 min, 1000 mM NaOAc in 100 mM NaOH; 46-60 min, 100 mM NaOH.

The determination of the sugar composition was performed using the same HPAEC system equipped with a Dionex CarboPac PA20 (3 mm ID x 150 mm) in combination with CarboPac PA20 guard column (3 mm ID x 30 mm) and Borate trap (4 mm ID x 50 mm) and separated

at 0.5 mL/min by isocratic elution with 3 mM NaOH for 20 min followed by applying a linear gradient from 0 to 700 mM NaOH in 20 min.

MALDI-TOF mass spectrometry

For MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) an Ultraflex workstation (Bruker Daltronics, Germany) was used. The mass spectrometer was calibrated with a mixture of malto-dextrins (mass range 365-2309). The samples were mixed with a matrix solution (1 μ l 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:water (300 μ l: 700 μ l). 2 μ l of the prepared sample and matrix solutions was put on a gold plate and dried with warm air.

^{13}C and ^1H NMR

Prior to NMR analyses, the samples were exchanged in 99.96% D₂O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D₂O (Cambridge Isotope Laboratories). NMR spectra were recorded at a probe temperature of 25°C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to internal acetone: δ = 2.225 ppm for ^1H and δ = 31.55 ppm for ^{13}C .

The 1 D ^1H proton spectra were recorded at 500.13 MHz using 8 scans of 8192 data points and a sweep width of 3000 Hz. The 1 D ^{13}C proton decoupled carbon spectra were recorded at 125.77 Hz using 100000 scans of 32768 data points and a sweep width of 31250 Hz.

The 2D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.

2D TOCSY spectra were acquired using standard Bruker pulse sequences with 110ms mixing time, respectively. For all homonuclear 2D spectra 512 experiments of 2048 data points were recorded using 32 scans per increment.

For the 2D HMBC spectrum (4) a standard gradient enhanced 2D- HMQC pulse sequence delivered Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms. For the HMBC experiment 1024 experiments of 2048 data points were recorded with 128 scans per increment.

RESULTS

Partial degradation of potato arabinogalactan

Arabinogalactan from potato was partially digested by endo-galactanase from *A. niger* to obtain (arabino)galacto-oligosaccharides of various chain lengths. The degradation was monitored by HPSEC and HPAEC analysis and the reaction was stopped after the formation of a broad range of oligosaccharides. It is emphasized that these oligosaccharides in the digest are not limit-digest products.

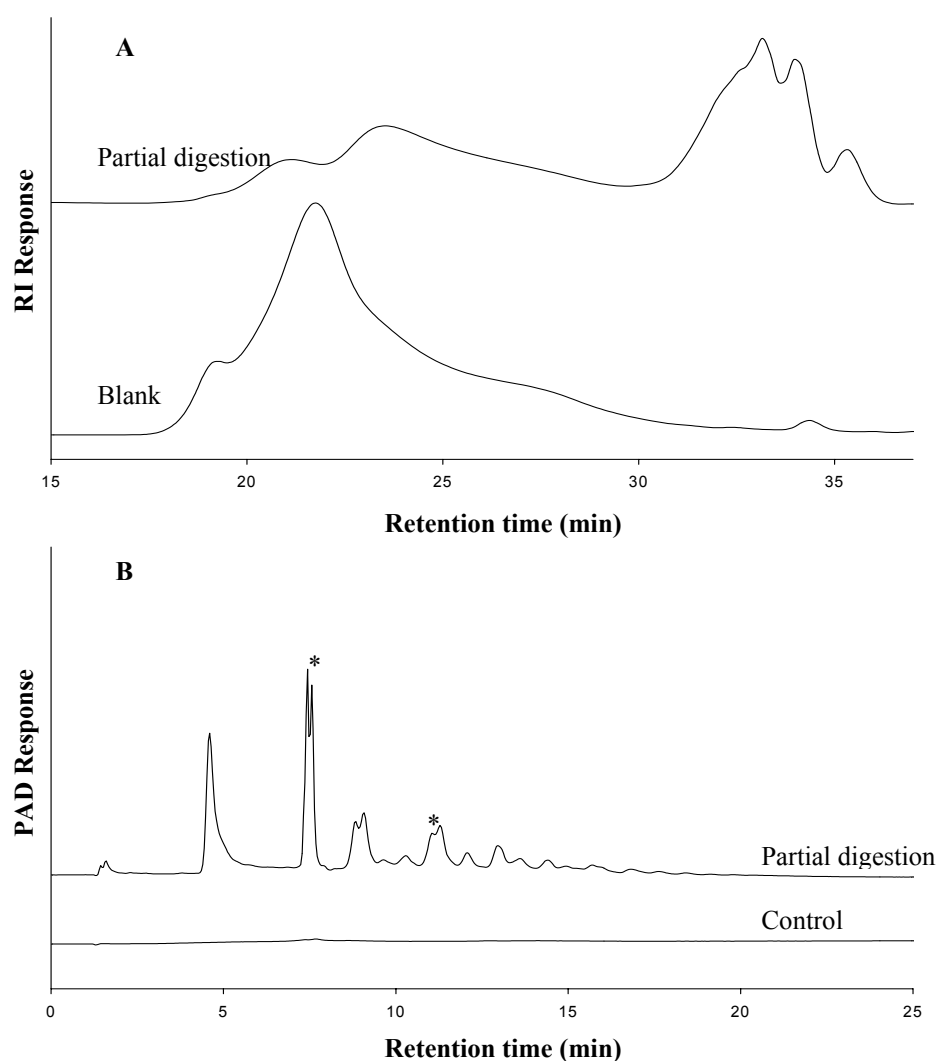


Figure 2.1: HPSEC (A) and HPAEC (B) profiles of partial degradation of potato arabinogalactan by an endo-galactanase from *A. niger*. * double peak.

HPSEC analysis showed a shift from a polymeric fraction to a mixture of oligomers. Part of the starting material was not degraded by the endo-galactanase, as shown in the HPSEC profile (Figure 2.1A). Interestingly, several oligomers seemed to elute as double peaks upon HPAEC analysis (Figure 2.1B). To assign the (β 1 \rightarrow 4)-galacto-disaccharide in the chromatogram of the digest, a commercially available (β 1 \rightarrow 4)-galacto-disaccharide derived from potato arabinogalactan was analyzed on HPAEC with the same gradient. Typically, this (β 1 \rightarrow 4)-galacto-disaccharide showed the same double peak at 8.2 and 8.7 min as observed in the digest described above.

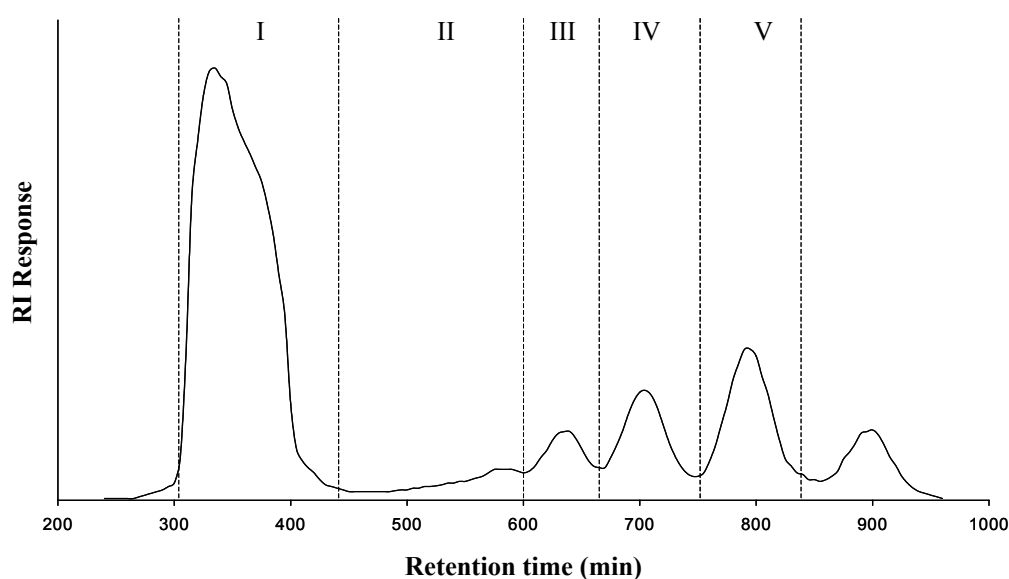


Figure 2.2: Bio-Gel P2 size-exclusion chromatography profile of arabinogalacto-oligosaccharides from the partial degradation of potato arabinogalactan by an endo-galactanase from *A. niger*. I: Pool of larger galactooligomers and undigested material, II: Pool of galactooligomers with a dp of 5-9, III: Pool of galactooligomers with a dp of 4, IV: Pool of galactooligomers with a dp of 3, V: Pool of galactooligomers with a dp of 2.

Fractionation of (arabino)galacto-oligosaccharides.

To investigate the nature of the products eluting in these double peaks, the digest of potato arabinogalactan was fractionated by Bio-Gel P2 chromatography. Fractions were pooled as indicated and characterized for the presence of various (arabino)galacto-oligosaccharides (Figure 2.2) by HPAEC and MALDI-TOF MS analysis. The monosaccharide compositions of the native potato arabinogalactan and the five pools were determined using HPAEC after methanolysis (Table 2.1).

Table 2.1: Sugar composition of potato arabinogalactan (PG) and fractions thereof.

Fraction	Monosaccharide composition (mol%)						Carbohydrate content ¹⁾	Gal/Ara
	Fuc	Rha	Ara	Gal	Glc	GalA		
PG	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
I	0.8 ± 0.0	15.3 ± 0.8	27.1 ± 0.3	30.3 ± 0.3	1.2 ± 0.1	25.4 ± 0.8	57.9 ± 2.8	1.1
II	2.0 ± 0.4	-	9.0 ± 0.7	88.5 ± 0.3	0.6 ± 0.0	-	89.1 ± 5.3	9.8
III	-	-	5.9 ± 0.1	94.1 ± 0.1	-	-	97.6 ± 3.6	16
IV	-	-	2.5 ± 0.2	97.5 ± 0.2	-	-	94.5 ± 2.9	39
V	-	-	-	100.0 ± 0.0	-	-	96.5 ± 2.9	n.a. ²⁾

¹⁾Expressed as % w/w.²⁾n.a.= not applicable.

Because of the presence of galacturonic acid and rhamnose, it was concluded that the potato arabinogalactan contained some pectic material. Pool I also contained this pectic material, besides arabinogalactan material with a degree of polymerisation of 10 and higher, as determined by MALDI-TOF MS (data not shown). In pools II, III, and IV mainly galactose and a small amount of arabinose was found. The Gal/Ara-ratio was calculated for the different pools (Table 2.1), and showed a shift from 1.1 in pool I to 39 in pool IV. This suggested that the endo-galactanase poorly degrades arabinosyl-rich regions of the arabinogalactan. Pool II contained different types of arabinogalacto-oligosaccharides with a degree of polymerisation of 5-9; examples of oligosaccharides present in this fraction are Gal₄Ara, Gal₅, Gal₄Ara₂, Gal₅Ara, Gal₆, Gal₅Ara₂, as visualized by Maldi-TOF MS (Figure 2.3). Pool III, IV and V contained tetrasaccharides, trisaccharides and disaccharides, respectively. Pool V contained the disaccharides with 100% galactose, suggesting that the double peaks observed in the HPAEC elution profile could be derived from two galactosyl dimers with different linkage composition.

Characterization of the dimer, trimer and tetramer fraction

To examine the presence of different linkage types in the galacto-oligosaccharides, the pools III, IV and V were incubated with a β -D-Galp-(1→4)- β -D-Galp-specific β -galactosidase from *Bifidobacterium adolescentis* (14). The reaction products were analyzed by HPAEC. Only one of the two disaccharides in the dimer fraction (pool V) was converted to galactose (Figure 2.4A). This suggested that the disappearing oligomer was the (β 1→4)-linked galacto-disaccharide, whereas the other dimer may contain a different linkage type. Digestion of the

trimeric (Figure 2.4B) and tetrameric (Figure 2.4C) fractions yielded galactose and the same indigestible galacto-disaccharide. This result showed that the tri- and tetramers contained only one linkage-type differing from (β 1 \rightarrow 4). Dimer pool V, digested by the β -galactosidase from *B. adolescentis*, was fractionated by Bio-Gel P2 size-exclusion chromatography to obtain the purified, non-digested galacto-disaccharide. To unambiguously prove the presence of two galactose dimers with different linkage types, the purified dimer was subjected to NMR analysis.

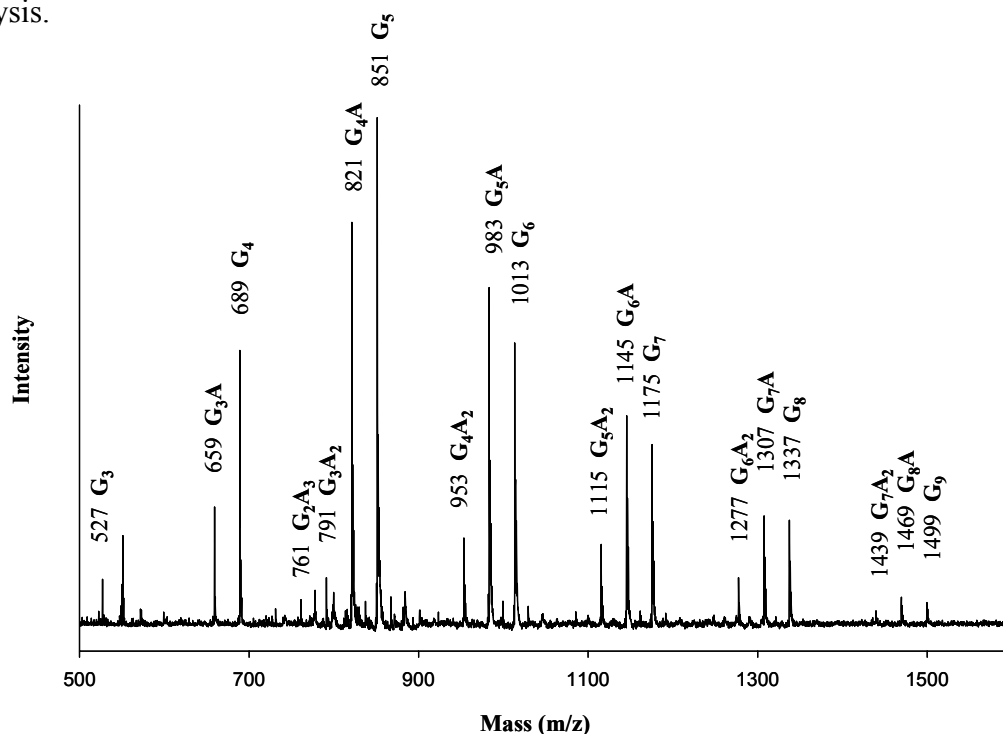


Figure 2.3: MALDI-TOF mass spectrum of Bio-Gel P2 pool II after fractionation of partially digested potato arabinogalactan. Masses of the sodium-adducted galacto-oligosaccharides are indicated (G = galactose; A = arabinose).

NMR analysis of the indigestible galacto-disaccharide

By recording a 2D homonuclear COSY and TOCSY spectrum it was possible to assign all the ^1H chemical shifts as shown in Table 2.2. From the differences with the values known for both α and β O-methyl-galactose (1), it was apparent that the oligomer consisted of two galactosyl residues connected by a (1 \rightarrow 3)-linkage, because proton 2, 3 and 4 of the reducing end moieties (**A** and **B**) showed a significant downfield glycosylation shift. The fact that also proton 2 showed a significant downfield shift, which is far less within a 4-linked galactose (3), indicated the presence of a (1 \rightarrow 3)-linkage.

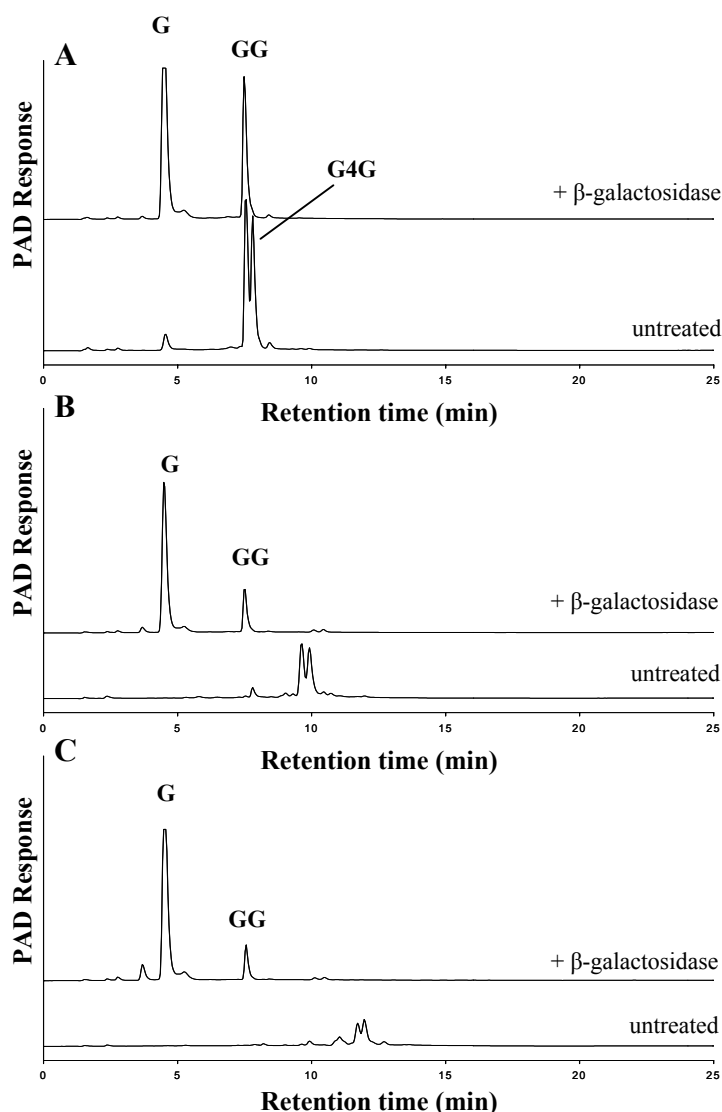


Figure 2.4: HPAEC profile for the Bio-Gel P2 pools V (A), IV (B), and III (C) after digestion with a β -D-Galp-(1 \rightarrow 4)- β -D-Galp-specific β -galactosidase from *Bifidobacterium adolescentis*. **G**: galactose; **GG**: galactodisaccharide with unknown linkage-type and **G4G**: β -D-Galp-(1 \rightarrow 4)- β -D-Galp.

The presence of this linkage type was further substantiated by the assignment of the ^{13}C chemical shifts (Table 2.2) from the 2D heteronuclear HMBC spectrum recorded. The inter-residue coupling of C-1 and H-1 of the terminal galactosyl residue (**C**) with C-3 and H-3 of both the α and β reducing end galactose (**A** and **B**), which is indicated in the segment shown in Figure 2.5A, proves the presence of a (β 1 \rightarrow 3)-linked galactose dimer. These findings already explain the double peak found in the HPAEC elution profiles shown in Figure 2.1B. It is suggested that the double peak in the HPAEC profile of the commercial preparation is also due to a (β 1 \rightarrow 3)-linked galactose dimer. The presence of this new linkage type in type I potato arabinogalactans raised the question whether the (β 1 \rightarrow 3)-linked galactosyl residue is the point of galactosyl side chain attachment or whether the new structural element is an integral part of the (β 1 \rightarrow 4)-linked backbone.

Table 2.2: ¹H and ¹³C chemical shifts of (β1→3) galactose dimer at 25°C relative to internal acetone at 2.225 ppm or 31.55 ppm, respectively.

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)	H-6'
A →3)α-Galp-OH	5.28 0.06 ²⁾ (93.6)	3.97 0.19 (68.9)	3.99 0.18 (81.0)	4.26 0.31 (70.5)	4.11 0.08 (71.6)	3.72 0.03 (62.5)	3.72 0.03 -
B →3)β-Galp-OH	4.63 0.1 (97.6)	3.65 0.20 (72.3)	3.79 0.20 (84.0)	4.2 0.31 (69.9)	3.73 0.08 (76.2)	3.76 ¹⁾ 0.12 (62.4)	3.76 ¹⁾ 0.041 -
C β-Galp(1→	4.6 0.07 (105.8)	3.62 0.17 (72.7)	3.67 0.08 (73.9)	3.94 0.05 (70.0)	3.69 0.04 (76.4)	3.77 ¹⁾ 0.13 (62.2)	3.77 ¹⁾ 0.05 -

¹⁾ Might be interchanged.

²⁾ Difference with values found for α and β O-methyl- galactose (1).

To examine this, similar NMR spectra were recorded of the mixture of tetramers present in pool III. Figure 2.4C indicated that one of these tetramers contained only (1→4)-linkages whereas the other contained a deviating linkage. From the COSY, TOCSY and HMBC spectra most of the different residues could be assigned until proton 5 and carbon 5 (Table 2.3). Only 2 terminal residues could be found as indicated by residue **G** and **F** in Table 2.3. These residues could be separated from the other residues due to the fact that none of these proton and carbon resonances show a considerable downfield shift when compared to the terminal residue of the dimer shown in Table 2.2. The fact that only 2 terminal residues could be found already indicates that the (1→3)-linked galactose is present in the backbone of the tetramer. The proton chemical shifts of both reducing galactosyl residues in their α anomeric configuration could be assigned completely as can be seen from Table 2.3 residues **A** and **B'**. The proton chemical shifts of residue **A** were the same as the ones found for the α reducing end of the dimer measured before. This together with the fact that residue **B'** showed a significantly less downfield shift of proton 2, demonstrated that residue **A** belongs to a reducing end derived from a (1→3)-linked galactose and **B'** is the α anomer of the (1→4)-linked reducing galactose. Actually, if residue **A** would be derived from a (1→3),(1→4)-linked galactose, a far larger downfield shift for proton 4, 3 and 2 would be expected (3). The chemical shifts of the (β1→3)-linked galactose (**D**) were also the same as the ones found for the same residue in the dimer (residue **B** Table 2.2). By recording 2D heteronuclear HMBC spectrum, see segment Figure 2.5B, further evidence for a (1→3)-linked galactose at the reducing end of the second tetramer could be obtained. In Figure 2.5B the carbon 3 signals of

both the ($\alpha 1 \rightarrow 3$) and ($\beta 1 \rightarrow 3$)-linked galactose ($\delta=80.8$ and 84.0 ppm) could be clearly separated from the carbon 4 signals of the ($1 \rightarrow 4$)-linked galactosyl residues around $\delta=79$ ppm.

Table 2.3: ^1H and ^{13}C chemical shifts of the mixture of 2 galactose tetramers at 25°C relative to internal acetone at 2.225ppm or 31.55ppm, respectively.

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)	H-6'
A $\rightarrow 3$) α -Galp-OH	5.28 0.06 ²⁾ (93.7 ¹⁾)	3.99 0.21 (68.7)	3.99 0.18 (80.8)	4.23 0.28 (70.5)	4.12 0.09 (71.4)	3.72 0.03 (61.8)	3.72 0.03 -
B' $\rightarrow 4$) α -Galp-OH	5.27 0.05 (93.7 ¹⁾)	3.89 0.11 (nd)	3.97 0.16 (74.8)	4.22 0.27 (80.2)	4.12 0.09 (71.2)	3.82 ¹⁾ 0.13 (61.8)	3.75 0.06 -
C $\rightarrow 4$) β -Galp-($1 \rightarrow$	4.66 0.13 (105.8)	3.68 0.23 (72.1)	3.78 0.19 (72.8)	4.19 0.30 (78.8, 78.5 ³⁾)	3.73 0.08 (75.8)	nd - (62.0)	nd - -
D $\rightarrow 3$) β -Galp-OH	4.63 0.1 (97.5)	3.65 0.20 (72.3)	3.81 0.22 (84.0)	4.18 0.29 (70.1)	3.73 0.08 (75.6)	nd (62.0)	nd -
E $\rightarrow 4$) β -Galp-OH	4.61 0.08 (97.8)	3.57 0.12 (73.5)	3.74 0.15 (74.0)	4.18 0.29 (79.1)	3.73 0.08 (75.6)	nd (62.0)	nd -
F β -Galp($1 \rightarrow$	4.59 0.06 (105.7)	3.59 0.14 (72.7)	3.65 0.06 (74.2)	3.9 0.01 (69.9)	3.68 0.03 (76.5)	nd (62.0)	nd -
G β -Galp($1 \rightarrow$	4.58 0.05 (105.7)	3.62 0.17 (72.7)	3.67 0.08 (74.2)	3.91 0.02 (69.9)	3.68 0.03 (76.5)	nd (62.0)	nd -

¹⁾ Might be interchanged.

²⁾ Difference with values found for α and β O-methyl- galactose (1).

³⁾ C4 = 78.5 ppm for both residues neighboring the terminal residues (G and F)

nd: not determined.

The ^{13}C chemical shifts of both residue **A** and **D** representing the α and β anomeric configuration of the ($1 \rightarrow 3$)-linked galactose, respectively, were the same as the ones found for the dimer shown in Table 2.2. Therefore the ($\beta 1 \rightarrow 3$)-linked galactose should be at the reducing end of the tetramer. Both the chemical shifts of the ($\alpha 1 \rightarrow 4$) and ($\beta 1 \rightarrow 4$)-linked galactose residue (**B'** and **E**) resembled the ones found in literature (1, 3, 5). All internal galactoses, residues **C**, show approximately the same shifts as the ($\beta 1 \rightarrow 4$)-linked galactose

residue except for their anomeric carbon and C-2. Due to extreme overlap, the different internal residues could not be distinguished.

However the 2nd and 3rd galactose within both tetramers could be separated by their C-4 chemical shift 78.5 ppm and 78.8 ppm, respectively. Both terminal residues (**G** and **F**) could be assigned due the up field signal of their carbon 4. From these results we concluded that the mixture consisted of the following 2 tetramers.

1. β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) α / β -Galp-OH

G/F
C
C
B'/E
2. β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 4) β -Galp(1 \rightarrow 3) α / β -Galp-OH

G/F
C
C
A/D

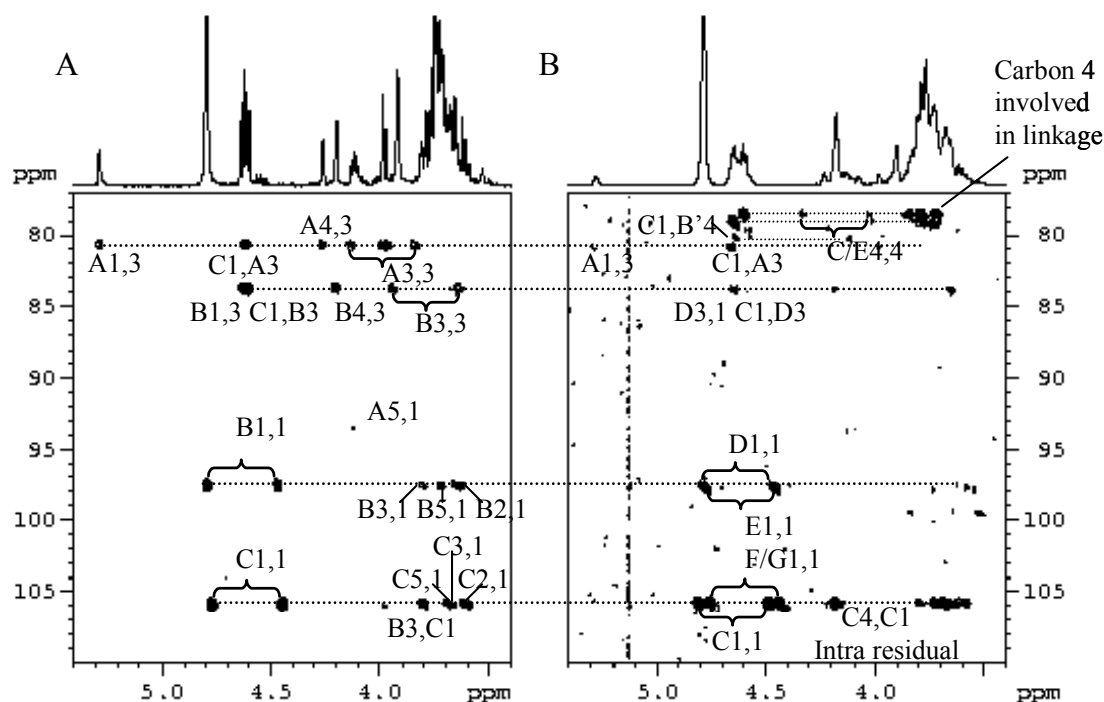


Figure 2.5: Segments of the 500-MHz 2D ^1H ^{13}C undecoupled HMBC spectrum of both the galactose dimer (A) and tetramer (B) recorded in D_2O at 70°C. 5A: **A** = \rightarrow 3) α -Galp-OH; **B** = \rightarrow 3) β -Galp-OH; **C** = β -Galp(1 \rightarrow . 5B: **A** = \rightarrow 3) α -Galp-OH; **B'** = \rightarrow 4) α -Galp-OH; **C** = \rightarrow 4) β -Galp-(1 \rightarrow ; **D** = \rightarrow 3) β -Galp-OH; **E** = \rightarrow 4) β -Galp **G/F** = β -Galp(1 \rightarrow . The code C1,1 stands for the coupling between **C** H-1 and C-1, C2,1 stands for the coupling between **C** H-2 and C-1 and B3, C1 stands for the long range coupling between **B** H-3 and **C** C1, etc.

Tetramer **2**, together with the known mode of action of endo-galactanase, showed that potato galactan not only contained (1→4)-linkages in its backbone, but also (1→3)-linkages. This is corroborated by the mode of action of the β -galactosidase from *B. adolescentis* towards this tetramer. Previous research showed that the β -galactosidase was not able to cleave the (β 1→4)-linkage of α -D-Galp-(1→3)- β -D-Galp-(1→4)- β -D-Galp, suggesting that the enzyme was only able to cleave (β 1→4)-linkages at the non-reducing end (14). Figure 2.4C showed that the β -galactosidase degraded tetramer **2** to galactose and (β 1→3)-galactobiose, confirming that the (β 1→3)-linkage was located at the reducing end of the oligosaccharide.

Estimation of the (β 1→4)/(β 1→3)-ratio in arabinogalactan from different sources.

To investigate if the new structural element is also present in other type I arabinogalactans and to estimate the (β 1→4)/(β 1→3)-ratio, arabinogalactans from different botanical sources, like potato, soybean, onion, and citrus were incubated with the endo-galactanase from *A. niger* to an end-point. These incubations were analyzed by HPAEC (Figure 2.6). It was found that all four arabinogalactans contained β -D-Galp-(1→3)- β -D-Galp, besides major products as arabinose, galactose, GalAra, and β -D-Galp-(1→4)- β -D-Galp. The end-point degradations of potato (Figure 2.6A), soybean (Figure 2.6B), and citrus arabinogalactans (Figure 2.6D) gave a similar HPAEC profile. The HPAEC profile of onion arabinogalactan showed an extra peak (Figure 2.6C*). The nature of this peak could not be revealed by digestion of the product with an arabinofuranosidase from *A. niger*, to liberate a possible arabinosyl residue, or a β -galactosidase from *A. niger*, which was able to degrade different types of galactosyl linkages. The end-point degradations were subsequently incubated with the β -D-Galp-(1→4)- β -D-Galp-specific β -galactosidase from *Bifidobacterium adolescentis* (14). The β -D-Galp-(1→4)- β -D-Galp was completely degraded to galactose, whereas the β -D-Galp-(1→3)- β -D-Galp was not. Based on these results, the amount of (β 1→3)- and (β 1→4)-linkages in the four different arabinogalactans was estimated, in which every liberated galactose represents a cleaved (β 1→4)-linkage (Table 2.4).

The highest amount of (β 1→3)-linkages was found in potato arabinogalactan. The (β 1→4)/(β 1→3) ratio was 163; the ratio for the other arabinogalactans was higher. The amount of (β 1→3)-linkages in arabinogalactan seems rather low, but it is possible that this amount is underestimated. The endo-galactanase seemed to be able to degrade (β 1→3)-

linkages (although at a slower rate), since the β -D-Galp-(1 \rightarrow 3)- β -D-Galp peak in the HPAEC diagram of the partially degraded arabinogalactan (Figure 2.1B) was approximately 4 times larger than that of the completely degraded arabinogalactan (Figure 2.6A). The ability of an endo-galactanase to degrade (β 1 \rightarrow 3)-linkages was also observed with an endo-galactanase from *Bifidobacterium longum* (unpublished results). Approximately 20% of the galactan backbone was substituted with arabinosyl side chains, which were only poorly degraded by the endo-galactanase. We did not take this part of the galactan into account when estimating the (β 1 \rightarrow 4)/(β 1 \rightarrow 3)-ratio. It is assumed that the (β 1 \rightarrow 3)-linkages are evenly distributed in the backbone, and consequently this ratio will not be affected.

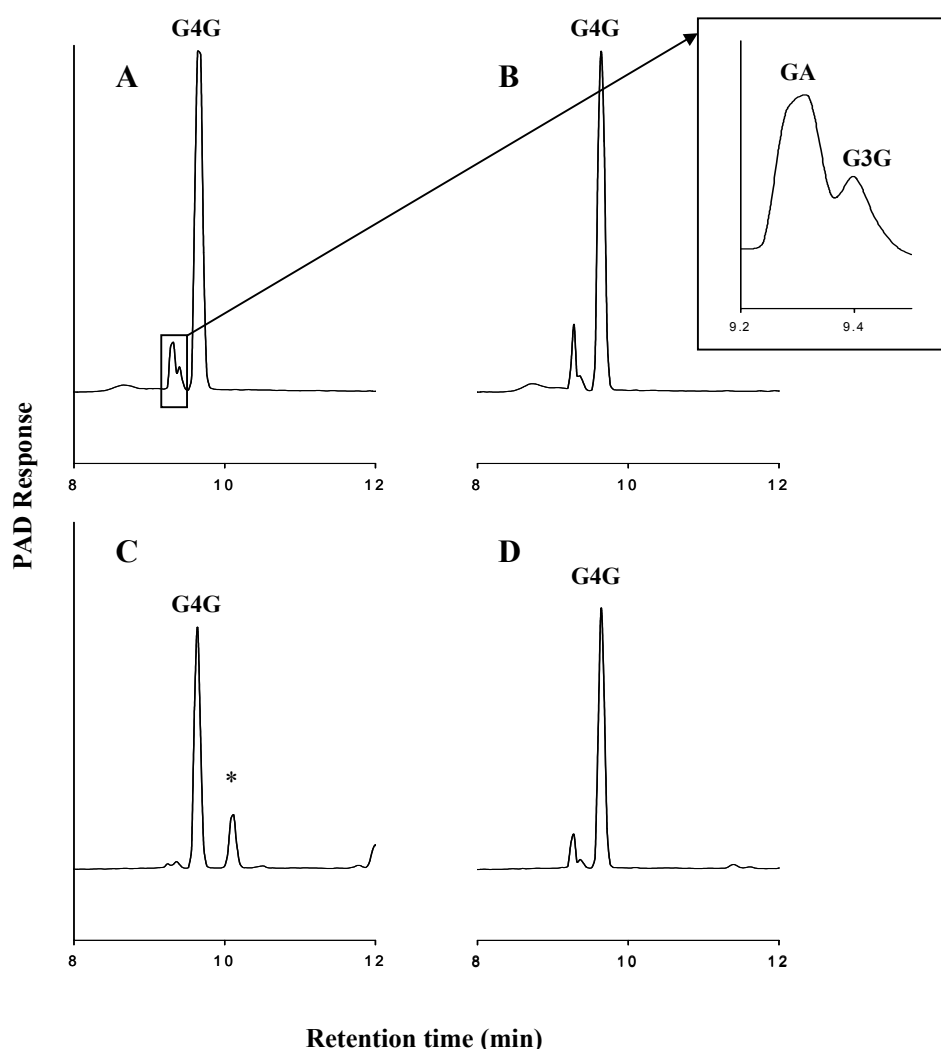


Figure 2.6: HPAEC profiles of the end-point degradation of arabinogalactans from different sources with endo-galactanase from *A. niger*. **A**: potato galactan; **B**: soybean galactan; **C**: onion galactan; **D**: citrus galactan. **G**: galactose; **A**: arabinose, **G4G**: β -D-Galp-(1 \rightarrow 4)- β -D-Galp; **G3G**: β -D-Galp-(1 \rightarrow 3)- β -D-Galp; * unknown peak.

Table 2.4: Estimation of the ($\beta 1 \rightarrow 4$)/($\beta 1 \rightarrow 3$)-ratio in different arabinogalactans.

	($\beta 1 \rightarrow 4$) ¹⁾	($\beta 1 \rightarrow 3$) ¹⁾	($\beta 1 \rightarrow 4$)/($\beta 1 \rightarrow 3$) ratio
Potato galactan	97.6	0.6	163
Soy galactan	96.4	0.5	193
Onion galactan	99.2	0.4	248
Citrus galactan	98.1	0.5	196

¹⁾Expressed as mol%.

DISCUSSION

It is generally accepted that the type I potato arabinogalactan contains ($\beta 1 \rightarrow 4$)-linked galactosyl residues in the backbone, substituted with ($\alpha 1 \rightarrow 5$)-linked arabinosyl residues (16, 19). In this study, we showed that the potato arabinogalactan also contained ($\beta 1 \rightarrow 3$)-linked galactosyl residues as an integral part of the backbone. The presence of peculiar structural elements in the arabinogalactan backbone was previously found for soybean arabinogalactans. For this type I arabinogalactan, it is known that the ($\beta 1 \rightarrow 4$)-linked galactosyl backbone can be interspersed with ($1 \rightarrow 5$)-linked arabinosyl residues (15).

In this research, we also demonstrated that the presence of ($\beta 1 \rightarrow 3$)-linked galactosyl residues is not restricted to the type I arabinogalactan from potato. Very different botanical sources such as soybean, onion, and citrus arabinogalactan also contained this structural element, which suggests that all type I arabinogalactans may contain ($\beta 1 \rightarrow 3$)-linkages as an intrinsic part of the backbone. From the combined *A. niger* endo-galactanase and *B. adolescentis* β -galactosidase treatment the ($\beta 1 \rightarrow 4$)/($\beta 1 \rightarrow 3$)-ratio was estimated as approximately 160-250.

The presence of ($\beta 1 \rightarrow 3$)-interruptions in a ($\beta 1 \rightarrow 4$)-polymer is also found in other cell wall polysaccharides, like ($\beta 1 \rightarrow 3$)($\beta 1 \rightarrow 4$)-D-glucans. These glucans in, for example, barley contain a ($\beta 1 \rightarrow 4$)/($\beta 1 \rightarrow 3$)-ratio of approximately 2.5 (21). This ratio is much lower than that found in type I arabinogalactans (a ratio of 163 was found in potato galactan), demonstrating that the ($\beta 1 \rightarrow 3$)-interruptions occur at a much higher frequency in the β -glucans.

For β -glucan synthesis the insertion of ($\beta 1 \rightarrow 3$)-glucosyl residues seems to be intentional; evidence is accumulating that the addition of these linkages is an intrinsic property of the β -glucan synthase (or synthesizing complex). Buckeridge et al. (6) found that β -glucan synthase

interrupts cellotriose or cellobiose units in a linear backbone structure with single (β 1→3)-linkages. The much lower frequency of (β 1→3)-interruptions in type I arabinogalactans compared to β -glucans suggests that these elements are inserted incidently. We speculate that the (β 1→3)-linkages in type I arabinogalactan are ‘errors’ in the biosynthesis. Since both (β 1→4) and (β 1→3)-galactosyltransferases are present in the same compartment of the Golgi apparatus (12), the (β 1→3)-galactosyltransferase might incidently use the growing (β 1→4)-galactan chain as an acceptor substrate and add a (β 1→3)-galactosyl unit. Subsequently, the (β 1→4)-galactosyltransferase continues the elongation of the nascent galactan. The variation in (β 1→4)/(β 1→3) ratio observed for the galactans from different botanical sources might be related to the ratio of the two galactosyltransferases. However, it is also possible that differences in biochemical properties of the transferases (such as K_m for UDP-gal, processive or distributive mode of action) underlies this variation.

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β -Galactosidase from *Bifidobacterium adolescentis* DSM 20083 prefers (β 1 \rightarrow 4)-galactosides over lactose

A β -galactosidase gene (β -Gal II) from *Bifidobacterium adolescentis* DSM 20083 was cloned into a *pbluescript* SK (-) vector and expressed in *Escherichia coli*. The recombinant enzyme was purified from the cell extract by anion-exchange and size-exclusion chromatography. β -Gal II had a native molecular mass of 235 kDa and the subunits had a molecular mass of 81 kDa, indicating that β -Gal II occurs as a trimer. The enzyme was classified as belonging to glycoside hydrolase family 42. The optimal pH was 6.0 and the optimal temperature was 50°C, using *p*-nitrophenyl- β -D-galactopyranoside as a substrate. The K_m and V_{max} for Gal(β 1 \rightarrow 4)Gal were 60 mM and 1,129 U/mg, respectively. The recombinant β -Gal II was highly active towards Gal(β 1 \rightarrow 4)Gal and Gal(β 1 \rightarrow 4)Gal-containing oligosaccharides, only low activity was observed towards Gal(β 1 \rightarrow 3)Gal, lactose, and Gal(β 1 \rightarrow 3)GalOMe. No activity was found towards Gal(β 1 \rightarrow 6)Gal, Gal(β 1 \rightarrow 4)Man, Gal(α 1 \rightarrow 4)Gal, Gal(α 1 \rightarrow 3)Gal(β 1 \rightarrow 4)Gal, cellobiose, maltose and sucrose. β -Gal II was inhibited at high substrate concentrations (100 mg/ml) and no transglycosylation activity was found. At lower substrate concentrations (10 mg/ml) only low transglycosylation activity was found; the Gal/[Gal(β 1 \rightarrow 4)]₂Gal peak area ration was 9:1.

Published as

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INTRODUCTION

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Carbohydrates can be degraded to low molecular weight oligosaccharides or monosaccharides by a wide range of depolymerizing enzymes. Bifidobacteria contain a large amount of carbohydrate modifying enzymes. The genome sequence from *Bifidobacterium longum* reveals that 5% of all genes corresponds to carbohydrate modifying enzymes (29). The growth of bifidobacteria can be selectively stimulated by the use of specific carbohydrates, which is claimed to be healthy for the host (8). Such carbohydrates are referred to as prebiotics.

As found in literature (1, 23, 26, 27) galactan or galacto-oligosaccharides seem to have a large potential to be fermented by bifidobacteria. For the degradation of these substrates, these microorganisms contain several β -galactosidases, which all have different properties (15, 20, 33). For example, Møller et al. (20) cloned three different β -galactosidases from *B. bifidum*. One of these β -galactosidases contains a signal peptide and a carbohydrate-binding domain. The other two β -galactosidases do not have this signal peptide and carbohydrate-binding domain. Van Laere et al. (33) found a β -galactosidase from *B. adolescentis*, which is only induced by larger galacto-oligosaccharides (degree of polymerization > 2), whereas another β -galactosidase was induced by lactose. The first enzyme was not able to hydrolyze lactose, in contrast to other known β -galactosidases from bifidobacteria (7, 14, 20, 25).

So far, not much is known about the enzymic machinery of bifidobacteria for galactose utilization. It may be expected that the various β -galactosidases provide bifidobacteria with flexibility in utilizing different types of galactans or galacto-oligosaccharides. In this paper the β -galactosidase from *B. adolescentis* induced by larger galacto-oligosaccharides, as described by Van Laere et al. (33), was cloned and further characterized with respect to substrate specificity.

MATERIAL AND METHODS

Bacterial strains, and growth conditions

Bifidobacterium adolescentis DSM 20083 was purchased from the Deutsche Sammlung von Microorganismen und Zellkulturen. The strain was grown anaerobically using MRS medium

pH 6.0 (Becton, Dickinson, Franklin Lakes, USA) supplemented with 0.5 g/l cysteine at 37°C. DNA cloning was performed using the *Escherichia coli* strain XL1 blue MRF' (Promega, Madison, USA). The *E. coli* strain was grown in Luria-Bertani (LB) broth or solidified LB medium (15 g agar/l) supplemented with 100 μ g/ml ampicillin when appropriate.

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

Chemicals, substrates, and enzymes

Chemicals were purchased from Sigma (St. Louis, USA) unless stated otherwise. Restriction enzymes and other enzymes used for DNA manipulation were obtained from MBI Fermentas (St. Leon, Germany) and were used according to the instructions of the manufacturer. The mixture of TOS was purchased from Borculo Domo Ingredients (Zwolle, The Netherlands) and was fractionated as described by Van Laere et al. (33). Transgalacto-oligosaccharides (TOS) were obtained by transgalactosylation of lactose with a β -galactosidase. The preparation of the [β -D-Galp-(1 \rightarrow 4)]_n-D-Galp oligosaccharides will be discussed in detail elsewhere (12). Briefly, they were obtained on a small scale (approximately 10 mg per oligosaccharide) by partial degrading potato galactan by an endo-galactanase from *A. niger*, followed by BioGel P2 (Bio-Rad, Hercules, USA) size exclusion chromatography. Three purified oligosaccharide fractions were obtained, which were designated as [β -D-Galp-(1 \rightarrow 4)]_n-D-Galp with n=2, n=3 and n>3. The molecular size of the products were verified by Maldi TOF MS. The β -galactosidase from *Aspergillus niger* was purchased from Megazyme (Bray, Ireland).

Isolation and cloning of the β -galactosidase β -Gal II gene

The β -galactosidase enzyme (β -Gal II) was isolated as described by Van Laere et al. (33). Internal amino acid sequences were determined by the Sequence Center of Utrecht University and was performed as described previously (31). From these internal amino acid sequences the primers GAL23F, GAL1R, GAL1F, and GAL31R were designed (5'-GCSTCSGGHGGYGAYTACAAYCCV, 5'-CTRTRCASGGSRVCGSATG, 5'-GAYAT-YGTSCCSYTBGCSTAC, and 5'-GARGARTTCAACATYYTSGGYGCSGARGCC,

respectively, in which Y represents T or C, S represents C or G, R represents A or G, H represents T, C or A, B represents T, C or G, and V represents C, A or G).

The genomic DNA of *B. adolescentis* DSM 20083 was isolated using a modified Marmur procedure as described by Johnson (17). The polymerase chain reaction (PCR) was carried out on the genomic DNA with primers GAL23F, GAL1R, GAL1F, and GAL31R and Taq Polymerase (HT Biotechnology, Cambridge, UK). The forth-coming DNA fragments were purified from the PCR mixture with a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The DNA fragments were ligated into a *pGEM* T-easy vector (Promega) and transformed into *E. coli* XL1 blue MRF' cells. The cells were grown during 16 h at 37°C on solid S-Gal/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 plasmid containing the largest PCR fragment (23F31R) was used for preparing a digoxigenin (DIG)-labeled DNA fragment according to the instructions of the manufacturer (Boehringer, Mannheim, Germany). The labeled PCR fragment was used for colony hybridization as described by Sambrook et al. (28) on a genomic library of *B. adolescentis* DSM 20083 (32). Detection of nucleotides was done according to the instructions of the manufacturer (Boehringer). The colonies hybridized with the labeled fragment were identified as black spots on an X-ray film. Two colonies were found, each containing a part of the β -Gal II gene. A PCR was carried out with the proofreading polymerase High Fidelity PCR Cloning Enzyme Easy A (Stratagene, La Jolla, USA) on the *B. adolescentis* genomic DNA, using two primers for full length amplification of the β -Gal II gene. Two *Xba*I restriction sites were introduced at the beginning and at the end of the gene for cloning purpose. Primers used were: CGF (5'-GGGGTCTAGAGCATAACCAGGGCGATATCGG) and CGR (5'-GGGGTCTAGATTACCGCCTTACCACGAGCACG). The PCR product (β -Gal II) was purified from the PCR mixture as described before and digested with *Xba*I. β -Gal II was cloned into an *Xba*I digested *pBluescript* vector (Stratagene) and transformed into *E. coli* XL1 blue MRF' cells.

Isolation and characterization of β -galactosidase β -Gal II

Cells from an *E. coli* cell culture (700 ml, LB-medium, 37°C, overnight) containing the β -Gal II gene were harvested by centrifugation (15 min; 8,000 x g; 4°C). The supernatant was removed and used for activity measurement; the cells were suspended in 90 ml 50 mM

sodium acetate buffer pH 4.5 and disrupted by sonic treatment (10 min; duty cycle 30%; Sonifier 250, Branson Ultrasonics, Danbury, USA) on ice. Subsequently, the suspension was centrifuged (15 min; 8,000 x g; 4°C), the supernatant was collected, and the pellet suspended in 45 ml 50 mM sodium acetate buffer pH 4.5 and a second sonic treatment was performed. This step was repeated two times. The cell-free extracts were pooled and applied onto a Q-Sepharose (Amersham, Little Chalfont, UK) anion-exchange column. Elution took place with a linear gradient of 0-0.5 M NaCl in 50 mM sodium acetate pH 4.5 at a flow rate of 57 cm/h. Fractions with the highest β -galactosidase 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 30 cm/h.

Protein concentration was determined by the method of Bradford (4) using bovine serum albumin (BSA) as a standard. 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 10-15% 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). The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Elution was performed with 20 mM potassium phosphate buffer pH 6.0 containing 0.15 M NaCl at a flow rate of 76 cm/h.

N-Terminal amino acid sequence determination was carried out at the Sequence Center of Utrecht University and was performed as described previously (31).

Enzyme assays

β -Galactosidase activity was measured by determining the hydrolysis of *p*-nitrophenyl- β -D-galactopyranoside (*p*NP-Gal) at 37°C after 10 min incubation. The reaction mixture (125 μ l) consisted of 2 mM potassium phosphate buffer pH 6.0, and 0.2 mg/ml *p*NP-Gal solution. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer pH 9.0, containing 2 mM EDTA. The color formation was measured at 400 nm. One unit (U) of activity was defined as 1 μ mol of galactose liberated per min under the specified conditions. The molar extinction coefficient under these assay conditions was 13,700 1/M*cm. This assay was also used to measure the enzyme's temperature optimum and stability between 4 and

80°C. For determination of the pH-optimum, McIlvain buffers (0.1 M citric acid/0.2 M disodium phosphate) in the range of pH 2.5-7.5 were used.

For kinetic experiments, β -D-Galp-(1 \rightarrow 4)-D-Galp (further referred to as Gal(β 1 \rightarrow 4)Gal) concentration was varied in the range of 0.05-8.0 mg/ml and measured by high-performance anion-exchange chromatography (HPAEC). The reactions were carried out in 2 mM potassium phosphate buffer pH 6.0 and 0.12 U/ml β -galactosidase for 60 min at 37°C. The incubations were stopped by heating the incubation mixtures for 5 min at 100°C. After centrifugation (5 min, 10,000 x g) the supernatant was analyzed by HPAEC using a ThermoQuest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column in combination with a CarboPac PA guard column (3 mm ID x 25 mm) and a Dionex ED40 PAD-detector (Dionex, Sunnyvale, USA). A flow rate of 1 ml/min was used with the following gradient of sodium acetate in 0.1 M NaOH: 0-15 min, 0-100 mM; 15-20 min, 100-200 mM; 20-21 min, 200-1,000 mM. Each elution was followed by a washing step of 5 min 1,000 mM sodium acetate and an equilibration step of 15 min 0.1 M NaOH.

The hydrolytic activity of β -Gal II towards different types of oligosaccharides was measured by HPAEC, using the same gradient as for the kinetic experiments. The incubations were performed with 1 mg/ml substrate under the same conditions as described above.

Transglycosylation activity was measured with 100 mg/ml TOS, 0.12 U/ml of β -Gal II, and 2 mM phosphate buffer pH 6.0. For the hydrolytic activity towards TOS, a concentration of 1 mg/ml was used, while the other conditions were kept similar to the transglycosylation assay. The reactions were stopped by heating the incubation mixtures for 5 min at 100°C, after which the reaction mixture was centrifuged (5 min, 10,000 x g) and the supernatant analyzed by HPAEC.

DNA sequencing and sequence analysis

An automated DNA sequencer 373 (Applied Biosystems, Foster City, USA) was used to determine the nucleotide sequence of the gene. The DNA sequence data were submitted to the GenBank nucleotide databases under the accession number AY359872. The BLAST2 program ((2), available at <http://www.ncbi.nlm.nih.gov/>) was used for searching sequence homologies.

RESULTS

Isolation of the β -galactosidase gene (β -Gal II) from *Bifidobacterium adolescentis*

The β -galactosidase β -Gal II purified by Van Laere et al. (33) was subjected to N-terminal sequencing. Since the N-terminus was blocked, the enzyme was digested with trypsin to prepare internal fragments for amino acid sequencing. Three sequences were obtained, namely ADIVPLAYD, EEFTILGAEAEAGEPGE, and IAFGGDYNP. Based on these, primers (GAL1F, GAL1R, GAL23F, and GAL31R) were designed, and a PCR was carried out on the genomic DNA of *B. adolescentis*. Three internal fragments of the β -Gal II gene were amplified. The largest fragment (23F31R) consisted of 1.5 kb and had a 100% overlap with the other two DNA fragments. To obtain the full-length gene a colony hybridization was performed on a genomic library of *B. adolescentis* DSM 20083 (32). Two positive colonies were found, one containing the 5' part of the β -Gal II gene and the other containing the 3' part. Primers were developed using the putative start and stop codon to amplify the full-length β -Gal II gene from the genomic DNA of *B. adolescentis*.

The β -Gal II gene consisted of an open reading frame of 2.1 kb. Approximately 10 nucleotides upstream of the start codon a ribosomal binding site AGGAG (19, 20) was found. The open reading frame did not start with ATG, but with GTG. As described by Lewin (19) the translation of the mRNA usually starts at AUG, but in bacteria also GUG and UUG are used as start codon. The open reading frame implies a molecular mass (Mw) of 78,106 Da and an isoelectric point (pI) of 4.8 of the translation product.

The β -Gal II gene showed homology to other β -galactosidase genes: 68% and 67% identity with two β -galactosidase from *B. longum* (GenBank accession no. AAN24101 and ZP_00121105, respectively), and 48% and 46% identity with two β -galactosidase from *B. infantis* (CAC14567 and AF192266, respectively). All these β -galactosidases were classified in glycoside hydrolase family 42 (GH42), according to Henrissat (10) at <http://afmb.cnrs-mrs.fr/CAZY/index.html>. Therefore β -Gal II was also classified as a member of GH42. In figure 3.1 an alignment of different GH42 β -galactosidases is shown. Based on the 3D-structure of the β -galactosidase from *Thermus thermophilus* (11) and sequence comparisons, the catalytic residues of β -Gal II were identified as E₁₆₁ and E₃₂₁ (corresponding to E₁₄₁ and E₃₁₂ of the β -galactosidase from *T. thermophilus*). As expected, these two amino acid residues are highly conserved in all GH42 members (Figure 3.1).

The three internal amino acid sequences, obtained from the tryptic digest were found in the cloned *β-Gal II* (Figure 3.1). These three sequences were specific for *B. adolescentis* *β-Gal II*, demonstrating unambiguously that the cloned *β-Gal II* gene encoded the *β*-galactosidase described by Van Laere et al. (33).

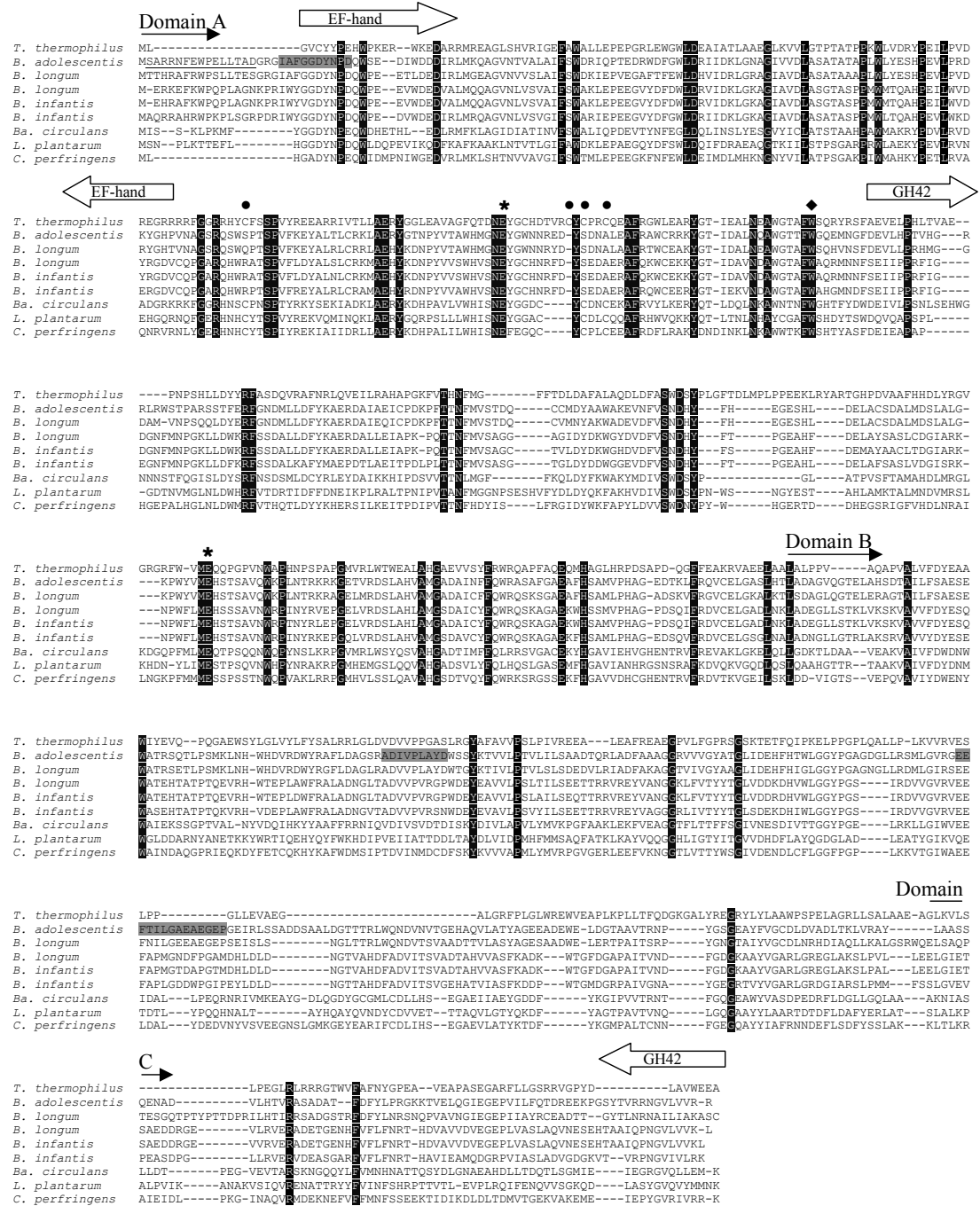
Signal peptide and transmembrane domain prediction using SignalP, described by Nielsen et al. (22) (<http://www.cbs.dtu.dk/services/SignalP/>), PSORT, described by Nakai and Kanehisa (21) (<http://psort.nibb.ac.jp/>), and SOSUI, described by Hirokawa et al. (13) (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>) showed that *β-Gal II* had neither a signal peptide, nor a transmembrane domain. These results suggest that *β-Gal II* is an intracellular enzyme.

The *β-Gal II* amino acid sequence was also analyzed for domain structures as described by Corpet et al. (5) (ProDom; <http://protein.toulouse.inra.fr/prodom/2002.1/html/form.php>) and Altschul et al. (2) (<http://www.ncbi.nlm.nih.gov/>). Using the first method, the protein consisted of two domains: a GH42 domain and an EF-hand calcium-binding domain (Figure 3.1). The amino acid sequence, analyzed according to the latter method contained only one domain, namely the GH42 domain.

Purification and physico-chemicals properties

The total *β*-galactosidase activity of the 700 ml cell culture was 1032 U in the cell-free extract, while the cell culture supernatant contained 399 U. The cell-free extract was used for the purification of the recombinant *β-Gal II* by anion-exchange chromatography and size-exclusion chromatography. The enzyme was pure after these chromatographic steps, as judged by SDS-PAGE and Coomassie Brilliant Blue staining. The specific activity of the purified enzyme was 526 U/mg, using *p*NP-Gal as substrate.

The N-terminal amino acid sequence of the recombinant *β-Gal II* was SARRNFEWPELLTAD. This amino acid sequence corresponded perfectly to residues 2 to 16 of the deduced amino acid sequence of the *β-Gal II* gene (Figure 3.1). This indicates that the first amino acid (methionine) was cleaved off during expression of the *β*-galactosidase in *E. coli*, this phenomenon was also found by Møller et al. (20) and Lewin (19).



The physico-chemical properties of the recombinant β -Gal II are given in Table 3.1. The K_m was 60 mM and the V_{max} 1129 U/mg towards the substrate Gal(β 1 \rightarrow 4)Gal, which is high compared with other β -galactosidases known. 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. The β -galactosidase had a native molecular mass of 235 kDa and the subunits had a molecular mass of 81 kDa, which corresponds well with the molecular mass calculated from the deduced amino acid sequence (78 kDa). This indicated that the recombinant β -Gal II occurred as a trimer.

To examine the dependence of the recombinant β -Gal II for metal ions, its activity towards Gal(β 1 \rightarrow 4)Gal was measured in the presence of various EDTA concentrations (0-1 mM). No effect on β -galactosidase activity was found.

Table 3.1: Physico-chemical properties of *Bifidobacterium adolescentis* β -Gal II

Parameter	β -Gal II value
K_m (mM) ^a	60
V_{max} (U/mg) ^a	1129
Deduced molecular mass (Da)	78,106
Molecular mass (kDa; SDS-PAGE)	81
Native molecular mass (kDa)	235
Proposed molecular structure	Trimer
Specific activity (U/mg) ^b	526
Optimum pH	6.0
Optimum temperature (°C)	50
Stability of the enzyme	Stable at 40°C, t $\frac{1}{2}$ at 50°C = 10 min

^a Gal(β 1 \rightarrow 4)Gal as substrate.

^b *p*-Nitrophenyl- β -D-galactopyranoside as substrate

Substrate specificity

The ability of the recombinant β -Gal II to hydrolyze different substrates was examined. The activity towards *p*-nitrophenyl- β -D-galactopyranoside (*p*NP-Gal) was already demonstrated. The reaction products obtained upon treatment of various oligosaccharides with the recombinant β -Gal II were analyzed by high-performance anion-exchange chromatography (HPAEC). The substrate specificity of *B. adolescentis* β -Gal II was compared with a

commercially available β -galactosidase from *Aspergillus niger*. This enzyme has not been classified to a GH-family. The same incubation conditions were used and the amount of enzyme, equivalent to 4 U towards *p*NP-gal, was added to the various incubations. HPAEC profiles of the degradation of Gal(β 1 \rightarrow 4)Gal, Gal(β 1 \rightarrow 6)Gal, and Gal(β 1 \rightarrow 3)Gal are shown in figure 3.2.

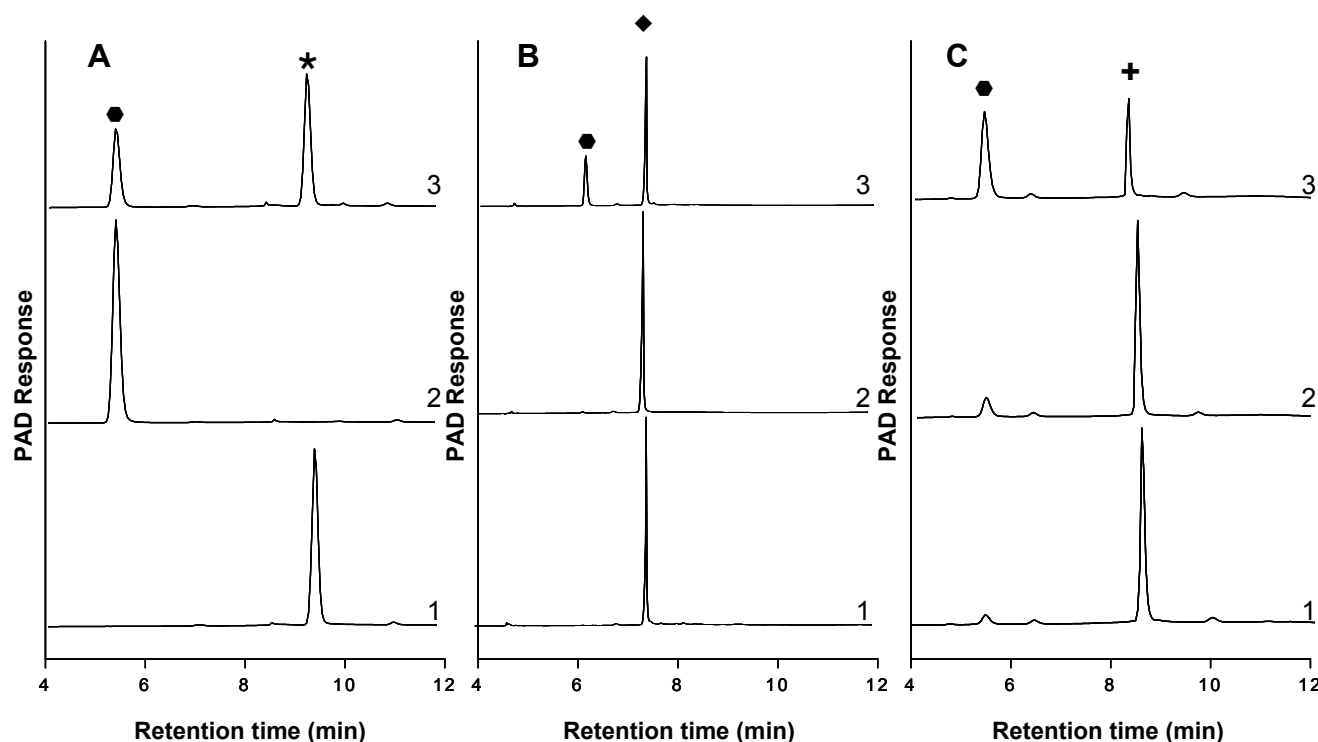


Figure 3.2: High-performance anion-exchange chromatography (HPAEC) of the degradation products of Gal(β 1 \rightarrow 4)Gal (A), Gal(β 1 \rightarrow 6)Gal (B), and Gal(β 1 \rightarrow 3)Gal (C). 1: Blank; 2: Digestion with recombinant β -galactosidase β -Gal II from *B. adolescentis*; 3: Digestion with β -galactosidase from *A. niger*. ●: Galactose; * Gal(β 1 \rightarrow 4)Gal; ◆ Gal(β 1 \rightarrow 6)Gal; + Gal(β 1 \rightarrow 3)Gal.

The recombinant β -Gal II degraded Gal(β 1 \rightarrow 4)Gal to galactose, but was not able to degrade Gal(β 1 \rightarrow 6)Gal. The β -galactosidase from *A. niger* was able to degrade both substrates to some extent. The Gal(β 1 \rightarrow 3)Gal was degraded by both enzymes, although the activity of the recombinant β -Gal II was much lower than the β -galactosidase from *A. niger*. The results of the hydrolysis of other oligosaccharides with the recombinant β -Gal II and the β -galactosidase from *A. niger* are summarized in Table 3.2. From this table it was concluded that both enzymes were able to degrade the same substrates, except the (β 1 \rightarrow 6)-linkages; the recombinant β -Gal II was not able to degrade these, whereas the β -galactosidase from *A.*

niger was. Both enzymes were not able to degrade Gal(α 1 \rightarrow 3)Gal(β 1 \rightarrow 4)Gal from which it was concluded that the enzymes cleave galactose from the non-reducing end of an oligosaccharide. The most striking result from this experiment was that the recombinant β -galactosidase β -Gal II had a much higher activity towards Gal(β 1 \rightarrow 4)Gal-linkages than the one of *A. niger*.

Table 3.2: Substrate specificity of *B. adolescentis* recombinant β -galactosidase β -Gal II and a β -galactosidase from *A. niger*.

Substrate ^a	<i>B. adolescentis</i> β -Gal II	<i>A. niger</i> β -galactosidase
<i>p</i> NP-Gal	+	+
Gal(β 1 \rightarrow 4)Gal	++++	++
Gal(β 1 \rightarrow 6)Gal	-	++
Gal(β 1 \rightarrow 3)Gal	+	++
Gal(β 1 \rightarrow 3)GalOMe	+	++
Gal(β 1 \rightarrow 4)Man	-	-
Gal(β 1 \rightarrow 4)Glc (Lactose)	+	+
Glc(β 1 \rightarrow 4)Glc (Cellobiose)	-	-
Glc(α 1 \rightarrow 4)Glc (Maltose)	-	-
Glc(β 1 \rightarrow 1)Fruc (Sucrose)	-	-
TOS	+++	++
(Gal(β 1 \rightarrow 4)) ₂ Glc	+++	++
(Gal(β 1 \rightarrow 4)) ₃ Glc	+++	+
(Gal(β 1 \rightarrow 4)) ₄ Glc	+++	+
(Gal(β 1 \rightarrow 4)) ₅ Glc	++	+
(Gal(β 1 \rightarrow 4)) ₂ Gal	++++	++
(Gal(β 1 \rightarrow 4)) ₃ Gal	++++	+
(Gal(β 1 \rightarrow 4)) _n Gal, n>3	+++	+
Gal(α 1 \rightarrow 3)Gal(β 1 \rightarrow 4)Gal	-	-
Gal(α 1 \rightarrow 4)Gal	-	-

^a Degradation of the substrate under the conditions used, as described in materials and methods. The score was based on the decrease in peak area of the substrate (-: 0-5% degradation of the substrate; +: 5-25% degradation of the substrate; ++: 25-50% degradation of the substrate; +++: 50-75% degradation of the substrate; ++++: 75-100% degradation of the substrate).

In general, chain elongation (transglycosylation) reactions are carried out with high concentrations of substrate (3, 6, 16). To determine the transglycosylation activity of the recombinant β -Gal II, an enzyme assay was performed with 100 mg/ml of TOS. For the hydrolysis activity towards TOS a concentration of 1 mg/ml was used, while the other conditions were kept similar to the transglycosylation assay. The reaction products were analyzed by HPAEC (Figure 3.3).

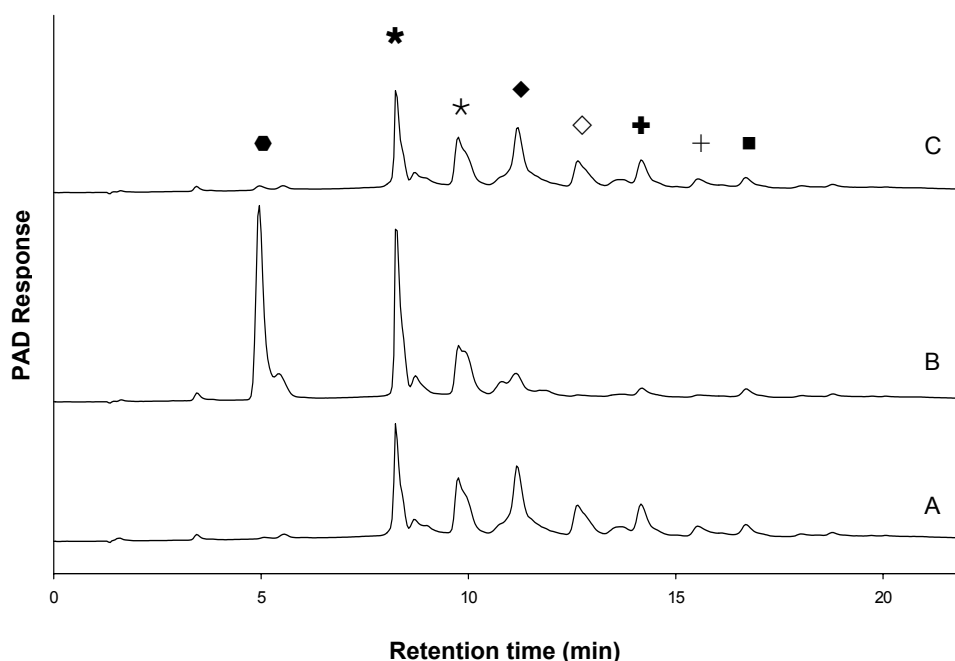


Figure 3.3: Substrate inhibition by transgalactooligosaccharides (TOS) of the *B. adolescentis* recombinant β -Gal II. A: TOS blank (1 mg/ml); B: Digestion with 1 mg/ml TOS; C: Digestion with 100 mg/ml TOS. This latter digestion was diluted to the same concentration as the blank for comparison. Identification of the peaks was in accordance to Van Laere et al. (2000); ●: Galactose and/or glucose; *: Lactose (Gal(β 1 \rightarrow 4)Glc); *: galactosyl dimers; ◆: (Gal(β 1 \rightarrow 4))₂Glc; ◇: galactosyl trimers; +: (Gal(β 1 \rightarrow 4))₃Glc; +: galactosyl tetramer; ■: (Gal(β 1 \rightarrow 4))₄Glc.

At a substrate concentration of 1 mg/ml the TOS mixture was degraded to the monomers galactose and glucose and the dimer lactose. At a concentration of 100 mg/ml TOS was used, no reaction occurred. An increase of trimer, tetramer, and pentamer peaks or the appearance of new peaks in the HPAEC profile were expected. However, neither hydrolysis nor transglycosylation occurred at a concentration of 100 mg/ml TOS (Fig. 3C). Similar results were found after incubation of the recombinant β -Gal II with 100 mg/ml *p*NP-Gal (data not shown). Transglycosylation activity was determined with Gal(β 1 \rightarrow 4)Gal at a substrate

concentration of 10 mg/ml. From the HPAEC results it can be concluded that the enzyme has relatively low transglycosylation activity, because the amount of galactose released from is much higher than the amount of transglycosylation product formed; the Gal:(Gal(β 1 \rightarrow 4))₂Gal peak area was 9:1.

DISCUSSION

Over the years several β -galactosidases from bifidobacteria have been isolated (7, 25, 30, 33) and cloned (15, 20, 24). β -Gal II differs from the ones known so far in that it is highly active towards Gal(β 1 \rightarrow 4)Gal-linkages and shows low transglycosylation activity, with galacto-oligosaccharides derived from lactose.

According to the classification of glycoside hydrolases (<http://afmb.cnrs-mrs.fr/CAZY/index.html>), the cloned β -galactosidases from bifidobacteria are classified in two families, namely GH42 and GH2. So far, no β -galactosidases from bifidobacteria in GH42 are extensively characterized. The recombinant β -Gal II is the first one, which is characterized in more detail. This enzyme had low hydrolysis activity towards lactose, which was also found for the recombinant β -gal III from *B. infantis* ((15); AF192266). Further characterization of other GH42 β -galactosidases from bifidobacteria is needed to conclude whether the enzymes within this family have similar substrate specificity as the recombinant β -Gal II. For GH2 only β -galactosidase β -gal I from *B. infantis* (AF192265) has been characterized with respect to substrate specificity (14). In comparison with β -Gal II (GH42), β -gal I shows high hydrolysis activity towards lactose. From many other β -galactosidases, which have been characterized no amino acid sequence information is available and it is unclear into which GH family they are classified.

Hidaka et al. (11) described the 3D structure of a GH42 β -galactosidase from *T. thermophilus*. This enzyme occurs as a trimer, of which each subunit comprises three domains, in which domain A contains the catalytic residues (Figure 3.1). Comparison of the deduced amino acid sequence of β -Gal II with the β -galactosidase from *T. thermophilus* showed that the catalytic residues were all conserved (Figure 3.1) as well as W₂₀₁ (W₁₈₂ in *T. thermophilus*), which is involved in galactose binding. It is expected that β -Gal II has a similar 3D-structure as the β -galactosidase from *T. thermophilus*. Domain structure analysis of β -Gal II with ProDom (5) revealed an EF-Hand calcium-binding domain. However, this calcium-binding domain was

not found in the 3D structure of the *T. thermophilus* β -galactosidase (11). Homology searches of β -Gal II with known EF-Hand sequences showed no homology (data not shown). From these results, it was concluded that β -Gal II did not have an EF-hand calcium-binding domain. The 3D structure of the β -galactosidase from *T. thermophilus* contains a metal-binding site, which is formed by four cysteines. As for the other *Bifidobacterium* β -galactosidases, the amino acid sequence of β -Gal II did not contain these cysteine residues (Figure 3.1), which might imply the lack of ion binding, and could explain that EDTA had no effect on the enzyme activity of β -Gal II.

As described before, bifidobacteria produce different types of β -galactosidases (15, 20, 33). With the publication of the genome sequence of *B. longum* (29) more information about the presence of carbohydrate modifying enzymes in bifidobacteria is available. For example, the genome reveals the presence of an extracellular endo-galactanase with a transmembrane domain. It is expected that this enzyme can degrade polymeric galactan into oligosaccharides, which might be transported into the bacterial cell (9, 18). Intracellularly, the galacto-oligosaccharides can be degraded by the different β -galactosidases present. It is known that *B. bifidum* contains β -galactosidases, which are not present in the *B. longum* genome sequence. This suggests that the various bifidobacteria species can have a different toolbox to utilize galactans or galacto-oligosaccharides. In *B. adolescentis* β -Gal II could play an important role in the degradation of prebiotic Gal(β 1 \rightarrow 4)Gal-oligosaccharides.

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An endo-galactanase from *Bifidobacterium longum* liberates galactotriose from type I galactans

A putative endo-galactanase gene (*YvfO*) classified into glycoside hydrolase family 53 was revealed from the genome sequence of *Bifidobacterium longum* NCC2705 (33). Since only 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. *YvfO* 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 weight of 329 kDa and the subunits had a molecular weight of 94 kDa, which indicated that the enzyme occurred as a tetramer. The optimal pH of endo-galactanase activity was 5.0 and the optimal temperature was 37°C, using 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 (β 1→4)-galactans and (β 1→4)-galacto-oligosaccharides, probably by a processive mechanism, moving towards the reducing end of the galactan chain after an initial mid-chain cleavage. *YvfO*'s mode of action was found to be different from that of an endo-galactanase from *Aspergillus aculeatus*. The enzyme seemed to be able to cleave (β 1→3)-linkages. Arabinosyl side chains in, for example, potato galactan hindered *YvfO*.

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INTRODUCTION

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 (33), 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 (β 1 \rightarrow 4)-linked (arabino)galactans with different bifidobacteria show that mainly *B. longum* strains were able to grow on these arabinogalactans (8, 10). 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 side-chains of galactans (arabinofuranosidases and arabinosidases) or galacto-oligosaccharides (β -galactosidases) (33). Interestingly, the genome sequence also suggests that *B. longum* contains an endo-galactanase (YvfO), which is predicted to be extracellular. This is rather exceptional, because only few endo-acting enzymes have been described in bifidobacteria so far (2, 22).

Most endo-galactanases described to date are able to degrade the (β 1 \rightarrow 4)-linked galactosyl backbone of type I arabinogalactans (6, 18, 19, 26, 35). Neither the substrate specificity of YvfO is clear, nor if it truly acts with an endo-mechanism. It is possible that this enzyme is an essential link in galactan utilization, together with β -galactosidases, arabinofuranosidases and transporters of galacto-oligosaccharides. To get more insight in galactan utilization by *B. longum*, we have cloned the *YvfO* gene, characterized this enzyme in detail, and compared its mode of action with an endo-galactanase from *Aspergillus aculeatus*.

MATERIAL AND METHODS

Bacterial strains, and growth conditions

Bifidobacterium longum NCC490 was kindly provided by Nestlé Research Centre (Lausanne, Switzerland). The strain was grown anaerobically using MRS medium pH 6.0 (Becton Dickinson, Franklin Lakes, USA) supplemented with 0.5 g/l cysteine at 37°C. DNA cloning was performed using the *Escherichia coli* strain XL1 blue MRF' (Promega, Madison, USA). 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 *YvfO* 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, USA), unless stated otherwise. Potato arabinogalactan was obtained as described by Van de Vis (36). Azurine-crosslinked galactan (AZCL-galactan) was purchased from Megazyme (Bray, Ireland). Endo-galactanase from *Aspergillus aculeatus* was purified by Van de Vis et al. (37). Arabinofuranosidase from *A. 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 transgalacto-oligosaccharides (TOS) was kindly provided by Borculo Domo Ingredients (Zwolle, The Netherlands), and was fractionated as described by Van Laere et al. (39). [β-D-Galp-(1→4)]_m-D-Galp and [β-D-Galp-(1→4)]_n-β-D-Galp-(1→3)-D-Galp oligosaccharides, with m=1–3 and n=1–2, were obtained as described by Hinz et al. (14).

Cloning of the endo-galactanase gene

Genomic DNA of *B. longum* was isolated using a modified Marmur procedure as described by Johnson (16). A polymerase chain reaction (PCR) was carried out on the genomic DNA with Easy A polymerase (Stratagene, La Jolla, USA), using two primers for amplification of the *YvfO* gene without the transmembrane domain (*Xba*I restriction sites were introduced at the beginning and at the end of the gene for cloning purpose). Primers used were (with the *Xba*I site underlined): 257F (5'-CCCCCCTCTAGACAAAGGAGAAAAAGCATGCG), and 257R (5'-CCCCCCTCTAGATCAGCTACCGGTATTGCTCAG). The forth-coming DNA fragments were ligated into a pGEM T-easy vector (Promega) and transformed into *E. coli* XL1 blue MRF' cells. The cells were grown during 16 h at 37°C on solid S-Gal/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 *YvfO* gene were cut out the plasmids with *Xba*I, purified and cloned into an *Xba*I digested pBluescript vector

(Stratagene). This vector was transformed into *E. coli* XL1 blue MRF' cells.

Isolation and characterization of endo-galactanase

E. coli cells, containing the *YvfO* gene, were grown overnight at 37°C, (1 l), and harvested by centrifugation (15 min; 8,000 x 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, 0.7 s off; Digital Sonifier, Branson, Danbury, CT) on ice. Subsequently, the suspension was centrifuged (15 min; 8,000 x g; 4°C), the cell free extract was collected, the pellet 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-free extracts were pooled and applied onto a Q-Sepharose (Amersham, Little Chalfont, UK) anion-exchange column, using a BioPilot pump system (Amersham). Elution was done with a linear gradient of 0–0.5 M NaCl in 50 mM sodium acetate pH 5 at a flow rate of 57 cm/h. Fractions with the highest endo-galactanase 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 endo-galactanase activity were pooled and concentrated with anion-exchange chromatography under the same conditions as described above.

Protein concentration was determined by the method of Bradford (5) using bovine serum albumin (BSA) as a standard. 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 10–15% 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), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Enzyme assays

Endo-galactanase activity was measured by determining the hydrolysis of AZCL-galactan (according to instructions of the supplier) at 37°C after 15 min 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 x g), the absorbance of the supernatant was measured at 595 nm. This absorbance was converted to the amount of reducing sugars according to the supplier. One unit (U) of activity was defined as 1 μ mol of reducing sugars liberated per min under the specified conditions. This assay was also used to measure the enzyme's temperature optimum, pH-optimum and for kinetic experiments. The temperature optimum was determined between 4 and 70°C. For determination of the pH-optimum, McIlvain buffers (0.1 M citric acid/0.2 M di-sodium phosphate) in the range of pH 2.6–7.6 were used. For kinetic experiments, the AZCL-galactan concentration was varied in the range of 0.08–16.0 mg/ml.

The mode of action of YvfO was determined by high-performance anion-exchange chromatography (HPAEC) and high-performance size-exclusion chromatography (HPSEC). The incubations were performed, in triplicates, with 5 mg/ml potato galactan in 5 mM sodium acetate buffer pH 5.0 and 3.3 μ U/ml enzyme at 37°C during different time intervals. The incubations were stopped by heating the incubation mixtures for 10 min at 100°C. After centrifugation (10 min, 10,000 x g) the supernatant was analyzed by HPAEC, HPSEC, and Nelson-Somogyi.

The substrate specificity was also measured by HPAEC in triplicates. 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 during 30 and 120 min. The incubations were stopped and analyzed as described above.

The influence of arabinosyl side chains on the hydrolytic activity of YvfO 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 during 24 hours. The incubations were stopped by heating the incubation mixtures for 10 min at 100°C. After cooling to 37°C, 3.3 μ U/ml YvfO was added, and the samples were incubated at 37°C during 48 hours. The incubations were stopped and analyzed as described above. All reactions were carried out in triplicates.

Analytical methods

HPAEC was performed using a Thermo-Quest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column in combination with a CarboPac PA guard column (3 mm ID x 25 mm) and a Dionex ED40 PAD-detector (Dionex, Sunnyvale, USA). A

flow rate of 1 ml/min was used with the following gradient of sodium acetate in 0.1 M NaOH: 0–40 min, 0–400 mM; 40–41 min, 400–1,000 mM. Each elution was followed by a washing step of 5 min 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 x 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 MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) an Ultraflex workstation (Bruker Daltronics, Germany) was used. The mass spectrometer was calibrated with a mixture of malto-dextrins. 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: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 (27), using galactose as standard.

DNA sequencing and sequence analysis

An automated DNA sequencer 373 (Applied Biosystems, Foster City, USA) was used to determine the nucleotide sequence of the gene. The DNA sequence data are available at 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.

RESULTS

Characterization of the endo-galactanase gene (*YvfO*) from *Bifidobacterium longum*

The *YvfO* 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. The open reading frame implied a molecular mass (Mw) of 91,366 Da and an isoelectric point (pI) of

4.19 of the translation product. YvfO was classified in glycoside hydrolase family 53 (GH53), according to Henrissat (13) at <http://afmb.cnrs-mrs.fr/CAZY/index.html>. A ClustalW comparison of four endo-galactanases, of which the 3D-structure is known (*Aspergillus aculeatus*, GenBank accession no. ASNGAL1A [Christgau, 1995 #299(32), *Bacillus licheniformis*, AAO31370 (4, 31), *Corynascus heterothallicus*, AAN99814 (21), *Humicola insolens*, AAN99815 (17, 21)), and YvfO from *B. longum* was performed (Figure 4.1). Based on the 3D-structures, the catalytic residues of YvfO were suggested to be E₁₉₂ and E₂₉₇. As expected, these two amino acid residues are conserved in all GH53 members (Figure 4.1). The alignment showed that the enzyme was twice as large as the other endo-galactanases.

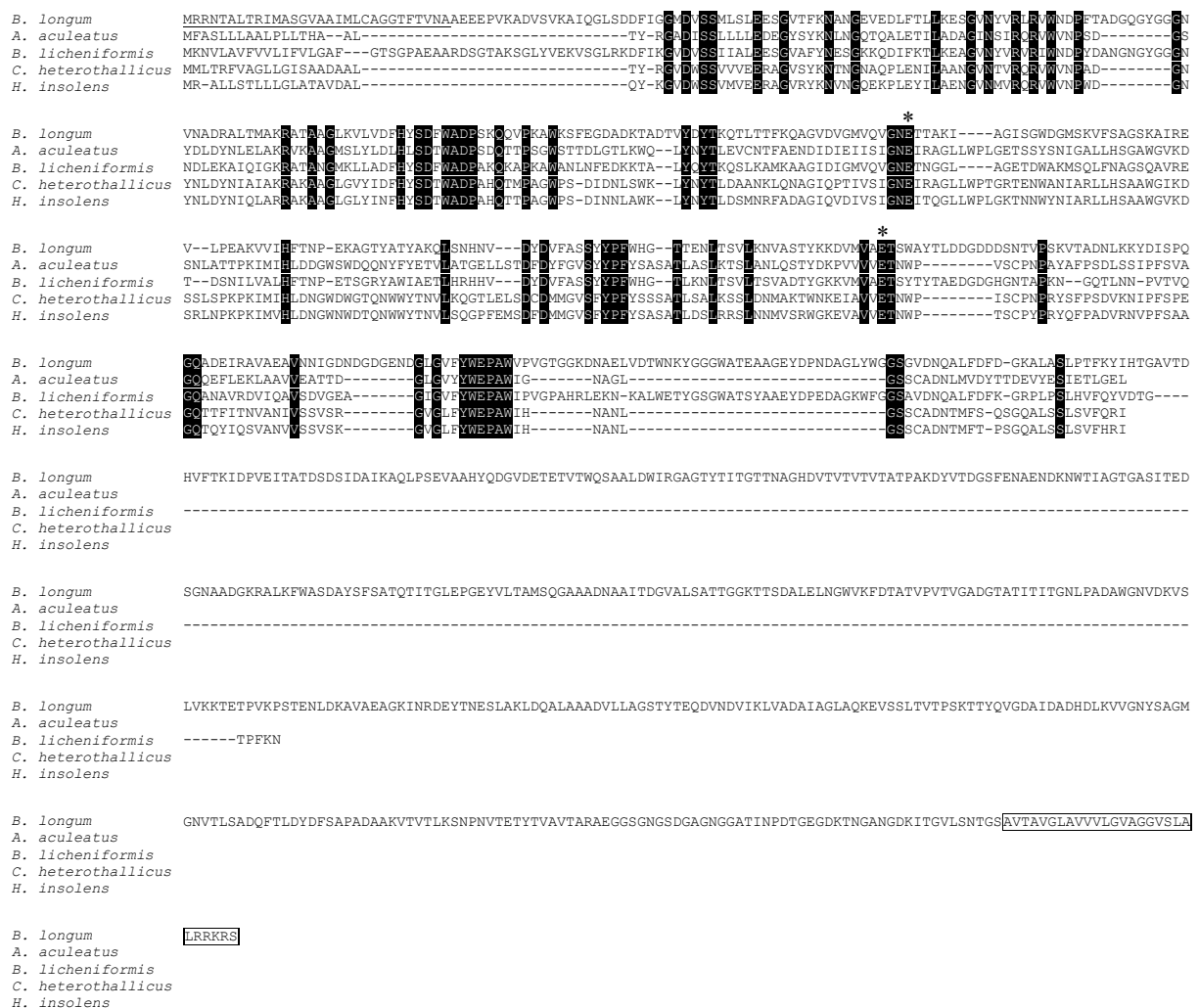


Figure 4.1: ClustalW comparison of the amino acid sequence of endo-galactanase from *Bifidobacterium longum* (YvfO) and endo-galactanases from *Aspergillus aculeatus* (accession no. ASNGAL1A), *Bacillus licheniformis* (AAO31370), *Corynascus heterothallicus* (AAN99814), *Humicola insolens* (AAN99815). Amino acid identity is indicated by vertical black shading, the catalytic residues E₁₉₂ and E₂₉₇ are indicated by asterisks. The signal peptide of YvfO is underlined, whereas its transmembrane domain is boxed.

Signal peptide and transmembrane domain prediction using SignalP (28) (<http://www.cbs.dtu.dk/services/SignalP/>), PSORT (25) (<http://psort.nibb.ac.jp/>), and SOSUI (15) (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>) showed that YvfO had a signal peptide and a transmembrane domain (Figure 4.1). These results suggested that YvfO is an extracellular enzyme, which is anchored to the cell membrane. The *YvfO* gene was cloned without the transmembrane domain.

Table 4.1: Physico-chemical properties of endo-galactanase (YvfO) from *Bifidobacterium longum*.

Parameter	YvfO 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 substrateTABLE 2. Sugar composition of potato arabinogalactan and tetramer fraction (obtained after partial degradation of potato galactan) as described by Hinz et al. (14)

Purification and physico-chemicals properties

The total endo-galactanase activity of the 1 l cell culture was 271 U in the cell-free extract, and 209 U in the cell culture supernatant. The cell-free extract was used for the purification of YvfO by anion-exchange chromatography and size-exclusion chromatography, because it contained most activity. The enzyme was pure after these chromatographic steps, as judged by SDS-PAGE and Coomassie Brilliant Blue staining. The physico-chemical properties of YvfO 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. YvfO 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 YvfO occurred as a tetramer.

Mode of action

The mode of action of YvfO towards 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 (14).

Table 4.2: Sugar composition of potato arabinogalactan (PG) and tetramer fraction, obtained after partial degradation of galactan as described by Hinz et al. (14).

Fraction	Monosaccharide composition (mol%)						Carbohydrate content ¹⁾	Gal/Ara
	Fuc	Rha	Ara	Gal	Glc	GalA		
PG	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

^aExpressed as % w/w

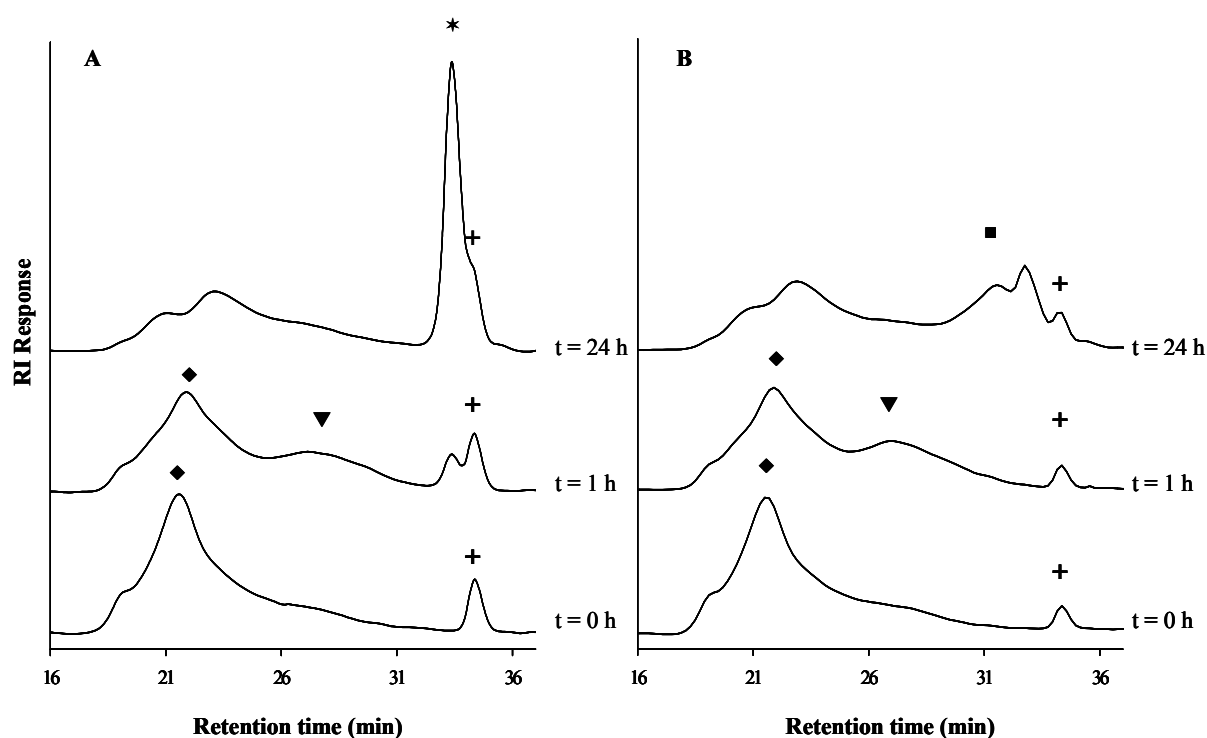


Figure 4.2: HPSEC profiles of potato arabinogalactan degradation after 0, 1, and 24 h of incubation by YvFO (A) and endo-galactanase from *A. aculeatus* (B). Symbols: ◆, polymeric potato arabinogalactan; ▼, degradation products of potato arabinogalactan with average molecular mass of 11 kDa; *, low molecular mass oligosaccharides (approximately 370 Da); +, acetate buffer; ■, higher molecular mass oligosaccharides (approximately 500–2,400 Da).

The galactan degradation was followed by HPSEC (Figure 4.2) and HPAEC (Figure 4.3). A shift in molecular mass of galactan occurred after 1 h of incubation, together with the appearance of a peak in the low molecular mass region (Figure 4.2A). 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 YvfO was compared with an endo-galactanase from *A. aculeatus*. Initially, this latter enzyme had a similar shift in molecular mass of the polymeric galactan after 1h (Figure 4.2B), but prolonged incubation showed the formation of larger oligomers than the ones observed after incubation with YvfO.

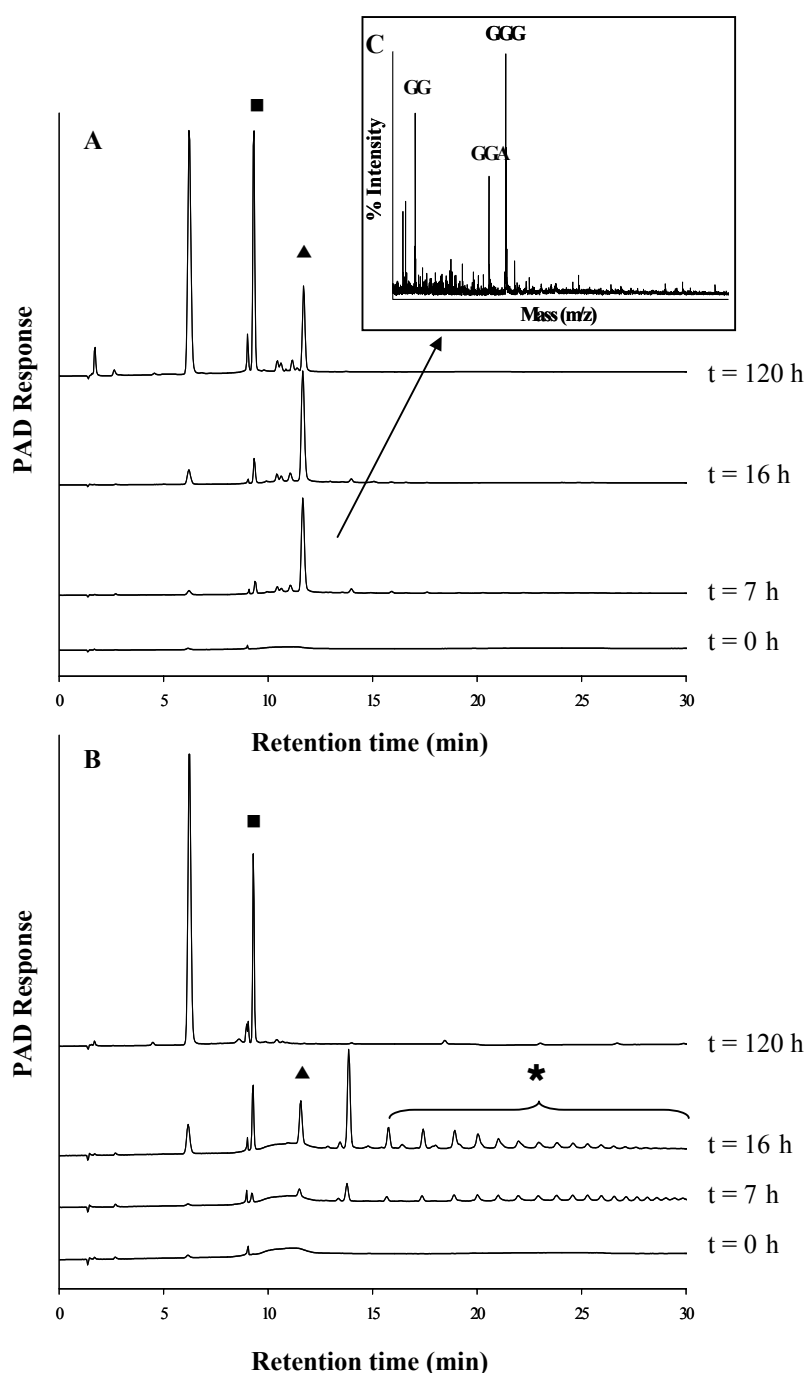


Figure 4.3: HPAEC profiles of the degradation of potato arabinogalactan after 0, 7, 16, and 120 h of incubation by YvfO (A) and endo-galactanase from *A. aculeatus* (B) and a Maldi-TOF MS profile of the potato arabinogalactan digest after 7 h incubation with endo-galactanase from *B. longum* (C). Symbols: ◆, galactose; ■, galactodisaccharide (β -D-Galp(1 \rightarrow 4) β -D-Gal); ▲, galactotrisaccharide (β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp); ●, galactotetrasaccharides (β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp); *, galactooligosaccharides with a degree of polymerization of ≥ 5 ; GG: galactobiose; GGA: arabinosyl-galactobiose; GGG: galactotriose.

HPSEC analysis revealed that 20 and 22% of the starting material was degraded after 1h, whereas at the end-point degradation (data not shown) 67 and 60% was degraded by YvfO and the endo-galactanase from *A. aculeatus*, respectively. HPAEC was used to determine the nature of the oligomers. The HPAEC profiles of the digests obtained with endo-galactanase degradation (Figure 4.3A) showed that YvfO had a preference to liberate galacto-trisaccharides. After prolonged incubation, also galactose and galactobiose were formed. Besides these products, some minor oligosaccharides were released, which probably correspond to galactobiose with an arabinosyl residue as determined by Maldi-TOF MS (Figure 4.3C). The degradation pattern of galactan by endo-galactanase from *A. aculeatus* appeared to follow a different pattern (Figure 4.3B).

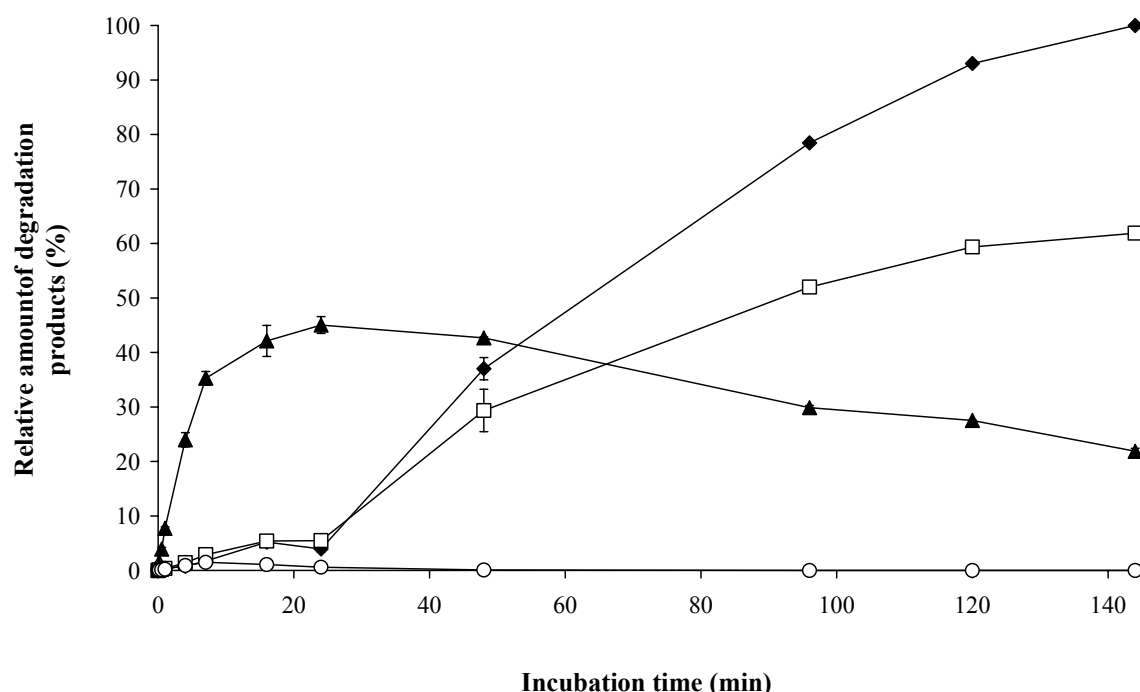


Figure 4.4: The formation of galactotrisaccharide (▲), galactodisaccharide (□), galactose (◆), and galactotetrasaccharide (○) during the degradation of potato arabinogalactan by YvfO in time.

After an incubation time of 16 hours a range of oligosaccharides with different degree of polymerization was 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 YvfO was monitored in time (Figure 4.4). The results from this experiment showed clearly that the enzyme initially liberated galactotrisaccharides from the polymeric galactan. After an incubation of 24 hours, the enzyme started to degrade

the trisaccharide to galactose and galactobiose. A very small amount of galacto-tetrasaccharide was found during the first 20 hours of incubation.

To compare the mode of action of YvfO and *A. aculeatus* endo-galactanase, the molecular weight of the galactan was plotted versus the amount of reducing sugars formed upon incubation for different periods of time (Figure 4.5). 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 YvfO was different from that of *A. aculeatus*. This latter enzyme was characterized as an endo-acting enzyme (37). YvfO showed a behaviour towards the potato galactan, which was clearly different from an endo-acting enzyme.

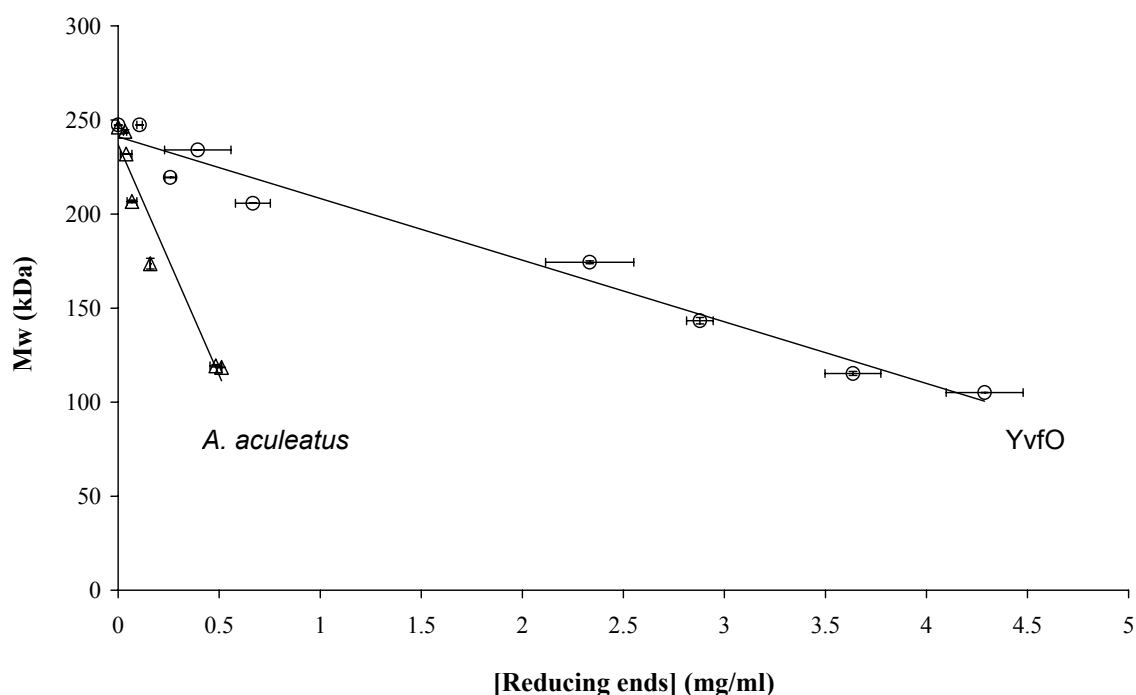


Figure 4.5: The amount of reducing sugars formed by the action of YvfO (○) and endo-galactanase from *A. aculeatus* (△) versus the reduction of molecular weight of the polymeric galactan.

Substrate specificity

As described previously, potato galactan contained a small amount of (β1→3)-linkages in the backbone (14). However, in none of the performed experiments with YvfO galactotriose with a (β1→3)-linkage was found. This might suggest that the (β1→3)-linkages were degraded by

the enzyme. Therefore, a galacto-oligosaccharide mixture, containing β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 3)- β -D-Galp and β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Galp, was incubated with YvfO. Table 2 shows the monosaccharide composition of this tetramer fraction. Only the trisaccharide Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Galp was found when both galacto-tetrasaccharides were completely degraded (Figure 4.6A).

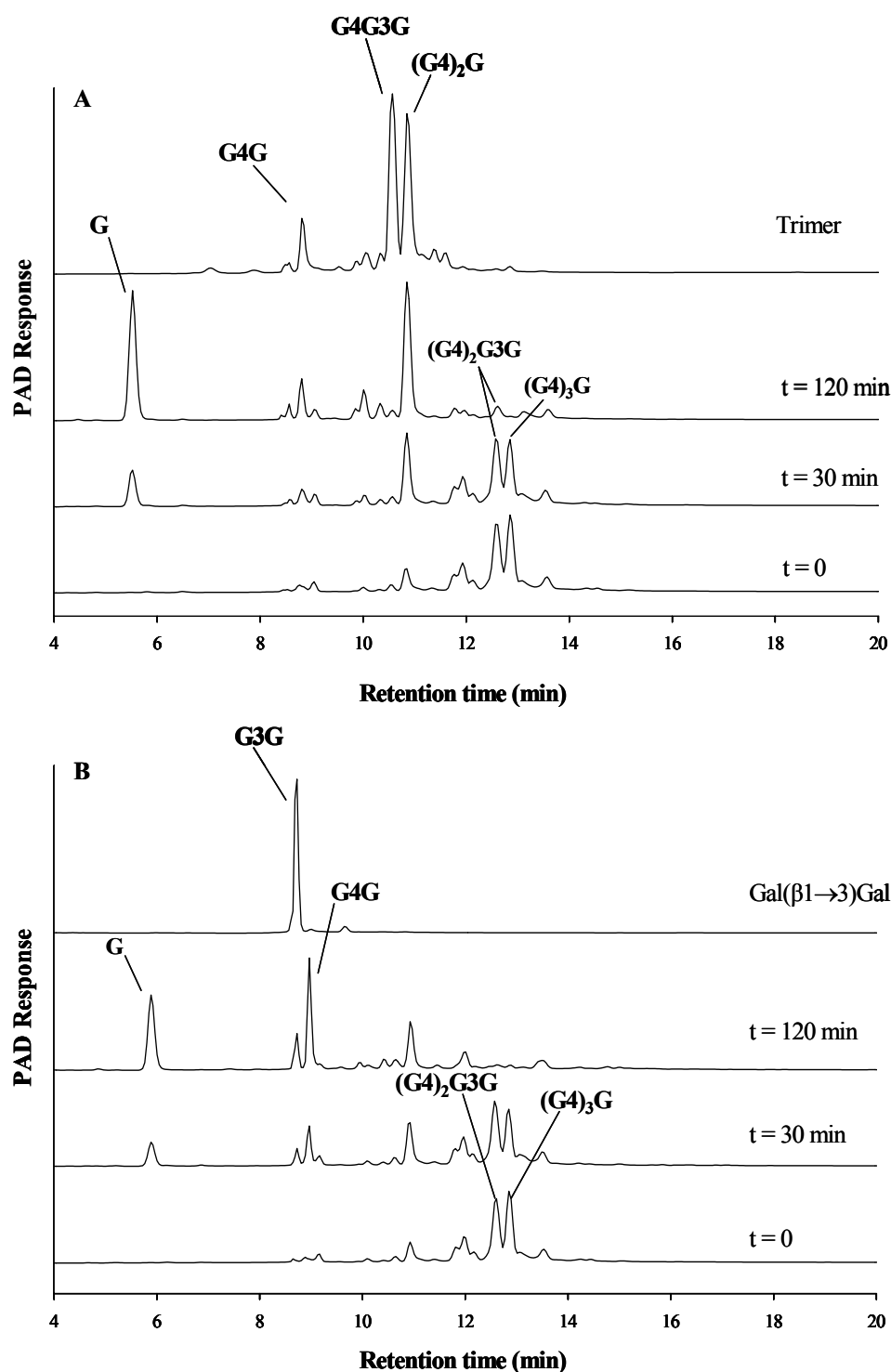


Figure 4.6: HPAEC diagrams of the degradation of the tetramer fraction (purified after partial digestion of potato galactan with endo-galactanase from *A. niger* (14)) with YvfO (A), and endo-galactanase from *A. aculeatus* (B). **G**: galactose; **G4G**: β -D-Galp(1 \rightarrow 4) β -D-Galp; **G3G**: β -D-Galp(1 \rightarrow 3) β -D-Galp; **(G4)₂G**: β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp; **G4G3G**: (β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 3) β -D-Galp; **(G4)₃G**: β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp; **(G4)₂G3G**: (β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 3) β -D-Galp).

From this result, it was concluded that YvfO is able to cleave the (β 1 \rightarrow 3)-linkage in the tetramer. Since a small amount of β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 3)- β -D-Galp is still available after an incubation of 120 min, it is suggested that the (β 1 \rightarrow 4)-linkage may be degraded faster than the (β 1 \rightarrow 3)-linkage. YvfO 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 (37). It is not clear whether the enzyme is hindered by the larger amount of side chains or whether it cannot degrade contiguous (β 1 \rightarrow 3)-linked galactosyl residues. The tetramer fraction was also treated with the endo-galactanase from *A. aculeatus* (Figure 4.6B). This endo-galactanase liberated mainly galactobiose and galactose from both the galacto-tetrasaccharides. This enzyme was less active towards the (β 1 \rightarrow 3)-linkage, since more disaccharide Galp-(1 \rightarrow 3)- β -D-Galp accumulated in contrast with the incubation with YvfO.

TOS (transgalactooligosaccharides) is a mixture of different types of β -galactooligosaccharides, derived after transglycosylation of lactose with a β -galactosidase, and can be used as a prebiotic. TOS was incubated with YvfO. The enzyme liberated galactotrisaccharides from the substrate, which were mainly pentasaccharides, resulting in an increased concentration of di- and trisaccharides (Figure 4.7). 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 Figure 4.3A and C the presence of trisaccharides containing arabinosyl residues was already indicated. The influence of the arabinosyl residues on the activity of YvfO was examined by pre-incubation 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 with YvfO. The amount of trimer liberated from the substrate increased with 13% when a pretreatment was performed (data not shown), which suggested that the enzyme is hindered by the arabinosyl side chains.

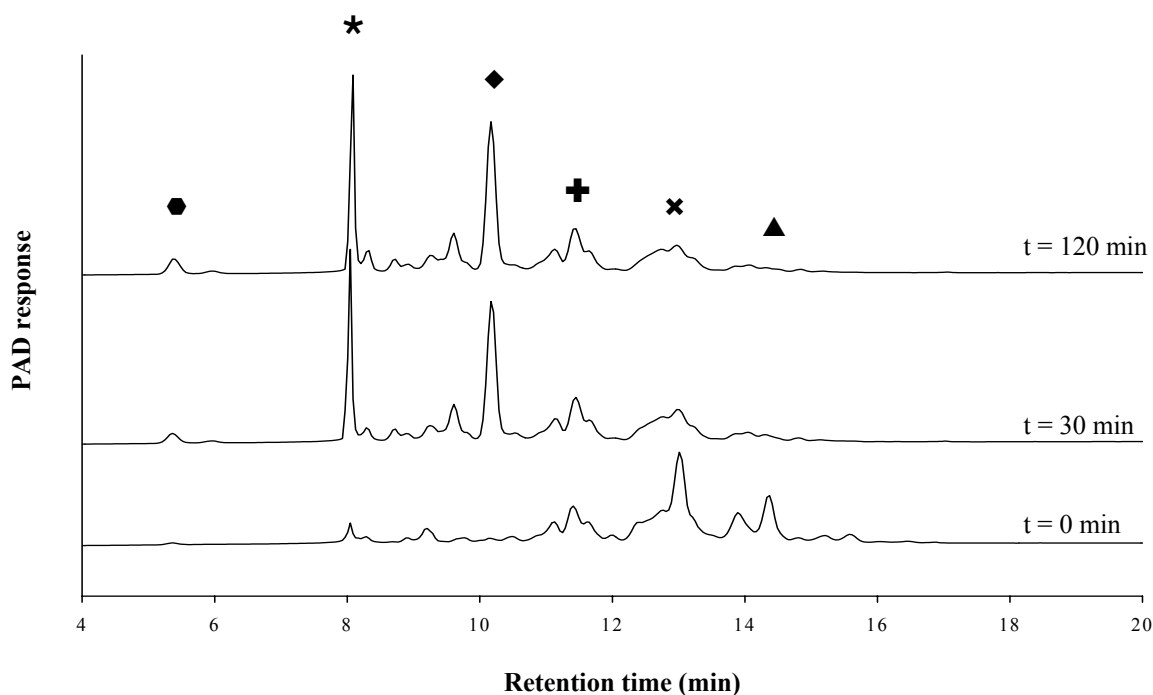


Figure 4.7: HPAEC profiles of TOS degradation with YvfO. The identity of several peaks in the TOS HPAEC profile are unknown, but oligosaccharides present include: $[\beta\text{-D-Galp-(1}\rightarrow\text{4)}]_n\text{-}\beta\text{-D-Galp-(1}\rightarrow\text{4)}\text{-}\beta\text{-D-Glcp}$, $[\beta\text{-D-Galp-(1}\rightarrow\text{4)}]_n\text{-}\beta\text{-D-Galp-(1}\rightarrow\text{6)}\text{-}\beta\text{-D-Glcp}$, $\alpha\text{-D-Glcp-(1}\leftrightarrow\text{1)}\text{-}\beta\text{-D-Galp}$, $\beta\text{-D-Galp-(1}\rightarrow\text{2)}\text{-}\alpha\text{-D-Glcp-(1}\leftrightarrow\text{1)}\text{-}\beta\text{-D-Galp}$, $[\beta\text{-D-Galp-(1}\rightarrow\text{4)}]_n\text{-}\alpha\text{-D-Glcp-(1}\leftrightarrow\text{1)}\text{-}\beta\text{-D-Galp}$, $\beta\text{-D-Galp-(1}\rightarrow\text{4)}\text{-}\alpha\text{-D-Glcp-(1}\leftrightarrow\text{1)}\text{-}\beta\text{-D-Galp-}[(1\rightarrow\text{4)}\text{-}\beta\text{-D-Galp}]_n$, with $n=1\text{-}4$ (11). ●: Galactose and/or glucose; *: galactosyl dimers; ◆: galactosyl trimers; +: galactosyl tetramers; ✕: galactosyl pentamers; ▲: galactosyl hexamers.

DISCUSSION

Several fermentation studies showed that *B. longum* was able to use polymeric arabinogalactan as a substrate (8, 10, 38). 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, YvfO, was able to liberate galacto-trisaccharides from type I arabinogalactan. Galacto-oligosaccharides with both ($\beta 1\rightarrow 4$) and ($\beta 1\rightarrow 3$)-linkages were degraded by YvfO. The enzyme was hindered by arabinosyl side chains of the type I arabinogalactan, and was not active towards type II galactans. Several galactanases are found in bacteria (6, 18, 19, 35), and *Aspergillus* spp. (7, 9, 20, 37). Most endo-galactanases described in literature initially produce a range of oligosaccharides with different degree of polymerisation, and after prolonged incubations only mono- and disaccharides remain. In this paper, we showed that YvfO acts with different mode of action.

The HPSEC profiles of the galactan degradation showed that YvfO initially seemed to act with an endo-mechanism (Figure 4.2). However, the results presented in Figure 4.5 suggested an exo-mechanism. Combining these results, we propose that YvfO acts with a processive mechanism, i.e. after an initial mid-chain (or endo) cleavage the enzyme remains attached to the galactan and liberates galacto-trisaccharides in an exo-fashion (3, 29). Because the enzyme liberated trisaccharides from the galacto-tetrasaccharides, and the (β 1 \rightarrow 3)-linkage was at the reducing end of the oligosaccharides, it was concluded that YvfO is moving towards the reducing end of a galactan chain. A similar mechanism was found for a galactanase from *Bacillus subtilis* (19). This latter enzyme initially liberated galacto-tetrasaccharides from citrus arabinogalactan, which were partially converted to tri-, di-, and monosaccharides during prolonged incubation.

The different mode of action of YvfO might be related to its C-terminal extension. Most endo-galactanases have a molecular mass of 30–50 kDa, whereas YvfO was approximately twice as large (Figure 4.1). A separate BLAST search with this C-terminal part revealed homology to bacterial endo-galactanases (*Enterococcus faecium*, ZP_00286096; *Thermotoga maritima*, NP_229006; *Streptomyces avermitilis*, NP_822499), and dextranase (*Paenibacillus* sp., AAQ91294). It is 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 (21, 31, 32). 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* (40) 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 YvfO is approximately 430 amino acids. Truncation of the *YvfO* gene can help to unravel the nature of this C-terminal part.

As mentioned before, YvfO 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 galacto-trisaccharides formed are directly transported into the bacterial cell (Figure 4.8A), where they can be further degraded by β -galactosidases (30, 34). 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 galacto-oligosaccharides has been suggested for *B. lactis* (12), since this bacteria had seemed to grow better on galacto-trisaccharides and galacto-tetrasaccharides than on mono- and disaccharides. It is unclear whether arabinosylated galacto-oligosaccharides (formed upon arabinogalactan degradation) can be taken up by *B. longum*.

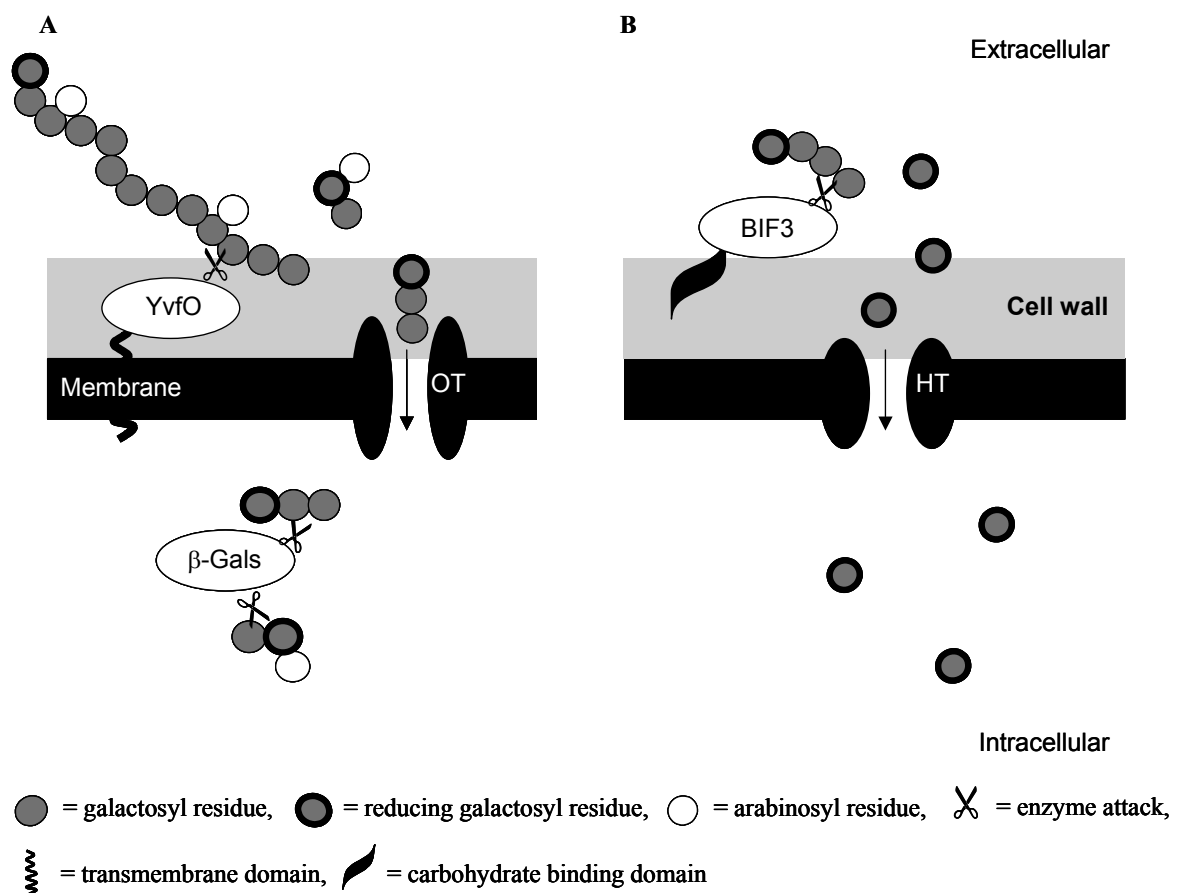


Figure 4.8: Schematic representation of the putative degradation of galactans and internalization of galacto-oligosaccharides in *Bifidobacterium longum* (A) and *Bifidobacterium bifidum* (B). YvfO: endo-galactanase YvfO from *B. longum*; BIF3: β -galactosidase BIF3 from *B. bifidum*; β -Gals: different β -galactosidases; OT: oligosaccharide-transporter; HT: hexose-transporter.

The presence of oligosaccharide-transporters is not necessarily common in bifidobacteria. It is known that *B. bifidum* contains a β -galactosidase (BIF3), which is expected to be located extracellularly (23), 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 (24). In this case, galacto-oligosaccharides might be degraded extracellularly to monomers, which can be internalized through the more common hexose-transporters, and no special oligosaccharides-transporter will be needed (Figure 4.8B). From the considerations above, it is evident that bifidobacteria can have different toolboxes to utilize galactans or galacto-oligosaccharides. It will be necessary to conduct more research to further unravel the mechanisms by which bifidobacteria degrade and take up carbohydrates.

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Increasing the transglycosylation activity of α -galactosidase from *Bifidobacterium adolescentis* DSM 20083 by site-directed mutagenesis

The α -galactosidase (AGA) from *Bifidobacterium adolescentis* DSM 20083 has a high transglycosylation activity. The optimal conditions for this activity are pH 8, and 37°C. At high melibiose concentration (600 mM), approximately 64% of the enzyme-substrate encounters resulted in transglycosylation. Examination of the acceptor specificity showed that AGA required a hydroxyl group at C-6 for transglycosylation. Pentoses, hexuronic acids, deoxyhexoses, and alditols did not serve as acceptor molecules. Disaccharides were found to be good acceptors.

A putative 3D-structure of the catalytic site of AGA was obtained by homology modeling. Based on this structure and amino acid sequence alignments, site-directed mutagenesis was performed to increase the transglycosylation efficiency of the enzyme, which resulted in four positive mutants. The positive single mutations were combined, resulting in six double mutants. The mutant His⁴⁹⁷Met had an increase in transglycosylation of 16%, whereas most of the single mutations showed an increase of 2-5% compared to the wild-type AGA. The double mutants Gly³⁸²Cys-Tyr⁵⁰⁰Leu, and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu had an increase in transglycosylation activity of 10-16%, compared to the wild-type enzyme, whereas the increase for the other double mutants was low (4-7%). The results show that with a single mutation (His⁴⁹⁷Met) the transglycosylation efficiency can be increased from 64 to 75% of all enzyme-substrate encounters. Combining successful single mutants in double mutations did not necessarily result in an extra increase in transglycosylation efficiency. The donor and acceptor specificity did not change in the mutants, whereas the thermostability of the mutants with Gly³⁸²Cys decreased drastically.

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INTRODUCTION

Prebiotic substrates like raffinose and stachyose are easily degraded by α -galactosidases from bifidobacteria (11, 20, 26, 35). In previous research (20, 32, 33), an α -galactosidase from *B. adolescentis* (AGA) was isolated, cloned and expressed in *Escherichia coli* cells. The enzyme was classified in glycoside hydrolase (GH) family 36. AGA possessed transglycosylation activity (32) and was able to elongate acceptor substrates, like melibiose, raffinose and stachyose, with (α 1 \rightarrow 6)-galactosyl residues at their non-reducing end (32, 33). However, not all enzyme-substrate encounters result in a transglycosylation reaction, since water can compete with the saccharides as acceptor molecule, resulting in a hydrolysis reaction.

Glycosyl transferases synthesizing (α 1 \rightarrow 6) galactosyl oligosaccharides also occur naturally and these enzymes do not seem to have hydrolytic activity. Interestingly, two different types of plant transferases occur in the same GH family as the *B. adolescentis* α -galactosidase (GH36) and the closely related family GH27. One of these transferases is stachyose synthases (GH36), which catalyzes the synthesis of stachyose by galactosyl transfer from galactinol to raffinose, and of verbascose by galactosyl transfer from galactinol to stachyose, as well as by self-transfer of the terminal galactosyl residue from one stachyose to another (25). The other transferases are galactan:galactan galactosyltransferases (GGTs) from GH-family 27. These GGTs are involved in the biosynthesis of the long-chain raffinose family oligosaccharides (RFOs) by catalyzing the transfer of an α -galactosyl residue from one RFO molecule to another (15). The amino acid sequence of these plant α -galactosyl transferases might be used to pinpoint important residues for transglycosylation reaction, which could subsequently be introduced in AGA by site-directed mutagenesis.

Several papers indicate that it is possible to enhance the efficiency to perform transglycosylation reactions by mutagenesis of an enzyme (17, 18, 21, 22). From the results of these papers, several possible factors have been suggested to promote the transglycosylation reaction of an enzyme. First, aromatic amino acids are involved in stabilizing the binding of the substrate through non-polar stacking interactions (21); replacement of these residues with non-aromatic residues might change the geometry of binding oligosaccharides, and therewith enhance transglycosylation (factor I). Second, Tyr, Trp, and His residues can form strong H-bonds with water and the substrate, thereby positioning these molecules in a manner that favours hydrolysis reactions (4) (factor II). Third, amino acids, capable of forming H-bonds, that are located further away from the catalytic site might favour transglycosylation by

trapping water molecules, which might therefore not be available for hydrolysis (16) (factor III). Fourth, a high hydrophobicity of the entrance of the catalytic site might reduce the amount of water entering the enzyme and lower the hydrolysis activity (19, 27) (factor IV). This factor probably only applies to enzymes which contain a pocket-like structure (6), because the water molecule can enter the catalytic site only at one position. Groove-like structures are more open and allow water molecules to enter the catalytic site at many places. Our goal was to improve the transglycosylation activity of the α -galactosidase from *Bifidobacterium adolescentis* DSM 20083 and therewith gaining more insight in the structure-function relationships underlying the transglycosylation reaction. The transglycosylation activity of this enzyme is characterized and amino acids close to the catalytic site are modified by site-directed mutagenesis. The *aga* gene was mutagenized according to the amino acid sequences of both the stachyose synthases and GGTs and the above mentioned possible factors suggested to be determinants of transglycosylation activity.

MATERIAL AND METHODS

Bacterial strains, and growth conditions

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 μ g/ml ampicillin, when appropriate.

The *E. coli* cells containing the α -galactosidase gene (*aga*) and the mutants were grown in LB broth or solidified LB medium supplemented with 100 μ g/ml ampicillin and 1 mM isopropyl β -D-thiogalactopyranoside (IPTG).

Chemicals, substrates and enzymes

Chemicals and substrates were purchased from Sigma (St. Louis, Mo., USA) unless stated otherwise. 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.

Cloning of the wild-type *aga* gene.

The wild-type α -galactosidase gene from *Bifidobacterium adolescentis* DSM 20083, was described by Van den Broek et al. (32). A polymerase chain reaction (PCR) was carried out

with *Pfu*-turbo polymerase (Stratagene, La Jolla, California) to obtain the *aga* open reading frame of the wild-type gene. Two primers were used for amplification of the *aga* gene (*Xba*I and *Hind*III restriction sites were introduced at the beginning and at the end of the gene, respectively, for cloning purpose). Primers used were (with the restriction sites underlined): AR1GAL (5'-GCGCTCTAGAGCAATGACGCTCATTCA), and AR2REVGAL (5'-CGCGAAGCTTTACTCA-GATGCGGACTA). The forth-coming DNA fragments were ligated into a *Xba*I/*Hind*III digested *p*Bluescript vector (Stratagene) or *p*ETBlue Perfectly Blunt Vector (Novagen, Darmstadt, Germany). The recombinant plasmid was transformed into *E. coli* XL1 blue MRF' cells for expression.

Construction of mutants

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to make selected point mutations according to the instructions of the manufacturer. For each single mutant, a sense/antisense primer pair was designed, which included silent mutations encoding restriction sites to verify the inserted mutations (sense primer, introduced mutations in boldface, codon for mutated amino acid italic, restriction sites underlined, and restriction enzymes in parentheses): Asp³⁸⁰Asn (5'-GGAACGCTTCGTCGTGA**A**CGATGCTGGTTC-CGCCGCCG-3') (*Nar*I); Asp³⁸¹Asn (5'-GGAACGCTTCGTCGTGGATA**A**CGGCTGGTC-CGCCGCCG-3') (*Nar*I); Gly³⁸²Cys (5'-TCGTGGATGAT**T**GCTGGTTCCGGAGCCCGCC-AAC-3') (*Ban*I); Phe³⁸⁴Leu (5'-GTGGATGATGGCTGG**C**TCGGAGCCCGCCGAGAC-3') (*Ban*I); Tyr⁴⁹²Ala (5'-CGGCGAACTCGGCATCGAT**T**GCCATCAAATGGGATCAC-3') (*Cla*I); Tyr⁴⁹²Gly (5'-CGGCGAACTCGGCATCGAT**T**GCCATCAAATGGGATCAC-3') (*Cla*I); Trp⁴⁹⁵Leu (5'-CGGCATCGAT**T**TACATCAAAT**T**GGATCACAACAAATACGTCA-CCG-3') (*Cla*I); Trp⁴⁹⁵Tyr (5'-GCTGGTCGGCATCGAT**T**TACATCAAAT**A**CGATCACAA-CAAATACG-3') (*Cla*I); His⁴⁹⁷Met (5'-CGGCATCGAT**T**TACATCAAATGGGAT**A**TGAACAAATACGTCAACCG-3') (*Cla*I); Asn⁴⁹⁸Cys (5'-CGGCATCGAT**T**TACATCAAATGGGAT-CACT**T**GCAAATACGTCAACCG-3') (*Cla*I); Tyr⁵⁰⁰Leu (5'-GGATC-ACAACAA**A**CTCGTC-ACCGAAGCGGTGTCGCCGCGGACCGG-3') (*Sac*II). The wild-type α -galactosidase gene (*aga*) was used as the template DNA. For each double mutant, a sense/antisense primer pair of the single mutants was used, accept for Tyr⁴⁹²Gly-His⁴⁹⁷Met (5'-CGAACTCGGC-ATCGAT**T**GCCATCAAATGGGAT**A**TGAACAAAT-3', template: mutant Tyr⁴⁹²Gly), and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu (5'-ATCGACTACATCAAATGGGAT**A**TGAACAA**A**CTCGTCACCGA-3', template: mutant His⁴⁹⁷Met).

Plasmid DNA was isolated by following the Qiagen plasmid purification method (Qiagen, Hilden, Germany). Positive colonies were selected according to the restriction pattern of the plasmids upon digestion with the specific restriction enzyme for the silent mutation. To verify if the point mutation was actually introduced, the DNA-nucleotide sequence of the plasmid was determined. After verification, the desired mutant plasmid was transformed to *E. coli* XL1 blue MRF' cells.

Isolation and characterization of mutants

Cells from an *E. coli* cell culture (1 l, LB-medium, 37°C, overnight) containing the wild-type or mutated *aga* genes were harvested by centrifugation (10 min; 3,000 x g; 4°C). The supernatant was removed and used for activity measurement; the cells were suspended in 40 ml 20 mM sodium phosphate buffer pH 7 and disrupted by sonic treatment (10 min; amplitude 30%, duty cycle 0.3 s on, 0.7 s off; Digital Sonifier, Branson) on ice. Subsequently, the suspension was centrifuged (10 min; 13,000 x g; 4°C), the cell free extract was collected, and the pellet suspended in 40 ml 20 mM sodium phosphate buffer pH 7 and a second sonic treatment was performed. The cell-free extracts were pooled and applied onto a Q-Sepharose Hiload 26/10 (Amersham, Little Chalfont, UK) anion-exchange column. Elution took place with a linear gradient of 0-0.5 M NaCl in 20 mM sodium phosphate pH 7 at a flow rate of 4 ml/min. Fractions with the highest α -galactosidase activity were pooled and further purified on a Superdex 200 PG Hiload 16/26 (Amersham) size-exclusion column. Elution was performed with 0.15 M NaCl in 20 mM potassium phosphate buffer pH 7.0 at a flow rate of 1 ml/min. Fractions with the highest α -galactosidase activity were pooled and concentrated with anion-exchange chromatography under the same conditions as described above.

Protein concentration was determined by the method of Bradford (3) using bovine serum albumin (BSA) as a standard. 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 8-25% gradient gels (Amersham).

Enzyme assays

Side activity of the purified enzymes was measured by determining the hydrolysis of *p*-nitrophenyl- α -D-glycosides (*p*NP- α -L-arabinopyranoside, *p*NP- α -L-arabinofuranoside, *p*NP- β -D-xylopyranoside, *p*NP- α -D-glucopyranoside, *p*NP- β -D-glucopyranoside, *p*NP- β -D-fucopyranoside, and *p*NP- α -L-rhamnopyranoside) at 37°C after 10 min incubation. The

reaction mixture (125 μ l) consisted of 2 mM potassium phosphate buffer pH 6.0, and 0.2 mg/ml *p*NP-glycoside solution. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer pH 9.0, containing 2 mM EDTA. The color formation was measured at 400 nm.

α -Galactosidase activity was measured by determining the hydrolysis of *p*-nitrophenyl- α -D-galactopyranoside (*p*NP- α -Gal) under the conditions mentioned above. One unit (U) of activity was defined as the amount of enzyme that liberates 1 μ mol of galactose per min under the specified conditions. The molar extinction coefficient of *p*-nitrophenol under these assay conditions was 13,700 M⁻¹cm⁻¹.

Optimal transglycosylation conditions of the wild-type *aga* were measured with 0.04 U α -galactosidase and 300 mM melibiose in McIlvain buffer (0.1 M citric acid/0.2 M di-sodium phosphate), in a total volume of 100 μ l. The enzyme's optimal transglycosylation pH determined with McIlvain buffers in the range of pH 3-8 and a temperature of 40°C during 4 h. For determination of the optimal transglycosylation temperature, temperatures between 20 and 70°C were used for incubation at pH 8 during 4 h. After incubation, the samples were 10 times diluted with McIlvain buffer pH 5, and were stopped by heating the incubation mixtures for 5 min at 100°C. All reactions were carried out in duplicate. After centrifugation (10 min, 10,000 \times g) the supernatant was analyzed by High-Performance Anion-Exchange Chromatography (HPAEC).

Acceptor specificity was measured with 300 mM melibiose, 300 mM acceptor substrate, 0.04 U α -galactosidase in McIlvain buffer pH 8, in a total volume of 100 μ l at 40°C during 7 hours. The incubations were diluted, stopped and analyzed as described before. All reactions were carried out in duplicate.

Transglycosylation activity was measured with 600 mM melibiose, 0.04 U α -galactosidase in 100 mM Tris-HCL buffer pH 8, in a total volume of 100 μ l at 40°C during 7 hours. The incubations were diluted, stopped and analyzed as described before. All reactions were carried out in triplicate.

The acceptor specificity of the wild-type and mutated *aga* was determined by the incubation of 300 mM melibiose, 300 mM acceptor and 0.04 U α -galactosidase at pH 8, 40°C and during 7 h. The incubations were diluted, stopped and analyzed as described before. All reactions were carried out in duplicate.

Analytical methods

HPAEC was performed using a Thermo-Quest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column in combination with a CarboPac PA guard column (3 mm ID x 25 mm) and a Dionex ED40 PAD-detector (Dionex Co., Sunnyvale, USA). A flow rate of 1 ml/min was used with a combination of linear gradients of sodium acetate and NaOH: 0-20 min, 16 mM NaOH; 20-25 min, 16-100 mM NaOH; 25-40 min, 0-10 mM NaOAc in 0.1 M NaOH; 40-45 min, 10-100 mM NaOAc in 0.1 M NaOH. Each elution was followed by a washing step of 5 min 1,000 mM sodium acetate in 0.1 M NaOH and an equilibration step of 15 min 16 mM NaOH.

Statistical analysis

Statistical analysis of the transglycosylation data was performed using General Linear Model in SPSS 10.0.7. Z-values $> |2|$ were considered as outliers. Simple contrasts were calculated to determine significant differences between the mutant enzymes with the wild-type AGA as the reference group at $\alpha < 0.05$.

DNA sequencing and sequence analysis

An automated DNA sequencer 373 (Applied Biosystems) was used to determine the nucleotide sequence of the gene. The DNA sequence data of *aga* are available at GenBank nucleotide databases under the accession number AF124596. The BLAST2 program ((1), available at <http://www.ncbi.nlm.nih.gov/>) was used for searching sequence homologies. The alignment of the amino acid sequences is made by use of the clustalW multiple alignment function with a Pam250 matrix and was manually edited. This alignment and the SWISS-MODEL Version 36.0003 program (14, 24, 29) (available from <http://www.expasy.org/swissmod/SWISS-MODEL.html>) were used for homology modeling.

RESULTS

Transglycosylation conditions for wild-type α -galactosidase from *Bifidobacterium adolescentis*

The wild-type α -galactosidase (AGA) from *Bifidobacterium adolescentis* was used to synthesize longer oligosaccharides, using melibiose as a donor substrate (Figure 5.1). The transglycosylation products were identified as α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)-D-Glcp,

and α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)-D-Glcp (data not shown). These results are in agreement with the native α -galactosidase from *B. adolescentis* (33). A third transglycosylation product was found at a retention time of 21 min, which is suggested to be α -D-Galp-(1 \rightarrow 6)-D-Galp, since this peak increased when both melibiose and galactose were incubated with the α -galactosidase (determination of acceptor specificity).

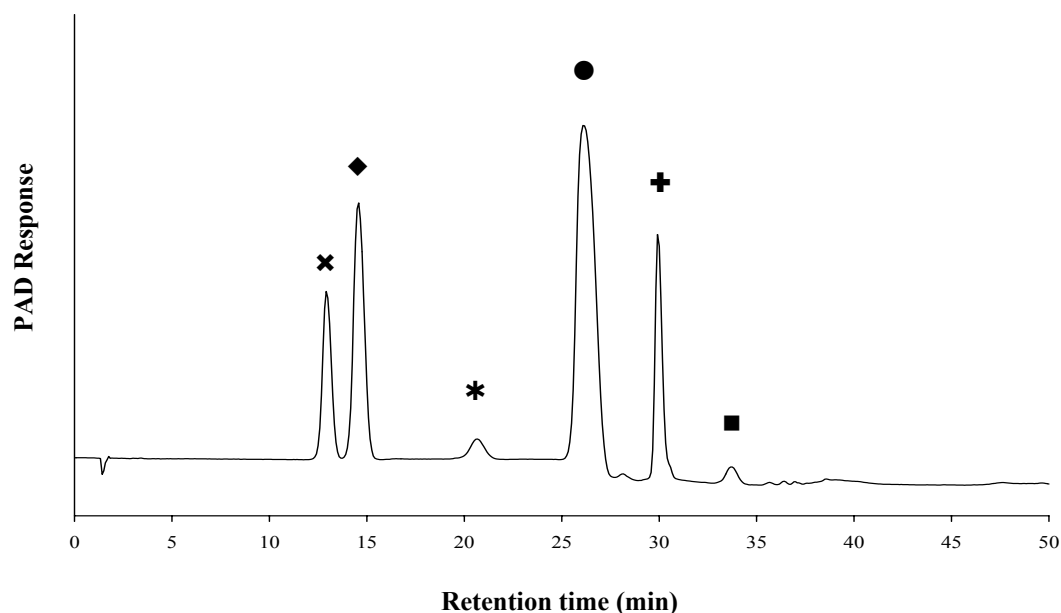


Figure 5.1: HPAEC diagram of the incubation of the wild-type α -galactosidase from *B. adolescentis* with melibiose. The reaction was carried out during 7 h at pH 8, and 37°C. The initial concentration of melibiose was 300 mM. **x**: galactose; **♦**: glucose; *****: tentative galactosyl dimer (α -D-Galp-(1 \rightarrow 6)- α -D-Galp); **●**: melibiose (α -D-Galp-(1 \rightarrow 6)- α -D-Glcp); **+**: galactosyl-melibiose (α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)- α -D-Glcp); **■**: galactosyl-galactosyl-melibiose (α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 6)- α -D-Glcp).

The proportion of hydrolysis and transglycosylation reactions catalyzed by AGA was determined as follows: the concentration of galactose represented the amount of hydrolysis, whereas the concentration of glucose represented the sum of hydrolysis and transglycosylation. The highest relative amount of transferase activity was found at pH 8 and 37°C (data not shown). At a melibiose concentration of 300 mM, approximately 50 of every 100 enzyme-substrate encounters resulted in a transfer reaction. At higher melibiose concentrations the transglycosylation activity reached a maximum of 69% (Figure 5.2).

Different acceptor substrates were examined (Table 5.1). D-Galactose, D-glucose, L-glucose, D-mannose, lactose, maltose, cellobiose, and sucrose were found to be good acceptors for α -galactosidase, when melibiose was used as donor. AGA used both the acceptor substrate and

melibiose as acceptor. Pentoses (*e.g.* Xyl), hexuronic acids (*e.g.* GalA), and deoxyhexoses (*e.g.* Rha) did not serve as acceptor molecules (Table 5.1). Also alditols were poor acceptors. Disaccharides, containing a hexose at the non-reducing terminus, were found to be good acceptors. From these experiments, it was concluded that AGA required a hydroxyl group at C-6 at the non-reducing end of the acceptor substrate to perform transglycosylation. In most cases, one sugar unit was linked to the acceptor molecule; the yield of longer oligosaccharides was very low.

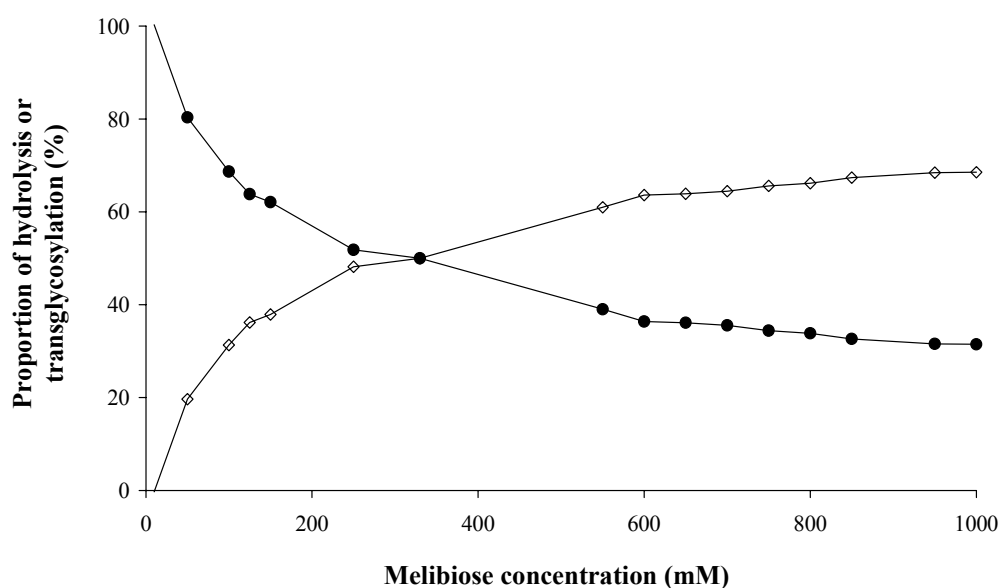


Figure 5.2: Influence of the melibiose concentration on the proportion of hydrolysis and transglycosylation activity of wild-type α -galactosidase. The reaction was carried out during 7 h at pH 8 and 37°C. ●: hydrolysis; ◇: transglycosylation.

Characterization of *aga* gene and design of single mutants

It was found in the literature that it is possible to enhance the transglycosylation activity of a glycoside hydrolase by mutagenesis (17, 18, 21, 22). Since AGA already possesses a high transglycosylation activity in comparison with other α -galactosidases (31), it seems an interesting enzyme for the synthesis of oligosaccharides. However, for more efficient elongation of oligosaccharides, it is important to enhance the transglycosylation activity and reduce the hydrolytic activity.

The 3D-structure of AGA is not known. It belongs to GH36, of which no 3D-structure is currently available. However, GH36 has a strong relationship with GH27 and consequently these families are grouped in the same clan (GH-D). Four 3D-structures from GH27 are

known: α -galactosidase from *Gallus gallus* (10), *Oryza sativa* (8), *Homo sapiens* (9), and *Trichoderma reesei* (13).

Table 5.1: Acceptor specificity for the transglycosylation reactions of the wild-type α -galactosidase from *B. adolescentis*. The reaction was carried out during 7 h at pH 8 and 37°C. The initial concentration of melibiose and that of the acceptor substrate were both 300 mM. The ability of α -galactosidase to use a substrate as acceptor is indicated with +. When α -galactosidase is unable to use a substrate as acceptor, this is indicated with –.

Substrate	Acceptor
D(+)-Galactose	+
D(+)-Xylose	-
Lactose	+
Maltose	+
D(+)-Glucose	+
Ethanol	-
D(+)-Mannose	+
Sucrose	+
L-Glucose	+
D(-)-Sorbitol	-
D-Galacturonzuur	-
L(+)-Rhamnose	-
D(+)-Cellobiose	+
L(+)-Arabinose	-

An amino acid alignment was made with AGA and four groups of enzymes, including the four α -galactosidases of which a 3D-structures is known (Figure 5.3). Group I contained bacterial GH36 α -galactosidases, group II GH36 plant stachyose synthases, group III GH27 α -galactosidases, and group IV GH27 galactan:galactan galactosyltransferases. Based on the 3D-structures and sequence comparisons, the catalytic residues of AGA were suggested to be Asp⁴⁹⁶ and Asp⁵⁶². The alignment showed that these catalytic residues and the residues Asp³⁸⁰ and Asp³⁸¹ were conserved in all enzymes. Two more amino acids were conserved in all enzymes, i.e. Trp³⁸³ and Lys⁴⁹⁴. Figure 5.3 shows three different regions of the alignment. Two regions show the regions around the catalytic amino acids of Asp⁴⁹⁶ (B) and Asp⁵⁶² (C). The other shows the region around the residues Asp³⁸⁰ and Asp³⁸¹ (A).

A putative 3D-structure of the catalytic site of AGA was constructed by homology modeling, using the structure of *O. sativa* (8) as a template (Figure 5.4). Of the four enzymes with a known 3D-structure, the one from *O. sativa* had the highest identity (23%) with AGA. Amino acids 371–584 were incorporated in the model. The putative 3D-structure of AGA showed that the residues Asp⁴⁹⁶, Asp⁵⁶², Asp³⁸⁰, and Asp³⁸¹ were all located in the catalytic site, at a distance of approximately 6–8 Å from each other. This indicated that, besides the catalytic residues Asp⁴⁹⁶ and Asp⁵⁶², also Asp³⁸⁰ and Asp³⁸¹ might be important for the catalytic activity of the enzyme. To conclude on this, two mutants were made by site-directed mutagenesis, in which the Asps was replaced by an Asns (Asp³⁸⁰Asn and Asp³⁸¹Asn). Lys⁴⁹⁴ might be involved in substrate binding, as the Lys residue in the α -galactosidase from rice (8), but mutagenesis of this residue was not further pursued since it was conserved in all enzymes. Special attention was devoted to Tyr, Trp, His, and Phe residues close to the catalytic residues, because these amino acids might play an important role in positioning the substrate molecule and catalytic water through stacking interactions and/or H-bonds (factors I and II). Mutation of these residues to non-aromatic amino acids might reduce the hydrolytic activity of AGA, and increase its transglycosylating activity. Combining the amino acid sequence and the putative 3D-structure of AGA, six aromatic residues were found, which were located in or close to the catalytic site (Figure 5.4). The putative 3D-structure shows that catalytic residue Asp⁴⁹⁶ is located on a strand containing amino acids 491–497 (indicated in red). This strand is schematically represented in Figure 5.5. The aromatic side chains of Tyr⁴⁹², His⁴⁹⁷, and Tyr⁵⁰⁰ are located in the catalytic cleft (Figure 5.4 and 5.5). These amino acids were subjected to site-directed mutagenesis to replace the aromatic side chain by a non-aromatic one, resulting in four mutants Tyr⁴⁹²Gly, Tyr⁴⁹²Ala, His⁴⁹⁷Met, and Tyr⁵⁰⁰Leu. The Gly residue in mutant Tyr⁴⁹²Gly was chosen because it was conserved in three stachyose synthases from GH36 (Figure 5.3). The Met in mutant His⁴⁹⁷Met was chosen because it is hydrophobic, non-aromatic, and present in other bacterial GH36 enzymes (Figure 5.3). The Leu and Ala in the mutants Tyr⁵⁰⁰Leu and Tyr⁴⁹²Ala, respectively, were chosen because they are hydrophobic and non-aromatic.

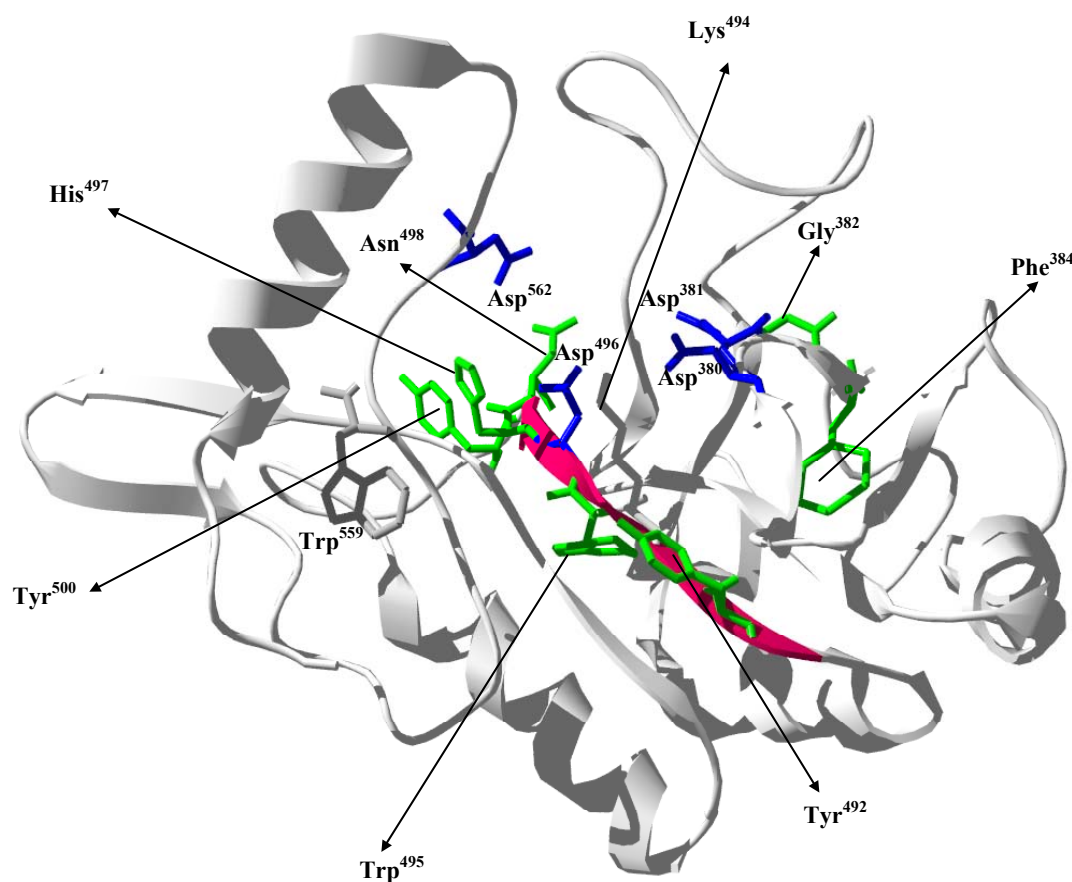


Figure 5.4: Putative 3D-structure of α -galactosidase AGA from *B. adolescentis*, obtained by homology modeling, using the 3D-structure of α -galactosidase from *Oryza sativa* as a template. The putative catalytic residues, Asp⁴⁹⁶ and Asp⁵⁶², and the residue involved in substrate binding, Asp³⁸⁰ and Asp³⁸¹ are indicated in blue. Amino acid residues which are subjected to site-directed mutagenesis are shown in green. Residues Trp⁵⁵⁹ and Lys⁴⁹⁴ are indicated in grey. The strand of amino acids 491-497 was colored red.

The three other aromatic residues Phe³⁸⁴, Trp⁴⁹⁵, and Trp⁵⁵⁹ are located near the catalytic site, but point away from the cleft. Therefore, they are less available for positioning the substrate or water. They might, however, be involved in capturing water molecules, and make them less available for hydrolysis (factor III). Two of these residues were modified to a hydrophobic, non-aromatic residue, Leu (Phe³⁸⁴Leu and Trp⁴⁹⁵Leu). Since GGTs are synthesizing enzymes, the Trp⁴⁹⁵ was replaced by a Tyr, because this Tyr is conserved in GH27 GGTs.

Two more mutants were created, based on the amino acid sequence of these GGTs. The residues Gly³⁸² and Asn⁴⁹⁸ of AGA were both replaced by a Cys residue (Figure 5.3 and 5.4). Cys residues are conserved at the corresponding positions of Gly³⁸² and Asn⁴⁹⁸ in GGTs.

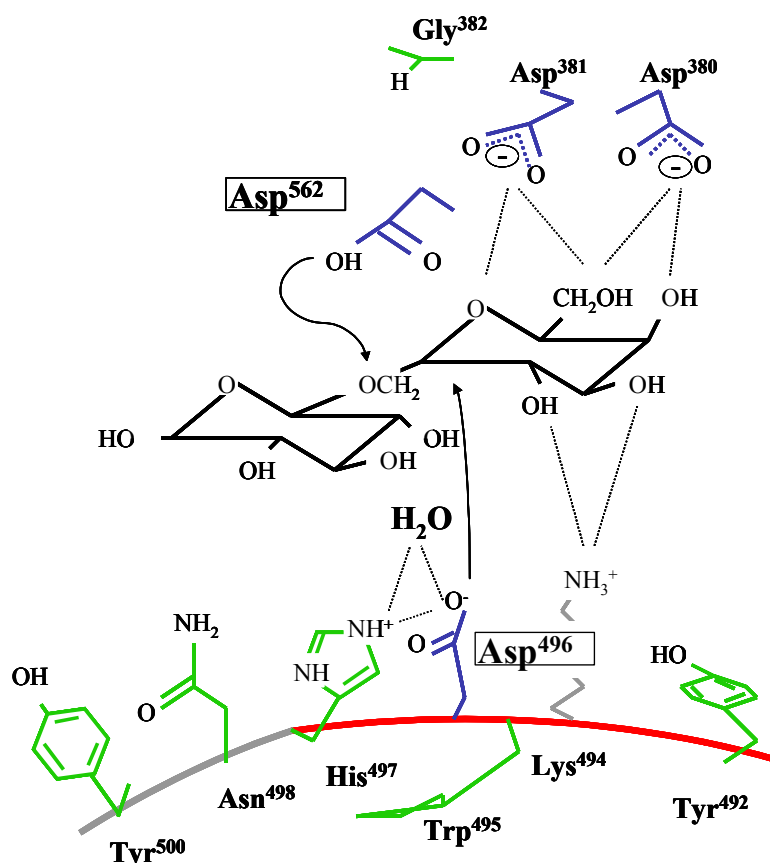


Figure 5.5: Schematic representation of the catalytic site of α -galactosidase AGA from *B. adolescentis* with melibiose as substrate based on the putative 3D-structure. The putative catalytic residues, Asp⁴⁹⁶ and Asp⁵⁶², and the residues involved in substrate binding, Asp³⁸⁰ and Asp³⁸¹ are indicated in blue. Amino acid residues subjected to site-directed mutagenesis are shown in green. Residue Lys⁴⁹⁴ is indicated in grey. The strand of amino acids 491-497 is colored red. The possible H-bonds are indicated with dotted lines. The action of the catalytic residues are indicated by arrows.

Hydrolysis and transglycosylation activity of mutants.

Eleven different single mutations in the *aga* gene were obtained: Asp³⁸⁰Asn, Asp³⁸¹Asn, Gly³⁸²Cys, Phe³⁸⁴Leu, Tyr⁴⁹²Ala, Tyr⁴⁹²Gly, Trp⁴⁹⁵Leu, Trp⁴⁹⁵Tyr, His⁴⁹⁷Met, Asn⁴⁹⁸Cys, and Tyr⁵⁰⁰Leu. These mutant genes were cloned and expressed in *E. coli* XL1 blue MRF' cells. Cell-free extracts were used for purification of the mutant α -galactosidases by anion-exchange chromatography and size-exclusion chromatography. The mutants were not completely pure after these chromatographic steps, as judged by SDS-PAGE and Coomassie brilliant blue staining. However, no activity towards a series of *p*NP-glycosides, other than *p*NP- α -Gal, was found. The *E. coli* cells itself did not show α -galactosidase activity. The α -galactosidase hydrolytic activity towards *p*NP- α -Gal and the transglycosylation activity towards melibiose

was determined. These results showed that the hydrolytic activity was lost in the mutants Asp³⁸⁰Asn and Asp³⁸¹Asn, suggesting that these residues were indeed involved in the catalytic activity of the enzyme. The hydrolytic activity towards *p*NP- α -Gal seemed to be unaltered in all mutant enzymes (data not shown). The mutants Gly³⁸²Cys, Tyr⁴⁹²Gly, His⁴⁹⁷Met and Tyr⁵⁰⁰Leu showed a higher transglycosylation efficiency than the wild-type enzyme (Figure 5.6), for the mutations Tyr⁴⁹²Gly, His⁴⁹⁷Met and Tyr⁵⁰⁰Leu this increase was significant ($P < 0.05$). Only mutant His⁴⁹⁷Met increased by 16% in transglycosylation activity under the conditions tested, whereas that of the other mutants increased by approximately 5%.

To enhance the transglycosylation activity further, double mutants were made. All four single mutations, which gave an increased transglycosylation, were combined to six double mutants: Gly³⁸²Cys-Tyr⁴⁹²Gly, Gly³⁸²Cys-His⁴⁹⁷Met, Gly³⁸²Cys-Tyr⁵⁰⁰Leu, Tyr⁴⁹²Gly-Tyr⁵⁰⁰Leu, Tyr⁴⁹²Gly-His⁴⁹⁷Met, and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu. The double mutants were also cloned and expressed in *E. coli* cells and purified as mentioned before. The hydrolytic activity towards *p*NP- α -Gal was determined and seemed to be unaltered in the mutant enzymes (data not shown). The transglycosylation activity of the double mutants increased significantly ($P < 0.05$). The mutants Gly³⁸²Cys-Tyr⁵⁰⁰Leu and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu increased to 10 and 16%, respectively, compared to the wild-type, whereas the other mutant enzymes had an increase of approximately 4-7% (Figure 5.6). This means that the best double mutant had approximately the same transglycosylation activity as single mutant His⁴⁹⁷Met.

Acceptor specificity and stability of mutants

The acceptor specificity of the mutants was determined. AGA was found to require a hydroxyl group at C-6 at the non-reducing end of the acceptor substrate. The transglycosylation activity of the mutants towards hexose (glucose), pentose (xylose), deoxyhexose (rhamnose) and hexuronic acid (glucuronic acid) was unaltered (data not shown).

Due to the mutations in the α -galactosidase gene, the folding of the enzyme might be changed and therewith the stability of the enzyme. The wild-type AGA was found to be stable for at least 24 h at temperatures below 50°C. The temperature stability of the double mutant enzymes was unaltered, except for the enzymes containing the mutation Gly³⁸²Cys. These were unstable at temperatures above 37°C.

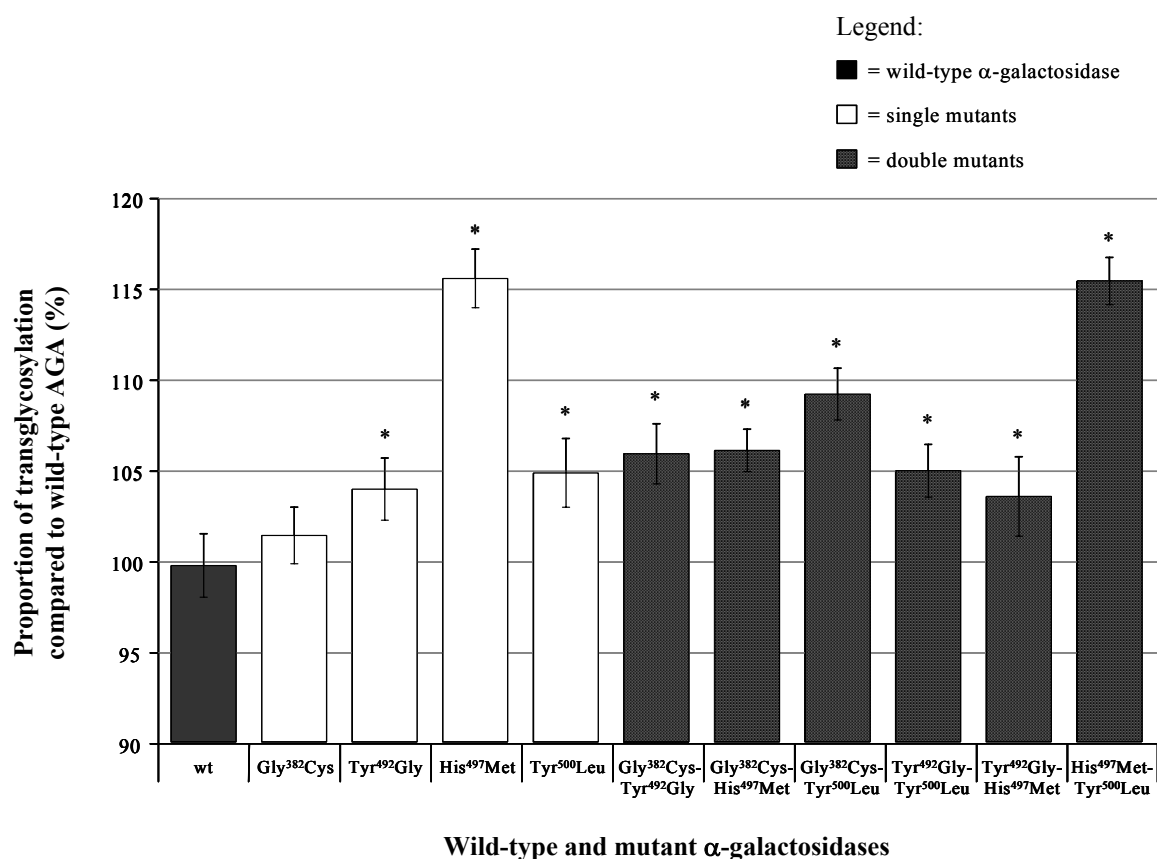


Figure 5.6: Percentage of transglycosylation of single mutants and double mutants made from *B. adolescentis* α -galactosidase. The reaction was carried out during 7 h at pH 8 and 37°C. The initial concentration of melibiose was 600 mM. * indicates a significant difference with the wild-type enzyme ($P < 0.05$).

DISCUSSION

This paper showed that it is possible to enhance the transglycosylation activity of AGA by site-directed mutagenesis. In the putative 3D-structure of AGA four Asp residues were found in the catalytic site of the enzyme. Two residues were identified as the catalytic residues, i.e. Asp⁴⁹⁶ and Asp⁵⁶². The two other residues, Asp³⁸⁰ and Asp³⁸¹, were subjected to site-directed mutagenesis; mutation from Asp to Asn resulted in an inactive enzyme. Therefore, it was concluded that all four Asp residues were involved in the catalysis, suggesting that the enzyme has a catalytic tetrad. Such a tetrad has also been described for β -glucanase from barley (5). There, the two non-catalytic residues of the catalytic tetrad were suggested to be involved in substrate binding (5). Catalytic triads were also found in different types of enzymes (2, 7, 28, 30). The residues Asp³⁸⁰ and Asp³⁸¹ probably play a key role in substrate

binding, through H-bonds between both Asp and the substrate (4, 5, 7, 23). They also may play a role in influencing the ionization status of the catalytic residues (4, 5, 7).

The transglycosylation efficiency was increased significantly in three single and six double mutants. The largest increase was found with the mutants His⁴⁹⁷Met and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu. As shown in Figure 5.5, His⁴⁹⁷ was located next to the catalytic Asp⁴⁹⁶. It is proposed that this His residue stabilizes the catalytic Asp via H-bonds with the substrate or water in a triangular arrangement, therewith positioning them, and stabilizing the transition state of the Asp and positioning the water molecule for hydrolysis. Replacing the His by a Met residue increased the transglycosylation efficiency of AGA, without altering the absolute activity of the enzyme. Apparently, the Met residue is unable to position the water molecule, thereby increasing the likelihood of other substrates to act as acceptors, which increases transglycosylation activity. These results correspond with factor II, mentioned in the introduction.

Two other mutations also showed a reasonable increase in transglycosylation activity, i.e. Tyr⁵⁰⁰Leu and Tyr⁴⁹²Gly. The Tyr residues were assumed to stabilize substrate binding through stacking interactions (factor I). Based on the putative 3D-structure of AGA, it was found that the residues Tyr⁵⁰⁰ and Tyr⁴⁹² were not located near the catalytic site (Figure 5.4 and 5.5), which makes stacking interactions with the substrate molecules unlikely. The residue Tyr⁴⁹² was, besides a Gly, also mutated to an Ala. This latter mutation decreased the transglycosylation activity, which suggests that changing the Tyr residue not necessarily results in a higher transglycosylation activity. It is unclear what the role of this Tyr residue can be. The stacchiose synthases from GH36 have a Gly residue at the position of Tyr⁴⁹². Residue Tyr⁵⁰⁰ was located on a coil in a low-similarity region of the putative 3D-model. Since modification of Tyr⁵⁰⁰ had a high impact on transglycosylation, it is suggested that the coil will be folded differently, more close to the catalytic site.

Using glycoside hydrolases to elongated oligosaccharides has an advantage compared to using glycosyl transferases, since low-cost donor substrates can be used. An increase in transglycosylation activity of 16% was reached in this research with only a single mutation. It is difficult to compare these results with results found in literature, because of the difference in calculation of the transglycosylation activity. Nevertheless, some of these results are discussed. For example, β -glucosidase CelB from *Pyrococcus furiosus* showed an increase in transglycosylation activity of approximately 17% after mutation of a Phe to a Tyr residue (16). After mutation, the transglycosylation activity of the enzyme accounted for 69% of all

enzyme-substrate encounters. Different mutants of neopullanase from *Bacillus stearothermophilus* showed an increase in transglycosylation between 17 and 21% (19), which resulted in a maximum of 52% of all enzyme-substrate encounters being a transglycosylation reaction. An α -amylase mutant from *Saccharomycopsis fibuligera*, in which a Tyr was replaced by a Trp residue, produced transglycosylation products from a gluco-heptasaccharide up to 25% of the total amount of oligosaccharides in the reaction mixture, whereas no transglycosylation products were formed by the wild-type enzyme (21). The increase in transglycosylation activity obtained with mutants of AGA was in the same order of magnitude as found in literature. Because wild-type AGA already contained a high transglycosylation activity, this activity could be increased to 75% of all enzyme-substrate encounters in mutant His⁴⁹⁷Met.

We also showed that AGA was able to use different acceptor substrates, as long as they contain a hydroxyl group at the C-6 position at the non-reducing end. This property of the enzyme could be used to make rare oligosaccharides. If used as prebiotics, these oligosaccharides might be less easy to ferment and reach the more distal areas of the colon (34), where the main disorders of the gut occur (12). The transglycosylation products formed in this research were mainly tri- and tetrasaccharides. To elongate oligosaccharides even further, it is necessary to improve the transglycosylation more.

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6

Concluding remarks

INTRODUCTION

The basis for the research described in this thesis, was the concept of pre- and probiotics. Galactans and galacto-oligosaccharides are known for their prebiotic potential, and commercial products containing these are available. The aim of this research was to investigate the mechanism of utilization of galactan and galacto-oligosaccharides, of which a prebiotic effect is known or suspected. Three galactan/galacto-oligosaccharide modifying enzymes were characterized to obtain more insight in this mechanism; a β -galactosidase, which was hardly active towards lactose, an extracellular and membrane-bound endo-galactanase, and an α -galactosidase, which possessed a high transglycosylation activity. This α -galactosidase was used for the synthesis of oligosaccharides. Subsequently, this enzyme was optimized for oligosaccharide synthesis by site directed mutagenesis.

In this chapter we review the results obtained in this research and discuss them in the context of the recent literature and their contribution to pre- and probiotics research. Some considerations about the application of pre- and probiotics will be discussed and future directions for research will be suggested.

DESIGN OF PREBIOTICS

The prebiotic 'effect' of oligo/poly-saccharides has been studied for several years, mainly by fermentation of the carbohydrates by bifidobacteria (11, 46) and/or by the enumeration of bifidobacteria in human and animals after intake of the carbohydrates (19). With this research, we embarked on another way of examining the prebiotic effect of carbohydrates: by characterizing the activity of carbohydrate modifying enzymes in bifidobacteria. Knowing more about the potential mechanism of carbohydrate utilization, would help us to understand why or if certain prebiotics can be utilized by bifidobacteria.

In vitro fermentation studies of poly- and oligosaccharides and pure cultures of 'beneficial' bacteria were in many cases used for the detection of prebiotic preparations. Nowadays, the *B. longum* genome sequence offers opportunities to rationalize prebiotic design, because a much clearer picture of the carbohydrate-degrading potential is available. More genome sequences of gut bacteria are available or will be soon (29, 39, 60). The unravelling of galactan utilization, as described in this thesis, is an example of a new approach for examine the prebiotic effect. In the genome sequence of *B. longum*, the gene for endo-galactanase YvfO

was identified. Characterization of the gene and the enzyme showed that YvfO was probably located extracellular and produced mainly trisaccharides from arabinogalactan type I outside the cell. It was suggested that the endo-galactanase acts with a processive mechanism, which may ensure that a substrate does not escape to the environment. In this way the enzyme can secure the substrate for itself. These results show that the available sequence information benefits from a thorough biochemical back-up, like the determination of the mode of action and the substrate specificity. The genome sequence showed the presence of several β -galactosidases, which were expected to be located intracellularly. It was therefore hypothesized that the formed trisaccharides were internalized by oligosaccharide transporters (Chapter 4, Figure 4.8). Although the genome sequence also reveals the presence of oligosaccharide transporters, it will be necessary to verify whether the extracellularly formed oligosaccharides can actually be internalized by *Bifidobacterium*. It is therefore important to establish a link between oligosaccharide structure and selectivity of the various transporter proteins, which will be a tremendous task.

The genome sequence of *B. longum* also revealed the presence of other enzymes involved in galactan/galacto-oligosaccharide utilization. An example is a putative galactoside acetyl transferase gene (47). *Escherichia coli* was found to contain a similar galactoside acetyltransferase and a maltose acetyl transferase (6, 31, 62). From analysis of these enzymes it was suggested that such a galactoside acetyltransferase might be involved in acetylation of non-metabolized galactosides. By acetylating them they will be transported out of the cell and are prevented to re-enter the cell (6, 31, 62). It remains unclear whether the micro-organism excretes the acetylated oligosaccharides or keeps them “in storage” in the periplasmic space. *Bifidobacteria* do not have a periplasmic space, therefore the “storage” of these oligosaccharides will be different from that of *E. coli*.

Most prebiotic ingredients used so far, are fermented in the proximal part of the colon. As most of the gut disorders are taking place in the more distal part of the colon (19), it might be important to modify oligosaccharides in such a way that they penetrate the gut further where they might influence the microbial composition in the more distal parts of the colon (61), for example longer or branched oligosaccharides, or acetylated oligosaccharides. Another advantage could be that a higher degree of polymerization and/or degree of branching might lead to less flatulent prebiotics (12). Different methods to modify oligosaccharides are possible. For example, the galactoside acetyltransferase might be an interesting enzyme to acetylate prebiotics. Although it has been found that acetylated xylo-oligosaccharides may

have a prebiotic effect (27), it is unlikely that the galactoside acetyltransferase can be used to synthesize acetylated galacto-oligosaccharides, because the expensive acetyl-CoA is needed as acetyl-donor, and there are cheaper, chemical alternatives. Acetylated oligosaccharides can also be obtained by the degradation of complex cell wall polysaccharides. Another approach is the use of carbohydrases with transglycosylation activity. It has been suggested that the carbohydrases of bifidobacteria (or, more in general, beneficial intestinal microbes) can be used for the production of prebiotic oligosaccharides, when they have transglycosylation activity. The rationale behind this is that oligosaccharides can be obtained, that can easily be utilized by bifidobacteria, because all the tools for degradation and uptake are in place. *Bifidobacterium* strains showed the highest growth rate on the oligosaccharide mixture produced by their own enzymes e.g. β -galactosidase (40). It is unclear by how many residues oligosaccharides should be elongated to penetrate the more distal parts of the gut. Alternatively, it is not clear whether longer oligosaccharides can still be internalized through the carbohydrate transporters. Research on synthesis and oligosaccharides transport should be linked.

The main disadvantage of using *Bifidobacterium* carbohydrases for elongating oligosaccharides is that they possess hydrolytic activity besides transglycosylation activity. In most cases, the former is more predominant than the latter. Different methods can be used to improve or optimize enzyme activities. Most of these methods are based on molecular genetic tools. Chapter 5 describes that the transglycosylation efficiency of α -galactosidase from *B. adolescentis* can be increased up to 16% by site directed mutagenesis compared to the wild type enzyme. Another example of increasing transglycosylation activity was given by Jørgensen et al. (26). The efficiency of transferase activity of a β -galactosidase from *B. bifidum* could be increased to 90% of all enzyme-substrate encounters by truncation of this enzyme at the C-terminal end by 580 amino acids. This C-terminal end contained a galactose binding domain. It was suggested that the truncated β -galactosidase may have a more open structure which facilitates transglycosylation (26).

HOW SPECIFIC SHOULD OLIGOSACCHARIDES BE?

It should be realized that there are still many aspects that should be approached with caution with respect to prebiotics. A large variation between the different *Bifidobacterium* spp. was found, which appear to have different enzyme systems for carbohydrate degradation. For

example, the ability to grow on arabinogalactans was mainly found with *B. longum* (11, 46). However, this bacterium is not able to grow on amylose or amylopectin, whereas several other bifidobacteria species, like *B. adolescentis* and *B. breve*, are (11, 46). So far, *B. longum* and *B. adolescentis* seem to be the only *Bifidobacterium* spp. with the ability to grow on arabinoxylan (9). Preliminary results with the genome sequence of *B. breve* UCC2003 revealed that this strain did not contain arabinoxylan degrading enzymes (60). Thus, oligosaccharides that stimulate the growth of one *Bifidobacterium* species does not necessarily stimulate other subspecies.

Besides the broad variation in carbohydrases between *Bifidobacterium* spp. themselves, there is always the question whether a rationally designed *Bifidobacterium* prebiotic is truly a selective substrate. It is proposed that more than 1300 bacterial species are present in the human intestine (22), which can all potentially compete with bifidobacteria for these substrates, and many faecal bacterial species have not yet been characterized (35). Table 6.1 shows the presence of carbohydrate modifying enzymes in the genome sequences of four different gut bacteria: *B. longum* NCC2705 (47), *Bacteroides thetaiotaomicron* VPI-5482 (65), *Escherichia coli* K12 (5), and *Clostridium perfringens* str. 13 (52). The table shows only the predicted carbohydrate hydrolyzing enzymes. Several other carbohydrate hydrolyzing enzymes, which are entitled as “hypothetical proteins”, are annotated as well, but no prediction of the enzyme type is given. These hypothetical proteins are not shown.

The bacteroides constitute the most abundant members of the intestinal microflora of mammals; they constitute approximately 30% of the total faecal flora (18). Typically, they are symbionts, but they can become opportunistic pathogens, being involved in intestinal putrefaction and production of carcinogens. Breakdown of complex plant polysaccharides such as pectin, (arabino)xylans, and (arabino)galactans is aided by the many enzymes these organisms produce (Table 6.1) and the many sugar transporters; this organism was found to contain 20 sugar-specific transporter proteins (65). It is not clear whether these transporters will also be able to transport oligosaccharides. Two less abundant members of the intestinal microflora are *E. coli* and clostridia, which can have pathogenic effects, like diarrhea, infections, liver damage and cancer. Table 6.1 shows that also these organisms contain carbohydrate degrading enzymes, even in high amounts (*C. perfringens*). In these organisms less putative carbohydrate transporters were found than in *Bacteroides thetaiotaomicron* (5, 52).

The most striking result from table 6.1 is that all four organisms seem to have α -galactosidase, β -galactosidase, and xylanase activity, which means that not only bifidobacteria are able to ferment galacto-oligosaccharides and xylans/xylo-oligosaccharides, but also pathogenic bacteria like *E. coli* and clostridia. These results were supported by *in vitro* fermentation results, showing the ability of these organisms to grow on the substrates mentioned (9, 45, 59). Enzymes that are not found in *C. perfringens* are arabinosidase, arabinofuranosidases and fructofuranosidases, and enzymes that are not found in *E. coli* are arabinosidase, and arabinofuranosidase. However, fermentation studies showed that clostridia were able to grow on fructo-oligosaccharides (FOS) (45, 59). The enzymes in the *C. perfringens* genome sequences that were annotated as “hypothetical proteins” might contain a fructofuranosidase, explaining the fermentation of FOS. Also *E. coli* was found to grow on FOS with fermentation studies (45), and rat studies (56). These results suggest that only arabinans, and arabino-oligosaccharides might be selective for bifidobacteria.

A remarkable result from the same table is that also a putative endo-galactanase was found in *Ba. thetaiotaomicron* and *C. perfringens*, which indicate that galactans might not be selective for bifidobacteria, which is mainly *B. longum*. It is unknown what the substrate specificity and mode of action of these enzymes is, and therefore it is unclear whether the same type of galactans can be fermented. The enzyme from *Ba. thetaiotaomicron* was classified in the same GH-family as the one from *B. longum* (GH53), whereas that from *C. perfringens* was classified as a GH16 enzyme. The deduced amino acid sequences were analyzed for the presence of signal peptides and transmembrane domains, based on sequence analysis using SignalP (36) (<http://www.cbs.dtu.dk/services/SignalP-2.0/>), PSORT (34) (<http://psort.nibb.ac.jp/>), and SOSUI (23) (http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html). The deduced amino acid sequence of the endo-galactanase from *Ba. thetaiotaomicron* showed the presence of a signal peptide but no transmembrane domain. Therefore, it is suggested that this enzyme is located extracellular, and not attached to the cell membrane. The deduced amino acid sequence of the *C. perfringens* enzyme revealed a signal peptide as well. The sequence analysis programs are not consistent in whether this enzyme contains a transmembrane domain.

Table 6.1: Sugar modifying enzymes in genome sequences of *Bifidobacterium longum* NCC2705 (47), *Bacteroides thetaiotaomicron* VPI-5482 (65), *Escherichia coli* K12 (5), and *Clostridium perfringens* str. 13 (52).

Enzyme	<i>B.</i> <i>longum</i>	<i>Ba.</i> <i>thetaitotaomicron</i>	<i>E.</i> <i>coli</i>	<i>C.</i> <i>perfringens</i>
β -Fructo-furanosidases	1		1	
Endo-arabinosidases	2	3		
α -Arabino-furanosidases	2	1		
Arabinosidases	3	2		
Endo- β -galactosidases/galactanases	1	1		1
β -Galactosidases	3	27	3	4
α -Galactosidases	2	3	1	2
α -1,6-Mannanases		6		
Endo- α -mannosidases		1		
α -Mannosidases	3	10		2
Xylanases	2	6	1	5
Xylosidases	1	13	1	
β -Glucuronidases		2	1	1
β -Glucosidases	1	4	1	1
Glucanases	1		1	
α -Glucosidases	1	13		4
Oligo-1,6-glucosidases	1			
α -Amylases		4	1	2
Isoamylase	2			
Pullulanases	1			3
Endo-polygalacturonases		2		
α -Rhamnosidases		4		
Sucrose phosphorylases	1			
Sialidases				2
Total	28	102	11	27

Another aspect concerning the specificity of prebiotic ingredients is that feed-crossing by other microorganisms in the gastro-intestinal tract (GIT) may play an important role (18). This means that various bacteria can cooperate in the degradation of carbohydrates. Using *in vitro* fermentation studies with pure cultures of selected microorganisms for determining if

substrates are prebiotic ingredients, do not reveal this. An example of feed-crossing might involve the extracellular, non-membrane-bound endo-galactanase from *Ba. thetaiotaomicron*. It is hypothesized that this bacterium excretes the endo-galactanase into the gut environment. Since it is probably not attached to the bacterial cell, it will degrade galactans in the gut environment. The galacto-oligosaccharides formed might therefore be available for other organisms, which are not able to degrade the polymeric galactans. This is in contrast with the endo-galactanase from *B. longum*, which seems to secure the substrate for itself by its processive endo-galactanase (Chapter 4).

In this paragraph, the presence of carbohydrate modifying enzymes of four gut bacteria (of which the genome sequence is known) was discussed. It is apparent that, when considering only these four microorganisms, it is already difficult to find specific prebiotics for bifidobacteria. With more than 1300 different species suggested to be present in the large intestine (22), the presence of many more carbohydrases may be anticipated, which probably makes it very difficult to target specific microorganisms with prebiotics.

DO PROBIOTICA/PREBIOTICA WORK?

As described in the chapter 1, it is claimed that pre- and probiotics have several health-promoting effects (17-19) like lowering cholesterol levels (4), immunostimulation (43), anticarcinogenicity (7, 42, 64), improved mineral absorption (48), and production of vitamins (14). In this paragraph we will discuss the scientific evidence for some of these health effects based on the results from different studies.

Health promoting effect of pre- and probiotics

The health promoting effects that are discussed in this paragraph, are immunostimulation, improved mineral absorption, and anticarcinogenicity. The mechanisms of immunostimulation by pro- and prebiotics remains unclear. It is suggested that peptidoglycan from the bacterial cell wall of the probiotics may be important for the function of the immune system (20). Immunostimulation by pre- and probiotics is examined by determining the composition and amount of immunoglobulin sIgA and cytokines (16). Most studies were performed in animal models. For example, it was found that pre- or probiotic supplementation induced significant immunomodulation in the intestine of rats or mice (38, 43). Roller et al. (43) found that this supplementation was only effective with synbiotics. In human studies, the effects found were

much lower. Treatment of children with Crohn's disease with *Lactobacillus rhamnosus* GG showed an increase in sIgA, but no clinical effect (32), whereas treatment of healthy men with *Lactobacillus casei* had no effect on immunostimulation (53).

Improved mineral absorption is another positive health effect that is claimed for pre- and probiotics. To date, the mechanism for this effect is still unclear. It was suggested that the production of short-chain fatty acids might be involved in stimulating mineral absorption (37). It was found that feeding oligofructose to rats increased the absorption of calcium (37). However, in humans this stimulation of mineral absorption by oligofructose was less pronounced (8, 15, 58).

Pre- and probiotics are also claimed to be involved in anticarcinogenicity. The development of colon cancer is a multi-stage process, that is initiated by DNA damage (64). The colonic microflora is able to generate carcinogens and tumor promoters. For example, the enzyme β -glucuronidase enhances the progression of aberrant crypt foci, from which cancer cells may develop (7, 41). In Table 6.1 it is shown that *Ba. thetaiotaomicron*, *E. coli*, and *C. perfringens* contain β -glucuronidases, which make them possible tumor promoters. Generally, lactobacilli and bifidobacteria have low activity of these kind of enzymes (7), which is also suggested from the genome sequence of *B. longum*, since no β -glucuronidases were found (Table 6.1). It has also been claimed that pre- or probiotics themselves have an anticarcinogenic effect, which is based on results with animal models. For example, Reddy et al. (42) showed that feeding rats with inuline, FOS or lyophilized *B. longum* cells had a inhibitory effect on chemically induced aberrant crypt foci. A similar result was found by Rowland et al. (44), who also found that a combination of inulin and *B. longum* cells resulted in an even higher inhibition. Human studies on these aspects do not show consistent results. Some studies show that an increase in amount of lactobacilli corresponds to a decrease in mutagen secretion, whereas others show no effect at all (41). Also, studies on examining the anticarcinogenic activity by a decrease in faecal β -glucuronidase activity did not give conclusive results (7).

Many of the effects, described above, were found with animal model studies. The effects found in animals are not always found in humans. The results obtained with human studies sofar seem to be inconsistent, and therefore it is still uncertain whether prebiotics have an effect in humans. One of the drawbacks of human studies is that another part of the colon is analyzed than the part where most of the fermentation takes place. Most of the prebiotics will be fermented in the proximal part of the colon (61), whereas the only area of the gut that is

easily accessible for investigation is the distal part of the colon, which is represented by the faeces. Faeces does not give information about the more proximal parts of the colon. A solution to overcome the drawbacks of animal models and feeding studies is, for example, simulation of the GIT in an *in vitro* model. An example of such a model is described by van der Werf and Venema (63). Another striking result from animal and human studies is that an increase of the number of lactobacilli or bifidobacteria in the faeces is only measured during the supplementation of the probiotics to the diet. After stopping the diet the amount of probiotics decreased to the initial level, suggesting that they are not colonizing the gut (22). An every day intake will be necessary to have the effects of pre- and probiotics.

Pre- and probiotics as therapeutic treatment

Besides a health promoting effect, pre- and probiotics can also have an therapeutic effect, like recovering from diarrhea. This latter is probably due to their effects on the immune system (38), competition with pathogenic bacteria for binding sites on epithelial cells, or through the production of bacteriocins (25). Human studies show that intake of *L. rhamnosus* GG can shorten the duration of diarrhea caused by rotavirus, whereas no consistent evidence was found for other probiotics (13, 55). Gastrointestinal infections with pathogens, like *E. coli*, *C. jejuni*, and *C. difficile* can also cause diarrhea. Lactobacilli and bifidobacteria showed an inhibitory activity towards some of these species, but the results displayed variations with the different strains (30).

Several studies showed that *L. rhamnosus* GG also decreased abnormal immune response (allergic reactions) (24, 28). It is thought that an allergy is due to an increased permeability of the intestinal barrier. It was suggested that adhesion of probiotics to the mucosal barrier helped to stabilize this and prevented absorption of antigens (55). As described before, *L. rhamnosus* GG stimulated the production of sIgA, which probably helped in increasing antigen elimination.

Another serious colon disorder is inflammatory bowel disease (IBD; collective term for ulcerative colitis (UC) and Crohn's disease). It is believed that bacteria play a role in the pathogenesis of this inflammatory bowel disease. The immune response in the intestine is conditioned by the endogenous microflora. Using probiotics can alter the endogenous microflora by competition for nutrients, adhesion sites, or producing antimicrobial metabolites (50). Probiotics were examined in clinical studies to be used as therapy for IBD. It was found that a non-pathogenic strain of *E. coli* had a positive effect towards achieving and

maintaining remission of UC, whereas *Saccharomyces boulardii* showed positive effects on Crohn's disease patients (55, 57). Mixtures of lactobacilli and bifidobacteria (55, 57) or synbiotics (49) also improved the remission of UC and Crohn's disease.

Do they work?

Besides positive effects of pre- and probiotics, also negative effects are found. Already in the mouth of humans prebiotics will be fermented by the lactic acid bacteria present there. During this fermentation lactic acid or short chain fatty acids may be produced, which can be involved in the development of dental caries (21). Since pre- and probiotics cause an increase in fermentation in the colon, more reaction products are formed in the colon, like gases as H₂, CH₄, and CO₂ (17). A large production of these gases may cause flatulence. It was suggested that a fast fermentation of FOS also resulted in a fast production of lactic acid and short chain fatty acids, which might be involved in colonic mucosal damage and translocation of salmonella in mice (56). Another negative effect of prebiotics is that non-digestible oligosaccharides or dietary fibre have a high water-binding capacity (10), which can cause diarrhea. Especially for prebiotics, the intake should be taken into account. A dosage higher than 20 g/day might induce these effects (57).

Pre- and probiotics have to survive the gastro-intestinal tract and arrive as functional substrates or viable organisms in the colon to give positive health effects. The commercial prebiotics, can survive the acid environment in the stomach and digestion in the small intestine (15, 18). The survival of probiotics is less promising. For example, Ananta et al. (3) found that several lactobacilli had a low survival in simulated gastric juice *in vitro*. But, the same authors showed that the survival rate of the probiotics can be increased in the presence of glucose, or applying sub-lethal stress to the strains before use. A question that arises is, whether it will be necessary to use live bacteria. Some studies show the same positive health effects with dead bacteria (22, 41). It should be stated that the studies with dead bacteria also give inconsistent results: some studies see an effect, whereas others do not (13, 41).

As described in this paragraph, pre- and probiotics may be involved in many health aspects. But, many of these health effects are only seen in animal models, and not in human studies, like the immunostimulation and cancer prevention. Especially, human studies in which healthy adults are used, did not show these health effects. Some of the human studies did not even show a change in the composition of the natural intestinal microflora (1, 2). However, studies that are carried out with patients, show certainly positive effects. For example, in

patients suffering from diarrhea the natural microflora is altered due to the diarrhea. Supplementation of probiotics can help to recover the microflora. It is not clear whether prebiotics can have the same effect in patients, since most of the studies are carried out with probiotics.

FUTURE PERSPECTIVES

From the previous paragraphs, it seems to be difficult to design specific prebiotics. For example, in the paragraph “How specific should prebiotics be?” it was discussed that the most widely used prebiotics (FOS, galacto-oligosaccharides, and xylo-oligosaccharides) are not specific for bifidobacteria, but can probably also stimulate bacteroides, enterobacteria and/or clostridia. It was also discussed that most of the probiotic effects are found in studies with patients, whereas studies with healthy adults show no effect. Moreover, it seems that the use of synbiotics might be more efficient than the use of pre- or probiotics alone (43, 44). Also for these synbiotic products, it is not clear what the mechanism for the positive effects is or why these seem to be more effective. An explanation might be that the prebiotics in the mixture keep the probiotics more stable, during the passage through the GIT. Increasing the probiotic stability was also found in the presence of glucose (3), possibly these results with monosaccharides might be extrapolated to prebiotics. Examination of synbiotics will face the same difficulties as that of pre- or probiotics. A helpful solution in determining the health effects of pre-, pro- and synbiotics can be biomarkers, however such biomarkers are not available yet (51).

As discussed before, the results of animal studies on the health promoting effects of pre- and probiotics show many positive effects, but the human studies with healthy subjects have not supported these findings until now. One of the problems of the research on pre- and probiotics is the large complexity of the large intestine. More and more genome sequences of human bacterial species become available, in which information about carbohydrate modifying enzymes can be found. This can lead to a better understanding of the carbohydrate fermentation in the gut, together with biochemical characterization of some of these enzymes. The putative metabolism of *B. longum* to utilize carbohydrates is schematically presented in Figure 6.1.

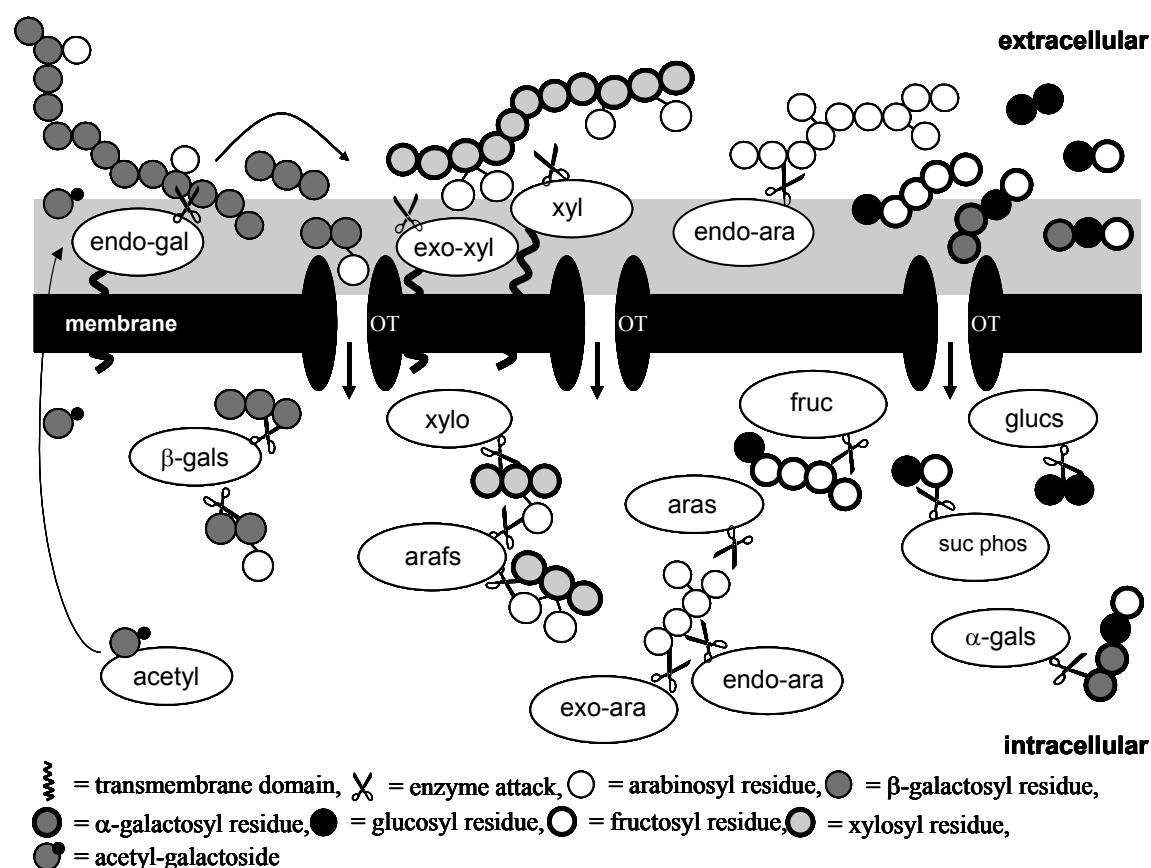


Figure 6.1: Schematic overview of the carbohydrate modifying enzymes in *B. longum* and the putative mechanism of utilizing carbohydrates. This figure is constructed based on information from this thesis and the genome sequence of *B. longum* NCC2705 (47). Endo-gal: endo-galactanase, β-gals: β-galactosidases, exo-xyl: exoxylanase, xylo: xylosidase, xyl: xylanase, arafs: arabinofuranosidases, exo-ara: exo-arabinosidase, endo-ara: endo-arabinosidase; aras: arabinosidases, fruc: fructofuranosidase, glucs: glucosidases, suc phos: sucrose phosphorylase, α-gals: α-galactosidases, acetyl: galactoside acetyl transferase, OT: oligosaccharide transporter.

This figure shows the carbohydrate modifying enzymes that are predicted from the genome sequence (Table 6.1). Amino acid sequence analysis revealed whether the enzymes were located intra- or extracellularly, and whether they were membrane-bound. The figure shows that besides the endo-galactanase also xylanases and an arabinosidase were probably located extracellularly. It is hypothesized that these extracellular enzymes degrade (arabino)xylans and arabinans to smaller oligosaccharides, which might be transported into the bacterial cell. Intracellularly, they probably will be degraded by intracellular enzymes. A biochemical characterization of the enzymes is needed to conclude on this. Constructing such schematic overviews of carbohydrate modifying enzymes in other microorganisms, like bacteroides or clostridia, etc., would help us to understand more about the digestion of carbohydrates in the

intestine, for example, to determine which organisms and enzymes are involved in feed-crossing. Also, it may assist in understanding why that certain groups of oligosaccharides are able to increase the number of bifidobacteria and lactobacilli in the faeces (19).

A new type of (potential) probiotics has been developed. These are genetically modified microorganisms, which can produce, for example, antigens *in vivo*. Steidler et al. (54) genetically engineered *Lactococcus lactis* in such a way that it synthesized IL-10 *in vivo*. IL-10 was shown to be important in decreasing inflammation of the colon (55). The engineered *Lactococcus* showed that this approach required lower doses of medicine than other treatments in mice models (54). Another example was described by Mercenier et al. (33). They constructed lactobacilli to facilitate the *in vivo* production of antigens to reduce the effects of *Helicobacter pylori* or rotavirus infections. *H. pylori* infection is associated with chronic gastritis, peptic ulcers, and gastric adenocarcinoma. The health effects of these second generation probiotics might also be effective in healthy humans. However, using such kind of genetically engineered microorganisms is still controversial, and much more research is needed to examine their safety.

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Summary

Nowadays, there is an increasing interest in positively influencing the intestinal microflora through the diet by the use of prebiotics. Galactans and galacto-oligosaccharides are known for their prebiotic potential, and commercial products containing these are available. So far, not much is known about the enzymic machinery of bifidobacteria for galactan utilization. For this, the galactan-modifying enzymes of bifidobacteria are of interest to us. Understanding the mechanism of galactan and galacto-oligosaccharide degradation may assist in the development of better prebiotics. The aim of this research was to investigate this galactan utilization mechanism. Three galactan/galacto-oligosaccharide modifying enzymes were characterized to obtain more insight in this mechanism; a β -galactosidase, which was hardly active towards lactose, an endo-galactanase, which was located extracellular and anchored to the cell membrane, and an α -galactosidase, which possessed a high transglycosylation activity. This α -galactosidase was used for the synthesis of oligosaccharides. Subsequently, the enzyme was optimized for oligosaccharide synthesis by site directed mutagenesis.

Galactans and arabinogalactans are plant polysaccharides, which can be divided in two groups, i.e. Type I and Type II. Chapter 2 describes a new structural element in Type I arabinogalactans. Arabinogalactan Type I from potato was partially degraded by endo-galactanase from *Aspergillus niger*. High-performance anion-exchange chromatography revealed that several of the oligomeric degradation products eluted as double peaks. To investigate the nature of these products, the digest was fractionated by Bio-Gel P2 chromatography. The pool that contained tetramers was treated with a $(\beta 1 \rightarrow 4)$ specific galactosidase from *Bifidobacterium adolescentis* to obtain a dimer with deviating linkage type. NMR-analysis revealed that the dimer contained a $(\beta 1 \rightarrow 3)$ linkage instead of the expected $(\beta 1 \rightarrow 4)$ -linkage. Using the same NMR techniques for the tetramer pool showed that it consisted of the following 2 galactosyl tetramers: β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4)-D-Galp and β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 3)-D-Galp. The fact that the deviating $(\beta 1 \rightarrow 3)$ -linked galactose was found at the reducing end of the tetramer showed that this deviating linkage is present within the backbone. The $(\beta 1 \rightarrow 3)$ -galactosyl interruption appeared to be a common structural feature of type I arabinogalactans with a frequency ranging from approximately 1 in 160 (potato, soy, citrus) to 1 in 250 (onion).

The cloning and characterization of a β -galactosidase from *Bifidobacterium adolescentis* DSM 20083 was described in chapter 3. The β -galactosidase gene (β -Gal II) was cloned into a *pbluescript* SK (-) vector and expressed in *Escherichia coli*. The enzyme was purified from the cell extract by anion-exchange and size-exclusion chromatography. β -Gal II had a native molecular mass of 235 kDa and the subunits had a molecular mass of 81 kDa, which indicated that β -Gal II occurred as a trimer. The enzyme was classified into glycoside hydrolase family 42. The optimal pH was 6.0 and the optimal temperature was 50°C, using *p*-nitrophenyl- β -D-galactopyranoside as a substrate. The K_m and V_{max} for β -D-Galp-(1→4)-D-Galp (referred to as Gal(β 1→4)Gal) were 60 mM and 1129 U/mg, respectively. The β -Gal II was specific towards (β 1→4)-linked galacto-oligosaccharides, but lactose was a poor substrate. The enzyme was inhibited at high substrate concentrations (100 mg/ml) and no transglycosylation activity was found. At lower Gal(β 1→4)Gal concentrations (10 mg/ml) only low transglycosylation activity was found; the Gal/[Gal(β 1→4)]₂Gal peak area ratio was 9:1, which makes the enzyme less appropriate for tailoring prebiotics.

The characterization of an endo-galactanase from *Bifidobacterium longum* was described in chapter 4. The gene of this endo-galactanase (*YvfO*), classified into glycoside hydrolase family 53, was revealed from the genome sequence of *B. longum* NCC2705. The deduced amino acid sequence suggested that this enzyme was located extracellularly, and anchored to the cell membrane. *YvfO* 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 weight of 329 kDa and the subunits had a molecular weight of 94 kDa, which indicated that the enzyme occurred as a tetramer. The optimal pH for endo-galactanase activity was 5.0 and the optimal temperature was 37°C, using 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 galacto-trisaccharides from (β 1→4)-galactans and (β 1→4)-galacto-oligosaccharides, probably by a processive mechanism, moving towards the reducing end of the galactan chain after an initial mid-chain cleavage. The enzyme seemed to be able to cleave (β 1→3)-linkages, but it is suggested that the (β 1→4)-linkage may be degraded faster than the (β 1→3)-linkage. Arabinosyl side chains in, for example, potato galactan hindered *YvfO*.

Chapter 5 describes that the α -galactosidase (AGA) from *Bifidobacterium adolescentis* DSM 20083 had a high transglycosylation activity. The optimal conditions for transglycosylation

were pH 8, and 37°C. At high melibiose concentration, approximately 64% of the enzyme-substrate encounters resulted in transglycosylation. Examination of the acceptor specificity showed that AGA required a hydroxyl group at C-6 for transglycosylation. Pentoses, hexuronic acids, deoxyhexoses, and alditols did not serve as acceptor molecules. Disaccharides were found to be good acceptors. AGA offers opportunities to tailor prebiotics, which might target the more distal part of the colon. However, besides transglycosylation, AGA also has hydrolysis activity, which makes oligosaccharide synthesis less efficient. Therefore, we embarked on improving the transglycosylation activity of AGA. A putative 3D-structure of the catalytic site of AGA was obtained by homology modeling. Based on this structure and amino acid sequence alignments, site-directed mutagenesis was performed to increase the transglycosylation efficiency of the enzyme, which resulted in ten positive mutants: 4 with a single mutation, and 6 with two mutations. Most of the mutations gave an increase in transglycosylation of 2-7% compared to the wild-type AGA, except for single mutation His⁴⁹⁷Met (16%), and double mutations Gly³⁸²Cys-Tyr⁵⁰⁰Leu (9%), and His⁴⁹⁷Met-Tyr⁵⁰⁰Leu (16%). The results showed that with only a single mutation (His⁴⁹⁷Met) the transglycosylation efficiency could be increased with 16%. Combining successful single mutations in double mutants did not result in an extra increase in transglycosylation efficiency. The acceptor specificity did not change in the mutants, whereas the thermostability of the mutants with Gly³⁸²Cys decreased.

The results described in this thesis suggest that galactan, and the longer galacto-oligosaccharides will be degraded extracellularly into trisaccharides by this endo-galactanase. The genome sequence of *B. longum* suggests that this bacterium does not have an extracellular β -galactosidase, which may indicate that this microorganism has a kind of translocation mechanism to internalize galacto-oligosaccharides. Intracellularly, the trisaccharides will be degraded by the different galactosidases present.

In chapter 6, it is discussed how specific prebiotics should be; also, the evidence for health effects of pre- and probiotics is elaborated. To get more insight into the complex system of the gut micro-flora, it is important to unravel the mechanism of carbohydrate utilization by micro-organisms in the large intestine. The results in this thesis show that detailed biochemical characterization of carbohydrate modifying enzymes can yield valuable information on how bifidobacteria acquire carbohydrates.

Samenvatting

Tegenwoordig staan prebiotica volop in de belangstelling. Prebiotica zijn niet-verteerbare ingrediënten die ervoor kunnen zorgen dat de ontwikkeling van een bepaalde groep darmbacteriën, zoals bifidobacteriën, positief beïnvloed wordt. Galactanen en galacto-oligosacchariden staan bekend om hun prebiotische werking en commerciële producten hiervan zijn verkrijgbaar. Er is echter op dit moment weinig bekend over de enzymatische afbraak en opname van dit soort galactose-bevattende koolhydraten door bifidobacteriën. Daarom zijn dergelijke galactaan-afbrekende enzymen uit bifidobacteriën interessant voor ons. Een beter begrip van de galactaan en oligosacchariden afbraak en opname, zal helpen om betere prebiotica te kunnen ontwikkelen. Het doel van dit onderzoek was het bestuderen van het galactaan en galacto-oligosacchariden gebruik door bifidobacteriën. Drie galactaan/galacto-oligosacchariden-afbrekende enzymen zijn gekarakteriseerd om hier meer inzicht in te krijgen: het eerste enzym was een β -galactosidase dat weinig activiteit heeft op lactose, het tweede enzym was een extracellulair endo-galactanase, en het derde enzym was een α -galactosidase met een hoge transglycosyleringsactiviteit. De efficiëntie van dit laatste enzym om prebiotische ingrediënten te maken is bestudeerd en verbeterd.

(Arabino)galactanen zijn plantaardige polysacchariden, die ingedeeld kunnen worden in twee groepen, nl.: Type I en Type II. In hoofdstuk 2 wordt een nieuw element in de structuur van Type I galactanen beschreven. Type I arabinogalactaan uit aardappel werd gedeeltelijk afgebroken door een endo-galactanase van *Aspergillus niger*. High-performance anion-exchange chromatography (HPAEC) liet zien dat verschillende oligomere afbraakproducten als dubbele pieken elueerden. Om de aard van deze producten te onderzoeken, werd het mengsel gefractioneerd met behulp van Bio-Gel P2 chromatografie. De tetramere fracties werden behandeld met een galactosidase uit *Bifidobacterium adolescentis*, dat specifiek is voor $(\beta 1 \rightarrow 4)$ -galactosyl bindingen, om een dimeer met afwijkend bindingstype te krijgen. NMR-analyse liet zien dat het dimeer een $(\beta 1 \rightarrow 3)$ -binding in plaats van een $(\beta 1 \rightarrow 4)$ -binding had. Dezelfde NMR techniek liet zien dat de tetrameer fractie 2 tetrameren bevatte, nl.: β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4)-D-Galp en β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 4) β -D-Galp(1 \rightarrow 3)-D-Galp. Het feit dat het afwijkende $(\beta 1 \rightarrow 3)$ -gebonden galactose aan het reducerende einde van het tetrameer gevonden werd, betekende dat dit bindingstype aanwezig

is in de hoofdketen van het galactaan. De aanwezigheid van dit nieuwe structuur element bleek ook in andere Type I galactanen voor te komen. De frequentie waarmee ze voorkomen, varieerde van 1 op 160 (in aardappel, soja en citrus vruchten) tot 1 op 250 (in ui).

Het kloneren en karakteriseren van β -galactosidase uit *Bifidobacterium adolescentis* DSM 20083 is in hoofdstuk 3 beschreven. Het β -galactosidase gen (β -Gal II) werd in een *pbluescript* SK (-) vector gekloneerd en tot expressie gebracht in *Escherichia coli*. Het enzym werd gezuiverd uit het celextract met behulp van anionenwisselings- en gelpermeatie chromatografie. β -Gal II had een natief molekuulgewicht van 235 kDa, en de subunits hadden een molekuulgewicht van 81 kDa. Dit is een indicatie dat het enzym als trimeer voorkomt. Het enzym werd in glycoside hydrolase familie 42 ingedeeld. De optimale pH was 6,0 en de optimale temperatuur 50°C, waarbij *p*-nitrophenyl- β -D-galactopyranoside als substraat gebruikt werd. De K_m en V_{max} voor β -D-Galp-(1 \rightarrow 4)-D-Galp (verder Gal(β 1 \rightarrow 4)Gal genoemd) waren respectievelijk 60 mM en 1129 U/mg. Het β -Gal II was specifiek ten opzichte van (β 1 \rightarrow 4)-gebonden galacto-oligosacchariden. Lactose werd slecht afgebroken. Het enzyme werd geremd door een hoge substraatconcentratie (100 mg/ml) en had hierbij geen transglycosyleringsactiviteit. Bij een lage Gal(β 1 \rightarrow 4)Gal concentratie (10 mg/ml) werd een kleine hoeveelheid transglycosyleringsactiviteit gevonden; de ratio voor de piekoppervlakten van galactose en het transglycosyleringsproduct [Gal(α 1 \rightarrow 4)]₂Gal was 9:1, hetgeen betekent dat het enzym niet geschikt is om prebiotica te verlengen.

In hoofdstuk 4 is de karakterisatie van een endo-galactanase uit *Bifidobacterium longum* beschreven. Het gen van dit endo-galactanase (*YvfO*), dat in glycoside hydrolase familie 53 was ingedeeld, werd gevonden in de genomsequentie van *B. longum* NCC2705. De aminozuurvolgorde suggereerde dat het enzym extracellulair en verankerd aan de celmembraan was. *YvfO* is zonder transmembraandomein gekloneerd in een *pbluescript* SK (-) vector en tot expressie gebracht in *Escherichia coli*. Het enzym is gezuiverd uit het celextract met behulp van anionenwisselings- en gelpermeatie chromatografie. Het gezuiverde enzym had een molekuulgewicht van 329 kDa en de subunits hadden een molekuulgewicht van 94 kDa. Dit is een indicatie dat het enzym als tetrameer voorkomt. De optimale pH voor endo-galactanase activiteit was 5,0 en de optimale temperatuur 37°C, waarbij AZCL-galactaan als substraat werd gebruikt. De K_m en V_{max} voor AZCL-galactaan waren respectievelijk 1,62 mM and 99 U/mg. Het enzym was in staat om galacto-trisacchariden van (β 1 \rightarrow 4)-galactanen en (β 1 \rightarrow 4)-galacto-oligosacchariden te splitsen, waarschijnlijk met een

processief mechanisme, bewegend in de richting van het reducerende einde na een initiële splitsing in het midden van de keten. Het enzym lijkt in staat te zijn om ($\beta 1 \rightarrow 3$)-bindingen te splitsen, maar ($\beta 1 \rightarrow 4$)-bindingen worden waarschijnlijk sneller afgebroken. Arabinose zijketens, in bijvoorbeeld aardappelgalactaan, hinderen YvfO.

Hoofdstuk 5 beschrijft de hoge transglycosyleringsactiviteit van het α -galactosidase (AGA) uit *Bifidobacterium adolescentis* DSM 20083. De optimale omstandigheden voor deze transglycosyleringsactiviteit waren pH 8 en 37°C. Bij een hoge melibiose concentratie (600 mM) resulteerde ongeveer 64% van de splitsingen in transglycosylering. AGA heeft een hydroxyl groep op C-6 van het acceptorsubstraat nodig voor transglycosylering. Ook disacchariden waren goede acceptoren. Pentoses, hexuronzuren, deoxyhexoses, en alditolen konden niet als acceptor gebruikt worden. Door zijn transglycosyleringsactiviteit biedt AGA mogelijkheden om prebiotica te verlengen. AGA heeft echter naast deze transglycosyleringsactiviteit ook hydrolyse activiteit, wat het verlengen van oligosacchariden minder efficiënt maakt. Daarom werd geprobeerd om de transglycosyleringsefficiëntie van AGA te verhogen. Met behulp van homology modeling is een mogelijke 3D-structuur van AGA's katalytische centrum gemaakt. Op basis van dit model en analyse van de aminozuurvolgorde, is site-directed mutagenese uitgevoerd om een verhoging van de transglycosyleringsactiviteit te bewerkstelligen. Dit heeft geresulteerd in tien positieve mutanten met verhoogde transglycosyleringsactiviteit: 4 met een enkelvoudige mutatie en 6 met een dubbelvoudige mutatie. De resultaten laten zien dat het mogelijk is om met slechts een mutatie (His⁴⁹⁷Met) de transglycosyleringsactiviteit met 16% te verhogen ten opzichte van het wild-type. De meeste mutaties gaven een verhoging van 2-7%. Het combineren van succesvolle enkelvoudige mutaties resulteerde echter niet in een extra verhoging van de transglycosyleringsactiviteit (mutaties Gly³⁸²Cys-Tyr⁵⁰⁰Leu leveren een verhoging van 9% op en His⁴⁹⁷Met-Tyr⁵⁰⁰Leu een verhoging van 16%). De acceptor specificiteit was niet veranderd in de mutanten, terwijl de thermostabiliteit van de mutanten, die de mutatie Gly³⁸²Cys bezaten, gedaald is.

Uit de resultaten van dit proefschrift kan opgemaakt worden dat galactanen en langere galacto-oligosacchariden extracellulair door het endo-galactanase kunnen worden afgebroken tot trisacchariden. De genomsequentie suggereert dat *B. longum* geen extracellulaire β -galactosidases heeft, wat erop zou kunnen wijzen dat dit microorganisme een soort mechanisme heeft om galacto-oligosacchariden intracellulair te krijgen, waar de

trisacchariden zullen worden afgebroken door de verschillende galactosidases die daar aanwezig zijn.

In hoofdstuk 6 wordt bediscussieerd hoe specifiek prebiotica moeten zijn, evenals de beoogde gezondheidsbevorderende effecten van pre- en probiotica. Om meer inzicht te krijgen in het complexe systeem van de darmflora, zal het belangrijk zijn om het mechanisme van koolhydraatgebruik door de darmflora op te helderen. De resultaten in dit proefschrift laten zien dat een gedetailleerde biochemische karakterisatie van koolhydraat afbrekende enzymen waardevolle informatie oplevert over hoe bifidobacteriën koolhydraten gebruiken.

Nawoord

Ik begin deze laatste pagina's van mijn proefschrift zoals zoveel anderen doen: het is af!! Heerlijk dat het af is. De laatste maanden waren nog even hard doorwerken, maar het is allemaal gelukt en daar ben ik erg blij mee. Het onderzoek kende een moeizame start, maar uiteindelijk is het allemaal toch erg snel gegaan. Ik heb met heel veel plezier aan dit onderzoek gewerkt en daarbij heb ik natuurlijk hulp gehad van verschillende mensen, die ik daarvoor graag zou willen bedanken.

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Sandra

Curriculum Vitae

Sandra Hinz werd geboren op 28 maart 1977 te Weert. Na het behalen van haar VWO-diploma in 1995 aan het Elzendaalcollege te Boxmeer, begon zij in september van dat jaar met de studie Levensmiddelentechnologie aan de toenmalige Landbouwniversiteit te Wageningen. In het kader van deze studie deed ze tijdens de doctoraalfase haar afstudeeronderzoeken bij de leerstoelgroepen Levensmiddelenchemie en Industriële Microbiologie. Daarnaast liep ze stage bij het Food Research and Development Centre te St-Hyacinthe in Canada en bij Schouten Products BV te Giessen. In november 2000 behaalde zij het doctoraal diploma.

Van januari 2001 tot en met januari 2005 deed zij promotie-onderzoek bij de leerstoelgroep Levensmiddelenchemie van de Wageningen Universiteit, onder begeleiding van prof. dr. A.G.J. Voragen en dr. J.-P. Vincken. Het onderzoek dat in deze periode werd uitgevoerd staat beschreven in dit proefschrift.

Sinds februari 2005 is zij werkzaam als postdoc bij de leerstoelgroep Levensmiddelenchemie van de Wageningen Universiteit.

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Addendum

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