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Enzymatic Production and Characterization of Konjac Glucomannan Oligosaccharides

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Supporting Information

ABSTRACT: Enzymes from a balanced human gut flora are promising tools to design prebiotic oligosaccharides. In this study, we investigated the action of enzymes from fecal bacteria on the complex polysaccharide konjac glucomannan (KGM). The oligosaccharides produced were compared to oligosaccharides from KGM digests with fungal *endo*-β-(1,4)-glucanase (EG) or *endo*-β-(1,4)-mannanase (EM). For this purpose, the oligosaccharides from the different digests were first studied for their structural characteristics like monosugar composition and exo-enzymatic degradability, as monitored by capillary electrophoresis with laser-induced fluorescence detection. Whereas the oligosaccharides produced by EG and EM were characteristic for the selectivity of the respective enzyme in cleaving the mannose-/glucose-sugar linkages of KGM, oligosaccharides produced by the fecal enzymes did not point to a sugar-selective degradation. The oligosaccharide fragments from the different digests indicated the KGM polysaccharide to be composed of a backbone composed of short mannose and glucose sequences, to which branches rich in mannose are attached.

KEYWORDS: Konjac glucomannan oligosaccharides, fecal enzymes, *endo*-β-(1,4)-mannanase, *endo*-β-(1,4)-glucanase, CE-LIF, prebiotics

INTRODUCTION

Oligosaccharides, intended to specifically stimulate gastrointestinal bacteria beneficial to the host's health, [1,2] are frequently added to food products. Commonly used oligosaccharides are galactooligosaccharides (GOS) and fructooligosaccharides (FOS), which have been studied extensively for their prebiotic potential.[3,4] These short-chain oligosaccharides are rapidly fermented in the colon. To prevent colonic diseases, which mainly take place in the distal part of the colon, more complex prebiotic oligosaccharides are needed that are able to survive the entire gastrointestinal transit.[5,6] The production of tailor-made oligosaccharides for this purpose is of great interest.[2] Complex polysaccharides can be used for the enzymatic production of a variety of oligosaccharide mixtures.

Glucomannan from *Amorphophallus konjac* C. Koch (KGM) represents an interesting polysaccharide for the production of structurally different oligosaccharides. KGM, which is composed of β-(1,4)-linked mannose and glucose units in a molar ratio of 1.5:1, enables the enzymatic hydrolysis by *endo*-β-(1,4)-glucanase (EG) as well as by *endo*-β-(1,4)-mannanase (EM).[7] The monosaccharide units are acetylated to an extent of 5−10%.[8] Branches are estimated to occur once upon every 10−11 hexose units in the main chain.[9] Little is known about the precise structure of KGM, such as the sequence of glucose and mannose within the backbone, precise length and distribution of the side chains, and location of acetyl groups, although several studies have been performed.[10−12] The complex structural composition makes the KGM polysaccharide and the oligosaccharides derived hereof potential substrates for reaching the distal colon, which is the desired target site for bioactive carbohydrates.[13] In a previous study, we tested KGM as well as two different KGM oligosaccharide sets, produced by EM and EG, for their in vitro fermentability by human gut flora.[7] The structural composition of the oligosaccharides was shown to influence the fermentability by human fecal bacteria. For the in vitro fermentation of the polysaccharide, the formation of an unknown oligosaccharide series, different from the series obtained by the glucomannan hydrolysis with fungal EG or EM, was described.[7]

No conclusions on the structural composition of the oligosaccharides, which were formed during in vitro fermentation, could be drawn.[7] The structural characteristics of oligosaccharides produced during the in vitro fermentation of polysaccharides provide information on the action of the enzymatic machinery of the colonic flora and have been recognized as a valuable tool for the design of target-specific prebiotic GOS.[14,15]

To evaluate the action of enzymes from fecal bacteria on KGM, the oligosaccharides produced were compared to two KGM digests produced by fungal EG and EM. For the structural characterization of the different oligosaccharide sets in the digests, oligosaccharides were fractionated according to their size and subsequently studied for their monosaccharide composition and degradability by exo-acting enzymes [*exo*-β-(1,4)-glucanase and β-(1,4)-mannosidase]. Their degradability was followed quantitatively

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by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF).[16] Supplementarily, the information on the structural characteristics of the oligosaccharide fragments could be used to draw some conclusions on the molecular structure of the KGM polysaccharide.

MATERIALS AND METHODS

Materials and Enzymes. The KGM polysaccharide was obtained from Kalys (St. Ismier, France). The KGM of the species *A. konjac* had a molecular mass of approximately 500 kDa, a mannose/glucose molar ratio of 1.5:1, and a degree of acetylation (DA) of 7.9%.[7] All chemicals used were of analytical grade.

EG VI (EC 3.2.1.4; GH family 7) and *exo*-β-(1,4)-glucanase III (EC 3.2.1.91) were purified from a crude cellulase preparation from *Trichoderma viride* (Maxazyme, DSM Food Specialties, Delft, The Netherlands) as described by Beldman et al.[17] EM (EC 3.2.1.78; GH family 5) was purified from a commercial *Aspergillus niger* enzyme mixture (Gamanase III, Novozymes, Bagsvaerd, Denmark) according to Düsterhöft et al.[18] β-(1,4)-Mannosidase from *Helix pomatia* (E.C.3.2.1.25)[19] was purchased from Sigma-Aldrich (St. Louis, MO).

The enzymes from human fecal bacteria originated from a supernatant, which was obtained from an in vitro fermentation of KGM after 24 h of fermentation time. The in vitro fermentation was performed with a mix of three human fecal samples, according to Albrecht et al.[7] The composition of the medium was adapted according to Sunvold et al.[20] The concentration of KGM at the start of the fermentation was 3 mg/mL, and the fermentation bottle was incubated with 200-fold diluted fecal slurry. The incubation volume was 20% (v/v) of the volume of substrate and medium present in the fermentation bottle. To prevent the loss of acetyl groups from KGM, the fermentation flasks were not autoclaved but pasteurized prior to fermentation (70 °C, 30 min). The fermentation liquid taken after 24 h of incubation was centrifuged (5 min, 2300*g*, room temperature), the bacterial cell material was discarded, and the supernatant containing the soluble enzymes from human fecal bacteria was stored at −40 °C.

Enzymatic Hydrolysis and Sample Preparation. The enzymatic digests of KGM using EG and EM were prepared according to Albrecht et al.[7] The enzymatic digests of KGM with enzymes from human fecal bacteria were prepared by adding 2 mL of the supernatant to 10 mL of a 1.5 mg/mL KGM solution in water prior to incubation for 24 h at 37 °C. Samples were mixed continuously during incubation (head-over-tail). Enzymes were inactivated (10 min, 100 °C), and the samples were centrifuged (5 min, 10000*g*, room temperature).

The KGM digests were fractionated into oligosaccharide pools on a BioGel P2 column (26 mm × 900 mm, 200−400 mesh; Bio-Rad, Hercules, CA). Five milliliters of the supernatants was applied onto the column and was eluted with Millipore water (1.5 mL/min) at 60 °C using an Akta Explorer system (GE Amersham, Uppsala, Sweden). The eluate was monitored by a refractive index detector (Shodex RI-72, Showa Denko K.K., Tokyo, Japan) before fractions (5 mL) were collected. The fractions were pooled according to the RI profile and freeze-dried.

For their characterization, 100 μL of the oligosaccharide pools (approximately 0.1 mg/mL) was incubated with 1.35 mU of *exo*-β-(1,4)-glucanase III or 19.7 mU of β-(1,4)-mannosidase. The incubation (24 h) with *exo*-β-(1,4)-glucanase III was performed in 20 mM sodium citrate buffer, pH 3.5, at 30 °C, and the incubation with β-(1,4)-mannosidase (24 h) was performed in 10 mM sodium acetate buffer at pH 4.5 at 45 °C, shaking conditions. The enzymes were inactivated by heating the samples for 10 min at 100 °C, and the hydrolysate was centrifuged (5 min, 10000*g*, room temperature) prior to analysis.

To qualitatively assay the fecal enzyme (FE) mixture, 100 μL of substrate (cellodextrin, maltodextrin, dextran, pectin, differently substituted galactomannans, GOS, and inulin) was incubated with 50 μL of FE mixture. A 350 μL amount of water was added, and the mixture was incubated for 24 h at 37 °C shaking conditions. The enzymes were inactivated by heating the samples for 10 min at 100 °C, and the hydrolysate was centrifuged (5 min, 10000*g*, room temperature) prior to analysis.

ANALYTICAL METHODS

Monosaccharide Composition. The enzymatic digests and the oligosaccharide pools derived thereof were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 121 °C. Subsequently, the acid was evaporated. The residue was repeatedly washed with methanol, followed by evaporation. The hydrolysates were redissolved in Millipore water and analyzed for their monosaccharide composition by HPAEC-PAD on a Dionex ISC 3000 system (Dionex, Sunnyvale, CA). The system was equipped with an analytical Carbopac PA−1 column (2 mm × 250 mm; Dionex) and a guard column (2 mm × 50 mm; Dionex). Separation was performed by elution with water, and 0.5 M sodium hydroxide was added postcolumn to the eluent for detection by PAD.[21]

Pools of DP2 were supplementarily reduced prior to acid hydrolysis to determine the monosaccharide units located at the reducing end. The pools were, therefore, treated with sodium borohydride in ammonium hydroxide as described elsewhere.[22]

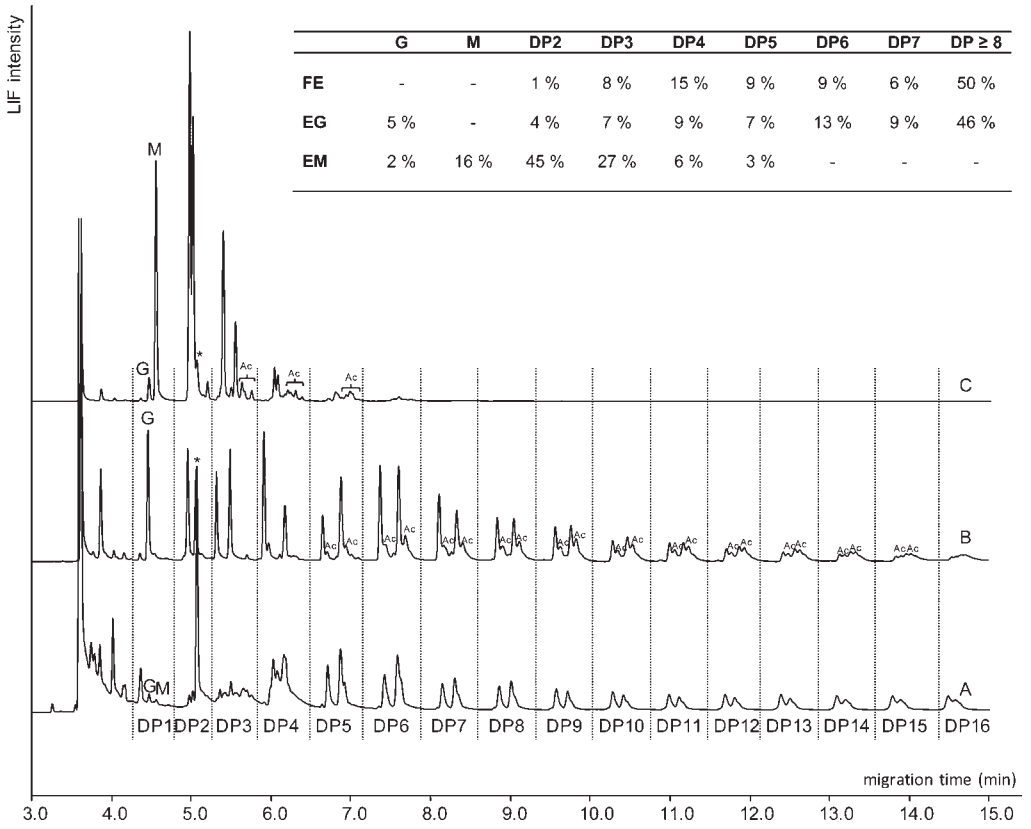
Degree of Acetylation (DA). Acetyl groups released from KGM during incubation with the enzyme mixture from fecal bacteria were determined by the analysis of acetic acid on a Dionex Ultimate 3000 HPLC system (Dionex). The system was equipped with an Aminex HPX 87H column (Bio-Rad) and a Shodex RI72 detector (Showa Denko K.K., Tokyo, Japan). The eluent used was 5 mM sulphuric acid (0.6 mL/min, 40 °C).

The molecular weight distribution of the polysaccharides and the enzymatic digests were determined by HPSEC using an Ultimate 3000 HPLC system (Dionex), equipped with an RI72 refractive index detector (Showa Denko K.K., Tokyo, Japan). Twenty microliter sample solutions (1 mg/mL) were injected, and separation was performed on three Tosoh TSK gel superAW columns in series (AW4000-AW3000-AW2500, each 6 mm × 150 mm; Tosoh Bioscience, Tokyo, Japan) in combination with a guard column (3.5 mm × 46 mm; Tosoh). For elution, 0.2 M sodium nitrate was used at a flow rate of 0.6 mL/min at 55 °C. The system was calibrated with pullulan standards (Sigma Aldrich; mass range 180 Da to 790 kDa).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). For MALDI-TOF MS of oligosaccharides, an UltraflexTreme TOF/TOF system (Bruker, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used. The measurement was performed in the positive mode. After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. Data from averaging 100 laser shots were collected, with the lowest laser energy applied to obtain sufficient spectra intensity. The mass spectrometer was calibrated with maltodextrin. Samples were prepared as described previously.[7]

CE-LIF. The sample preparation for CE-LIF, which includes the derivatization of the carbohydrates with the fluorescent dye APTS (9-aminopyrene-1,4,6-trisulfonate), was done using the ProteomeLab Carbohydrate Characterization kit (Beckman Coulter, Fullerton) as described previously.[7] Separation of the derivatized carbohydrates was performed using a ProteomeLab PA 800 CE system (Beckman Coulter) equipped with a polyvinyl alcohol-coated capillary (50 μm × 50.2 cm). Detection was done with a LIF detector (Beckman Coulter) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm after 40 cm capillary length.

Samples were loaded hydrodynamically (7 s at 0.5 psi, representing approximately 9 nL of sample solution) on the capillary, which was kept at 25 °C. Separation was performed in the reversed polarity mode (30 kV, 20 min) in a 25 mM acetate buffer (pH 4.75) containing 0.4%



	G	M	DP2	DP3	DP4	DP5	DP6	DP7	DP ≥ 8
FE	-	-	1 %	8 %	15 %	9 %	9 %	6 %	50 %
EG	5 %	-	4 %	7 %	9 %	7 %	13 %	9 %	46 %
EM	2 %	16 %	45 %	27 %	6 %	3 %	-	-	-

Figure 1. CE-LIF electropherograms of konjac glucomannan hydrolyzed by (A) FE, (B) EG, and (C) EM. *, maltose (internal standard); Ac, acetylated oligosaccharides. The inlay represents relative proportions of oligosaccharides per DP within the digest.

polyethylene oxide. Between sample runs, the capillary was rinsed with water (5 min, 30 psi) and with separation buffer (2 min, 30 psi).

RESULTS AND DISCUSSION

Production of Oligosaccharides from the KGM Polysaccharide. A digest of KGM with a mixture of soluble enzymes from an in vitro fermentation of KGM with human inoculum (FE) was produced in this study. As the aim of the study was to obtain oligosaccharides and end-point incubation of KGM with FE would result in a complete degradation of the intermediately formed oligosaccharides, the incubation was stopped after 24 h. To compare the hydrolysis of the KGM chain by FEs with the hydrolysis of KGM by fungal enzymes, EG and EM digests of KGM were included (end-point incubations), which were available from our previous research.[7] The oligosaccharides resulting from the different KGM digests could be distinguished by CE-LIF, and the proportions of the DPs to the total digests were calculated (Figure 1 and the table in Figure 1).

A repeating pattern, composed of two peaks per DP, was observed for the FE digest, with oligosaccharides DP ≥ 8 contributing to 50% to the total digest (Figure 1A). The DPs have been confirmed by MALDI-TOF MS. Monomers and dimers were present in trace amounts only in the FE digest. Although only soluble enzymes were present in the enzyme mixture from fecal bacteria, the degradation profile of KGM (Figure 1A) was comparable to the oligosaccharide profile, which was previously obtained during in vitro fermentation.[7] During in vitro fermentation also, cell-associated bacterial enzymes may have been involved. Acetylated oligosaccharides were present in trace amounts only in the FE digest, as investigated by MALDI-TOF MS (results not shown). The lack of acetylated oligosaccharides in the FE digest points to the activity of acetyl-esterases present in the FE mixture. The activity of acetyl-esterases was proven by the decrease of pH (ΔpH = 0.6) and the presence of free acetic acid in the incubation liquid.

Enzymes of the fecal mixture were assayed qualitatively. A broad range of substrates (cellodextrin, maltodextrin, dextran, pectin, differently substituted galactomannans, GOS, and inulin) was incubated with the FE mixture. Mannanase and glucanase activity could be proven. On the other hand, substrates not composed of a glucose or mannose backbone were not degraded.

The oligosaccharides resulting from the EG digest comprise a large DP range (DP > 15; Figure 1B), with oligosaccharides of DP ≥ 8 contributing to 46% of the total digest. Acetylated oligosaccharides, as previously determined by MALDI-TOF MS before and after saponification of the oligosaccharides,[7] are indicated in Figure 1B. Although showing an oligosaccharide pattern similar to the FE digest, most peaks of the EG digest showed different migration times as compared to the FE digest.

Mannose (16%), dimers (45%), and trimers (27%) are the main degradation products in the EM digest (Figure 1C). Tetramers and pentamers are minor enzymatic hydrolysis products (6 and 3%, respectively). This can be explained by the mannose/glucose ratio of the polymer (mannose/glucose 1.5:1) and the ability of EM from *A. niger* to cleave mannose−mannose and mannose−glucose linkages.[23] Acetylated oligosaccharides, as previously determined by MALDI-TOF MS[7] and deacetylation experiments, are indicated in Figure 1C.

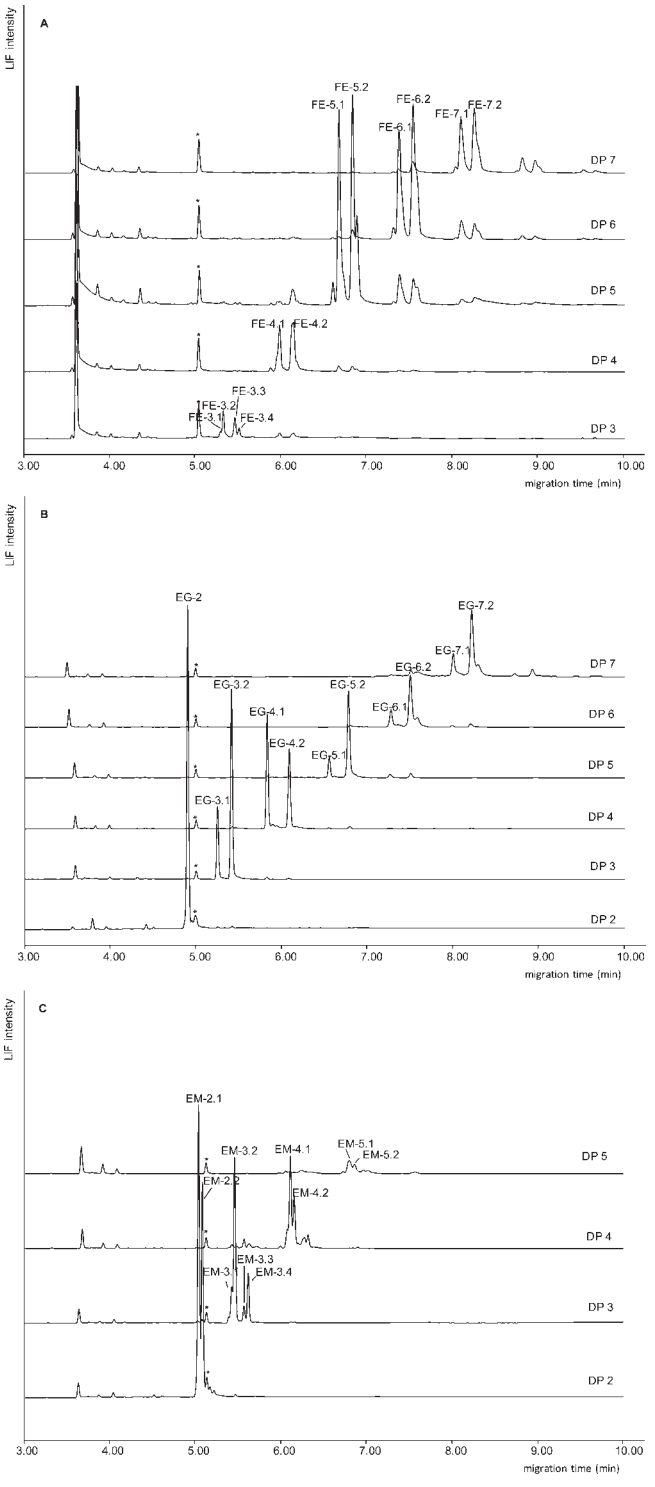


Figure 2. CE-LIF electropherograms of BioGel P2-pools from konjac glucomannan digest with (A) FE, (B) EG, and (C) EM. *, maltose (internal standard). The peaks within the respective pools are indicated.

Characterization of Enzymatic Hydrolysis Products from KGM. SEC of the enzymatically digested polysaccharide resulted in pools of distinct DPs for DP2−DP7, with minor contamination of the preceding and following DP, as was determined by MALDI-TOF MS (data not shown). CE-LIF electropherograms of the pools from the FE, EG, and EM digest, which were used for further investigation, are shown in Figure 2. Tri- and tetrasaccharides present in the respective pools of the FE digest (Figure 2A) were separated more clearly as compared to the total digest (Figure 1A), most probably due to impurities present in the total digest.

Table 1. Molar Ratios of Mannose (M) and Glucose (G) in Pools of Enzymatic Konjac Glucomannan Digests

	M/G		
	FE digest	EG digest	EM digest
DP1		0:1	8.8:1
DP2		0.9:1	2.3:1
DP2 after reduction[a]		*6.4:1*	*0.7:1*
DP3	1.3:1	1.4:1	0.9:1
DP4	1.5:1	1.4:1	1.0:1
DP5	1.5:1	1.9:1	1.1:1
DP6	1.5:1	2.3:1	
DP7	1.5:1	2.4:1	

[a] Supplementary, for dimers, the ratios after sample reduction are indicated. Only the sugar located at nonreducing end is detected.

Oligosaccharides decorated with acetyl groups were not present in the oligosaccharide pools of the EG and EM digest. Their presence in the total digests has previously been stated (Figure 1).[7] Thus, a lack of stability during the fractionation procedure is indicated for the acetyl groups.

Monosaccharide Composition of KGM Pools. For an overview, monosaccharide composition analysis of the oligosaccharide pools was performed. The molar ratio of mannose/glucose for the oligosaccharide pools is presented in Table 1. For each digest, a characteristic trend in mannose/glucose ratio was observed.

The oligosaccharide pools of the FE digest showed a constant mannose/glucose ratio (1.5:1, mannose/glucose) up to DP7. The initial mannose/glucose ratio of 1.5:1 in the KGM polysaccharide chain is thus maintained. A nonselective hydrolysis of KGM by FE can therefore be concluded.

Although the EG digest showed a homologous series of peaks, which is also found for the FE digest, the trends in mannose/glucose ratio were not similar. The molar ratio for the EG digest was 1.4 mol mannose per mol glucose for DP3 and DP4. The mannose/glucose ratio significantly increased to 2.4:1 for DP7.

Increased mannose ratios were expected for the higher M_w pools of the EG digest since mannose is present to a larger extent than glucose in the KGM polysaccharide, and EG is only able to cleave the glycosidic linkage between glucose−glucose and glucose−mannose but not between mannose−mannose and mannose−glucose.[24]

The mannose ratio in the DP3−DP5 oligosaccharide pools of the EM digest was constantly low and equal to glucose (0.9−1.1 mol mannose per mol glucose). DP5 is the highest M_w oligosaccharide pool produced by the hydrolysis of KGM by EM. With a mannose/glucose ratio of 1.1:1 for DP5, sequences of >2 glucose units are indeed not expected to occur in the KGM polysaccharide.[23] Taking into account monomeric mannose, which was present in considerable amounts in the total digest (16% of the total peak area; table in Figure 1), these results highlight the good accessibility of the enzyme to the glucomannan chain, as was indicated before.[23] Summarizing, for both EG and EM digest, a more selective enzymatic degradation of the KGM than for the FE digest was indicated by the monosaccharide compositions of the oligosaccharide pools.

Table 2. Composition of Dimer Pools from EG and EM Digest of Konjac Glucomannan, as Determined by Monosaccharide Composition Analysis[a]

	peak	structure	% of pool
EG digest	2	M-G	~100
		G-G	traces
EM digest	2.1.	G-M	57
	2.2.	M-M	43

[a] The relative proportion of the respective structure in the pool is indicated. Peak numbers are according to Figure 2.

Structural Composition of Dimers from EG and EM Digests. Before characterizing higher M_w oligosaccharides from the FE, EG, and EM digests, we first confirmed the composition of the dimers as present in the EG and EM digest. For these dimers, a theoretical assignment has previously been established,[7] as discussed below.

The sugar compositions of the dimer pools were also determined after reduction with sodium borohydride to determine the sugar unit located at the reducing end (Table 1). Combining this information with the number and proportion of compounds present in the respective EG and EM pool (Figure 2B,C) enabled the assignment of the dimers. M-G was assigned as the component present in the dimer pool of the EG digest (M = mannose; G = glucose; Figure 2B, peak EG-2), which is in agreement with the structure hypothesized in our previous study.[7] Supplementarily, a small proportion of G-G can be assumed to be present, due to the ratio of 0.9 mannose per mol glucose.

G-M (EM-2.1) and M-M (EM-2.2) were assigned for the EM digest (Figure 2C), and the assignment is in agreement with previous data.[7] The ratios of 2.3 mannose per mol glucose before reduction and 0.7 mol mannose per mol glucose after reduction in the EM digest match with the CE-LIF peak proportions of EM-2.1 (G-M; 57%) and EM-2.2 (M-M; 43%). The structural assignments as made for the dimer pools are summarized in Table 2.

Characterization of KGM Oligosaccharide Pools with exo-Enzymes. Knowledge of the monosaccharide composition is not sufficient for the assignment of oligosaccharides of DP > 2, due to the increase in structural possibilities. The hydrolysis of the oligosaccharides by *exo*-enzymes, which results in a sequential release of mono- and disaccharide units located at the nonreducing end will provide additional structural information. As KGM is composed of glucose and mannose, a cellobiohydrolase [*exo*-β-(1,4)-glucanase III (*exo*-glucanase) from *T. viride*][17] and a β-(1,4)-mannosidase (β-mannosidas; from *H. pomatia*)[19] were used. To first understand the action of these enzymes on KGM, the trimer pools of the EG and EM digests were used. A theoretical assignment has previously been established for these oligosaccharides and will be discussed below. Subsequently, the information obtained was used for the assignment of the oligosaccharide structures as present in the FE digest.

Mode of Action of exo-β-(1,4)-Glucanase III (exo-Glucanase) and β-(1,4)-Mannosidase (β-Mannosidase) toward KGM Trisaccharides. The dimers and monomers, which resulted from the incubation of the trimer pools with the *exo*-enzymes, are listed in Table 3. The dimers M-G, G-M, and G-G can, however, hardly be distinguished by CE-LIF if present together. The respective electropherograms, on which Table 3 is based, are shown in Figure S1 in the Supporting Information.

Table 3. Hydrolysis Products from *exo*-Glucanase and β-Mannosidase Incubated on Trisaccharide Pools from Konjac Glucomannan EG, EM, and FE Digest (for the Respective Electropherograms, see Figure S1 in the Supporting Information)[a]

	peak	*exo*-glucanase	β-mannosidase	structure	% of pool	M/G
EG digest	3.1	(G, M-G)	M, G G-G	M-G-G	26	1.4:1
	3.2			M-M-G	74	
EM digest	3.1		M, G-M	M-G-M	12	0.9:1
	3.2	M, G-G		G-G-M	64	
	3.3		M	M-M-M	6	
	3.4			G-M-M	18	
FE digest	3.1		M, G, G-G,[b]	M-G-G	8	1.5:1
	3.2		G-M[b]	M-G-M	42	
	3.3			M-M-G	36	
	3.4			G-M-M	14	

[a] Peak numbers are according to Figure 2. The M/G ratio was calculated based on the relative proportion of the respective structure in the pool. (), no complete degradation. [b] If comigrating, M-G, G-M, and G-G cannot be distinguished.

The trimer pool of the EG digest is composed of two structures (Figure 2B), previously theoretically assigned as M-G-G (EG-3.1) and M-M-G (EG-3.2).[7] EG-3.1 was only slightly degraded by *exo*-glucanase, whereas EG-3.2 was not at all degraded. For the EM digest, G-G-M, M-M-M, and G-M-M were previously assigned to the compounds EM-3.2, EM-3.3, and EM-3.4, respectively (Figure 2C).[7] Only G-G-M (EM-3.2) was degraded by *exo*-glucanase.

Thus, *exo*-glucanase can cleave off the terminal disaccharide unit G-G attached to a reducing mannose unit. A release of the terminal M-G unit, attached to a reducing glucose unit, is limited. G-M linked to a reducing mannose unit cannot be hydrolyzed by *exo*-glucanase.

Except for G-G-M (EM-3.2) and G-M-M (EG-3.4), all other structures were degraded by β-mannosidase. Thus, β-mannosidase was shown to be able to remove terminal mannose units, independent of being β-linked to a mannose or glucose unit. β-Mannosidase completely hydrolyzes repeating mannose units (e.g., M-M-G to 2 M and G or M-M-M to 3 M), which was concluded from the quantitative amounts of mannose released in the digested pools (data not shown).

Although not detected by us before,[7] a fourth structure (M-G-M, Figure 2C, peak EM-3.1) was assigned in the DP3 pool of the EM digest, due to its resistance to *exo*-glucanase digestion and its degradation to M and G-M by β-mannosidase. The presence of M-G-M in a KGM digest produced by EM from *A. niger* has been previously described.[11]

Information on the mode of action of the *exo*-enzymes was thus obtained by hydrolyzing the trisaccharide pools. Simultaneously, the mono- and disaccharides resulting from the hydrolysis of the trisaccharide pools confirm our previous theoretical assignments of the oligosaccharide structures.[7] The structural assignment of the trisaccharides is in agreement with the monosaccharide composition as mentioned in Table 1, considering the relative proportions of the individual oligosaccharides within the pools (Table 3).

The trisaccharides FE-3.1, FE-3.2, FE-3.3, and FE-3.4 of the FE digest were assigned as M-G-G, M-G-M, M-M-G, and

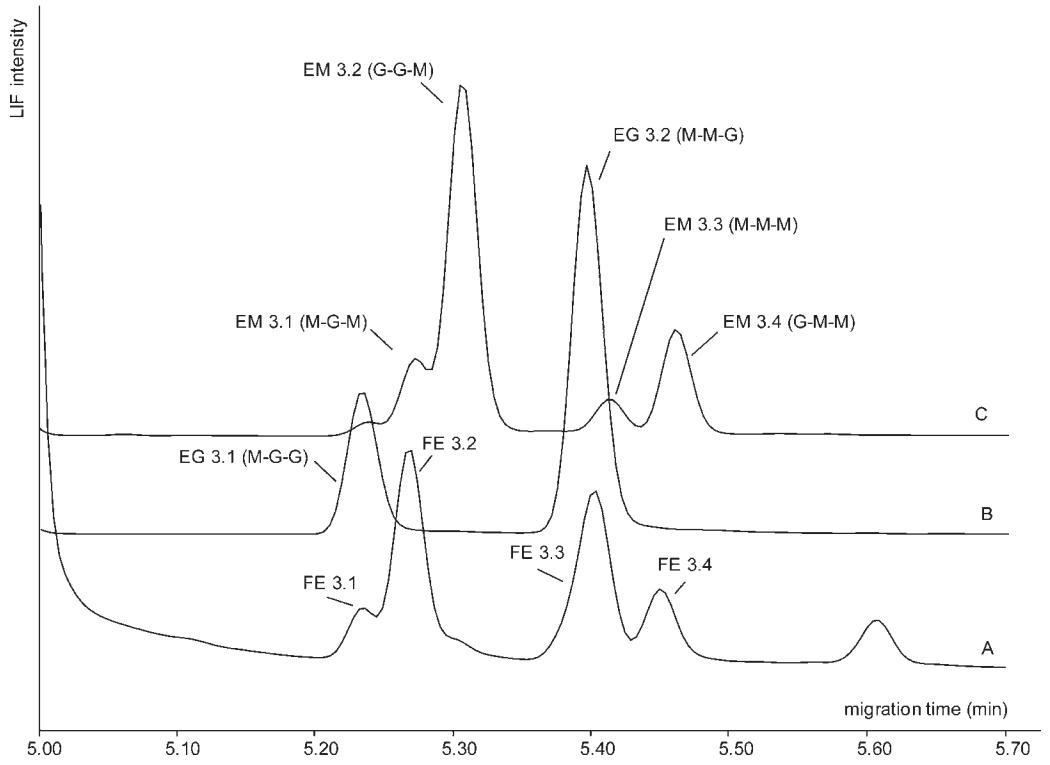


Figure 3. CE-LIF electropherograms of DP3 pools of konjac glucomannan hydrolyzed by (A) FE, (B) EG, and (C) EM. The peaks within the respective pools are indicated.

Table 4. Degradability of Different KGM-Oligosaccharide-Pools upon Incubation with *exo*-glucanase and *β*-mannosidase (% Represents Decrease of Respective Peak Area upon *exo*-Enzyme Incubation as Compared to the Nonincubated Samples)

	FE digest		EG digest		EM digest	
	exo-glucanase (%)	*β*-mannosidase (%)	*exo*-glucanase (%)	*β*-mannosidase (%)	*exo*-glucanase (%)	*β*-mannosidase (%)
DP3	0	86	7	97	64	31
DP4	23	43	59	86	95	72
DP5	22	34	68	±50[a]	49	50
DP6	85	38	81	83		
DP7	96	54	91	84		

[a] Deviating enzyme batch.

G-M-M, respectively (Figure 3 and Table 3). The presence of both glucose and mannose as reducing end units in the FE digest indicates a multiple enzyme system and a different mode of action of the bacterial enzymes on the KGM polysaccharide as compared to the fungal enzymes.

Assignment of Higher M_w KGM-Oligosaccharides Based on Their Degradation with exo-Enzymes. Knowing the structure of the various DP2 and DP3 peaks and the mode of action of the enzymes allowed the use of the *exo*-enzymes for the structural investigation of higher M_w KGM oligosaccharides. To subsequently draw some conclusions on the structural composition of the KGM polysaccharide, attention was paid not only to the investigation of oligosaccharides from the FE digest but also to the oligosaccharides in the EG and EM digest. The KGM pools investigated were degraded to different extents by the *exo*-enzymes. Table 4 summarizes the decrease in total initial peak area of the oligosaccharides in the pools upon incubation.

FE Digest. For the FE digest, the susceptibility to hydrolysis by *exo*-glucanase increased with increasing size of the oligosaccharides (Table 4). Oligosaccharides of the DP5 pool showed the lowest degradability by *exo*-glucanase as well as by *β*-mannosidase. This low degradability may be deduced to a complicated structural composition and may thus explain the predominant presence of DP5 oligosaccharides during the in vitro fermentation.[7] Interestingly, all FE pools degraded by *exo*-glucanase showed the presence of G-G-M, M-G-M, M-M-G, and G-M-M, as exemplified by the hydrolysis of the DP4 pool in Figure 4A. These trisaccharides are the components of the DP3 pool from the FE digest (Table 3). The homologous peak series observed for the FE digest can thus be explained by the presence of a number of core structures, which are extended, either linear or branched. Clearly, a variety of structures are present.

β-Mannosidase digestion of the FE pools of a given DPx resulted in the emergence of mainly oligosaccharides of DPx-1 and mannose, as exemplified for the hydrolysis of DP4 in Figure 4A.

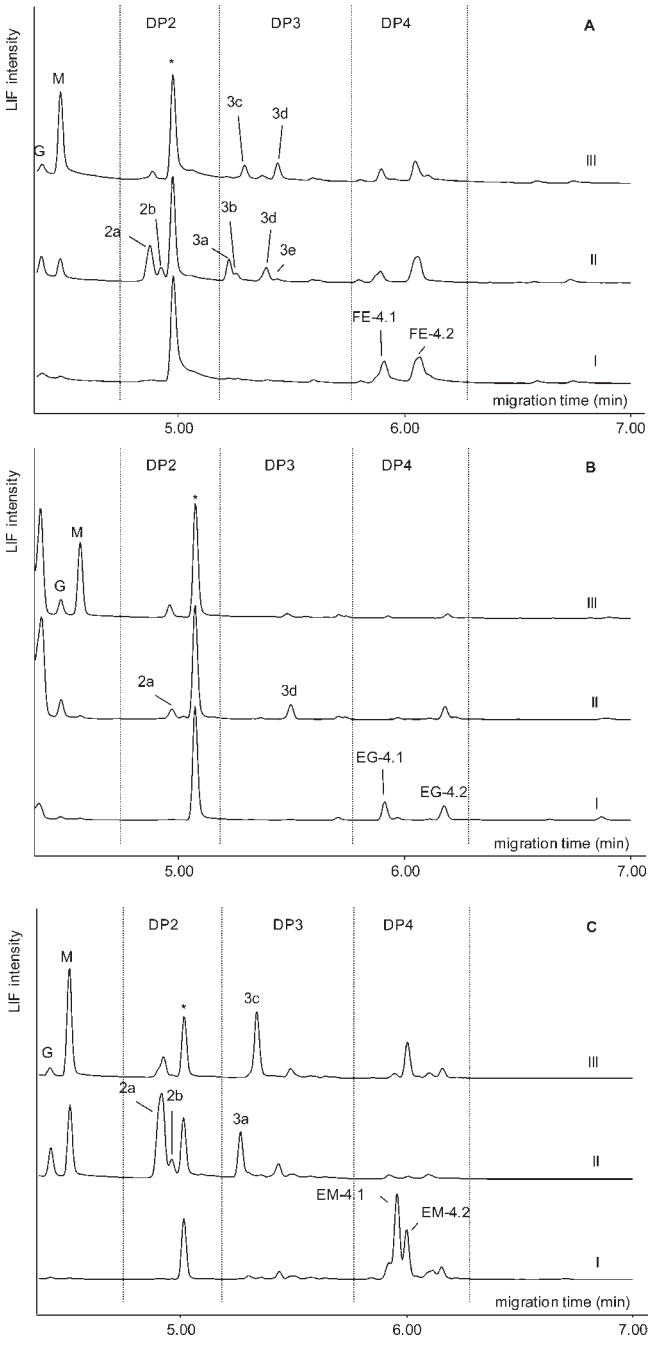


Figure 4. Action of exo-glucanase (A/B/C-II) and β-mannosidase (A/B/C-III) on tetrasaccharides resulting from konjac glucomannan digest with (A-I) FE, (B-I) EG, and (C-I) EM. FE-4.1/-4.2, EG-4.1/-4.2, and EM-4.1/-4.2 are according to Figure 2. Peaks: 2a, G-G/G-M/M-G; 2b, M-M; 3a, M-G-G°; 3b, M-G-M°; 3c, G-G-M°; 3d, M-M-G°; and 3e, G-M-M°. °, structures may be linear or branched. *, maltose (internal standard).

The action of β-mannosidase is thus hindered by the presence of a subsequent glucose unit and indicates the frequent occurrence of mannose−glucose repeats in the oligosaccharides of the FE pool. The structural proposition of the tetrasaccharides is given in Table 5.

Considering the ratio of 1.5 mannose per mol glucose, which represents the distribution as present for the intact KGM polysaccharide, the FE mixture seems to act less specific on the KGM chain as compared to the fungal enzymes.

Table 5. Structural Propositions for Tetrasaccharides from FE, EG, and EM Digest of Konjac Glucomannan[a]

	peak	structural proposition
FE digest		X-(M-G-G) or [X]-(M-G-G)
		X-(M-G-M) or [X]-(M-G-M)
		X-(M-M-G) or [X]-(M-M-G)
		X-(G-M-M) or [X]-(G-M-M)
EG digest	4.1	M-[M]G-G
	4.2	M-M-G-G/M-M-M-G
EM digest	4.1	[M]G-G-M
	4.2	G-M-G-M

[a] Peak numbers are according to Figure 2. The sugar unit written in brackets indicates a branch attached to the respective sugar unit in the backbone. X/[X]-(X-X-X): M or G attached to trisaccharide core structure in linear or branched way.

EG Digest. For the EG pools, the degradability of oligosaccharides with *exo*-glucanase and β-mannosidase significantly increased with increasing DP (Table 4). The degradability by *exo*-glucanase was surprising, since high mannose ratios were found for the higher M_w EG pools (Table 1). A simple structural extension by mannose units at the nonreducing end of the oligosaccharides present in the preceding pool is thus not the case. The profiles of the tetramer pool, composed of EG-4.1 and EG-4.2, after digestion with *exo*-glucanase and β-manosidase, are shown in Figure 4B.

The presence of a branched oligosaccharide was assumed for EG-4.1, as *exo*-glucanase resulted in the appearance of glucose, dimers, and a trimer at the migration time of M-M-G, whereas β-mannosidase resulted in a complete degradation to monomers and dimers.

exo-Enzymes may act differently on branched structures than on linear structures. Nevertheless, the good degradability by both enzymes indicates the presence of mannose at the nonreducing ends and a core unit, which contains glucose for DP-4.1, e.g., M-[M]G-G (the sugar unit written in brackets indicates a branch attached to the respective sugar in the backbone; Table 5). For DP-4.2, a single or combined presence of M-M-G-G or/and M-M-M-G was assumed (Table 5), as concluded by its resistance to *exo*-glucanase digestion and degradation by β-mannosidase (Figure 4B). An increased presence of branched oligosaccharides rich in mannose may explain the presence of the homologous series up to DP > 15, as observed for the EG digest.

EM Digest. DP4 and DP5 pools were present in the EM digest, but they did not significantly contribute to the total digest (Figure 1). As compared to the abundance of mono-, di-, and trimers, these DP pools are thus less dominant end products of the hydrolysis of KGM by EM. Equal proportions of glucose and mannose were found for the EM-tetramer pool (Table 1). The oligosaccharides in the DP4 pool (EM-4.1 and EM-4.2) are well-degraded by *exo*-glucanase (Figure 4C). A branched structure was assumed for EM-4.1 ([M]G-G-M, Table 5), due to the presence of G-G-M in the β-mannosidase digest. Additionally, a compound at the migration time corresponding to M-G-G was present in the *exo*-glucanase digest, which may correspond to the branched trisaccharide [M]G-G. The dominant presence of dimers in the *exo*-glucanase digest was assumed to result from EM-4.2. EM-4.2 was not degraded by β-mannosidase and was thus assigned as G-M-G-M (Table 5). No conclusions could be drawn on the structural composition of oligosaccharides present in the DP5 pool.

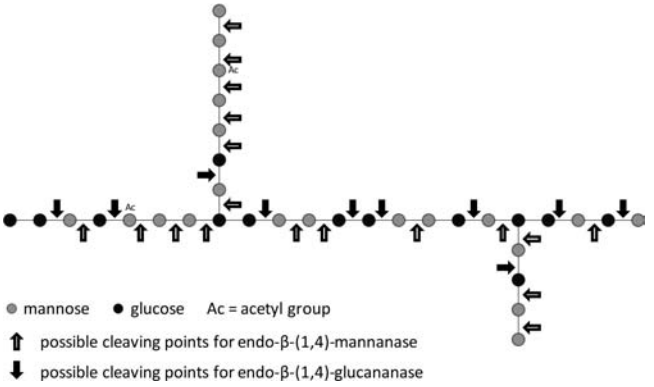


Figure 5. Excerpt of the konjac glucomannan polysaccharide and possible enzymatic cleaving points as hypothesized based on the oligosaccharide fragments obtained in this study (Table 6). Acetyl groups are placed arbitrarily on the mannose units, and possible hindrance to enzymatic hydrolysis has not been taken into account.

Table 6. Summary of Oligosaccharide Fragments from EG, EM, and FE Digests, as Characterized in This Study[a]

	EG	EM	FE
DP1	G	G, M	
DP2	M-G	G-M	
	G-G	M-M	
DP3	M-G-G	M-G-M	M-G-G
	M-M-G	G-G-M	M-G-M
		M-M-M	M-M-G
		G-M-M	G-M-M
DP4	M-[M]G-G	[M]G-G-M	X/[X]-(M-G-G)
	M-M-G-G/	G-M-G-M	X/[X]-(M-G-M)
	M-M-M-G		X/[X]-(M-M-G)
			X/[X]-(G-M-M)

[a] X/[X]-(X-X-X): M or G attached to trisaccharide core structure in linear or branched way.

Characterization of the KGM Polysaccharide. A hypothetical structure of the KGM polysaccharide is shown in Figure 5, as based by the oligosaccharide fragments obtained in our study and the enzymatic cleaving sites deduced hereof. Table 6 summarizes the respective oligosaccharide fragments obtained. The enzymatic cleaving sites of the FE mixture are not indicated in Figure 5. On the basis of the oligosaccharide fragments obtained (Table 6), though, a nonspecific cleaving mechanism of the FE mixture can be assumed. Acetylation was not taken into account for the oligosaccharides presented in Table 6. KGM is an acetylated polysaccharide (DA 5−10%).[8] The distribution of the acetyl groups has not yet been determined. An attachment of the acetyl group to the mannose unit can be assumed (Figure 5) due to the presence of higher M_w acetylated oligosaccharides in the EG digest (Figure 1), which are rich in mannose.

In the literature, different opinions on the structure of the KGM polysaccharide exist. A blockwise distribution of mannose units within the KGM polysaccharide chain was claimed by Shimahara et al.,[10] whereas a random distribution was proposed by Cescutti et al.[11] The significantly increased ratio of mannose within oligosaccharides DP > 4 in the EG digest as observed in our research supports the existence of a blockwise distribution of mannose units within the KGM polysaccharide chain. Ramification of the KGM backbone with side chains rich in mannose would explain the homologous peak series of DP > 15 as obtained for the EG digest. It would also justify the good degradability of the polysaccharide by EM to mainly monomers, dimers, and trimers. The small hydrolysis products and the equal mannose/glucose ratio in the DP pools for EM confirm the absence of more than two sequential glucose units in the KGM chain as suggested by Shimahara et al.[10] Repeating glucose/mannose-containing core structures in the KGM main chain, to which mannose-rich branches are attached, are assumed by us, as indicated by the structural assignments M-[M]G-G (EG-4.1) and [M]G-G-M (EM-4.1). Our research supports the attachment of the side chains to glucose units in the backbone, as was proposed by Katsuraya et al.,[12] but disagrees with the findings of Maeda et al.[9] and Smith et al.,[25] who propose branching at both glucose and mannose units in the polysaccharide backbone.

Evaluation and Implication of the Hydrolysis of KGM by FE. For the degradation of KGM by human gut bacteria, an enzyme machinery is activated, which results in the presence of a broad range of nonacetylated oligosaccharides. The FE mixture seems to act in a less selective way on the KGM chain as compared to the fungal enzymes tested. This can be concluded from the ratio of 1.5 mannose per mol glucose for the oligosaccharides produced, which represents the sugar distribution of the intact KGM polysaccharide. A multienzyme system expressed by the fecal bacteria is indicated by the presence of KGM hydrolysis products of consecutive mannose−glucose units. Gut bacteria adapt their enzyme expression to the substrates provided, as has been previously concluded from an in vitro fermentation of arabinoxylan.[26]

Surprisingly, in the FE digest, mono- and dimers were absent. With the absence of mono- and dimers and the presence of a large range of oligosaccharides obtained for the FE digest and the in vitro fermentation,[7] endo-acting enzymes, which have different modes of action on KGM as compared to the fungal enzymes used in this study, may therefore be prevailing. The oligosaccharides observed may represent suitable intermediate degradation products, which serve as nutrients for gut bacteria. Bacterial cross-feeding has been recognized as gastrointestinal utilization mechanism for polysaccharides.[27]

Monitoring and characterizing intermediately formed oligosaccharides during in vitro fermentation provide insight into the degradation mechanism of complex polysaccharides in the gastrointestinal tract. The structural composition of oligosaccharide mixtures influences their utilization by fecal bacteria, as was previously observed.[7] Oligosaccharides produced by EM, which are of small size carrying a glucose unit at their nonreducing end, resisted more to in vitro fermentation than oligosaccharides produced by cellulase.[7] The *endo*-enzymes of the fecal microbiota show a different mode of action on KGM, and only the determination of the prebiotic index (PI; defined as PI = [(Bifidobacteria/total bacteria) − (Bacteroides/total bacteria) + (Lactobacilli/total bacteria) − (Clostridia/total bacteria)]) would give information on the prebiotic value of the different oligosaccharide mixtures.[28] Prebiotic oligosaccharides produced by FE would require the characterization and identification of these enzymes for their subsequent large-scale use for the production of oligosaccharides. The production of functional oligosaccharides by the enzymes from health beneficial bacteria or the gut microbiota already has been recognized for the production of GOS,[14,15] although not yet commercially applied.

■ ASSOCIATED CONTENT

Supporting Information. Action of *exo*-enzymes on trisaccharides resulting from different konjac glucomannan digests. This material is available free of charge via the Internet at http://pubs.acs.org.

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