Structural Microheterogeneity of Kefiran from Kefir Grains

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Abstract The structure of kefiran from kefir grains was reinvestigated, because 6-*O*-substituted galactose was detected in methylated derivatives of kefiran, which was not expected to occur from the structure proposed by KOOIMAN³⁾. Hydrolysis of kefiran with *Trichoderma reesei* cellulase released only glucose and a pentasaccharide. By methylation analysis and ¹³C-n.m.r. spectroscopy, the pentasaccharide structure was elucidated as β -D-Glc-(1 $\rightarrow$ 2)- β -D-Gal-(1 $\rightarrow$ 4)- α -D-Gal-(1 $\rightarrow$ 3)- β -D-Gal-(1 $\rightarrow$ 4)-D-Glc, and this coincided with the structure proposed by KOOIMAN³⁾. However, methylation analysis of kefiran itself and ¹³C-n.m.r. spectra suggested the presence of an additional structure including 6-*O*-substituted galactose. Kefiran is considered to be constructed not only by the structure proposed by KOOIMAN³⁾ but also by another repeating unit including 6-*O*-substituted galactose.

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Key words: chemical structure, kefiran, kefir grains, microheterogeneity, pentasaccharide

Kefir grains are granular conglomerates which are used for making kefir, a popular fermented milk in Northern and Eastern Europe. Microorganisms are held in the grain together with insoluble proteins and a polysaccharide composed of equimolar glucose and galactose^{1,2)}, which were collectively called kefiran by LA RIVIÈRE *et al.*¹⁾. KOOIMAN³⁾ proposed a structure of kefiran made up by the repeating of the pentasaccharide unit named kefirose.

In a previous paper⁴⁾, we became aware of the discrepancy between the published structure of kefiran and our results obtained by methylation analysis of kefiran. We have detected 0.20 mole of 2,3,4-tri-*O*-methylgalactose as an additional trimethylgalactose in methylated derivatives of kefiran other than 2,3,4-tri-*O*-methylglucose, 3,4-di-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, and 2,3,6-tri-*O*-methylglucose which are detectable in molar ratios of 1 : 1 : 1 : 1 : 1 : 1. This suggests the presence of 6-*O*-substituted galactose in the structure of kefiran.

In the present paper, the structure of kefiran proposed by KOOIMAN³⁾ has been reinvestigated in detail by methylation analysis, ¹³C-n.m.r and enzyme analysis.

Materials and Methods

Kefir grains

Kefir grains were purchased from Christian Hansen's Laboratory (Copenhagen, Denmark).

The kefir grains were inoculated in sterilized reconstituted skim milk at 5% (w/v), then incubated at 20°C for 24 h. Harvested kefir grains were inoculated again in the next lot of skim milk and incubated in the same way. This procedure was repeated until sufficient amount of kefir grains was obtained.

Chromatography and electrophoresis

Thin-layer chromatography (t.l.c.) was done via the multiple ascending technique on silica gel 60 (Merck, No. 5553) in a solvent system of ethylacetate-acetic acid-water (2 : 1 : 1, v/v/v). Sugars were detected by spraying with 5% sulfuric acid in methylalcohol and heating.

Zone electrophoresis was carried out at 16.5 V/cm for 3 hours in 0.1 M sodium tetraborate (pH 9.3) on Toyo GA-100 glass fiber filter paper (6 × 60 cm). The filter paper was cut into strips 1 cm long which were then eluted with distilled water. The carbohydrate in the eluted solutions was determined via the phenol-sulfuric acid method⁵⁾.

Paper electrophoresis of oligosaccharide was carried out at 10 V/cm in 0.1 M sodium tetraborate (pH 9.3) on the Whatmann 3 MM filter paper (15 × 46 cm). Sugars were detected via the silver nitrate reagent⁶⁾.

The gel filtration of polysaccharide was performed as described previously⁴⁾.

Isolation and purification of kefiran

The method for isolating kefiran from kefir grains was as follows. Kefir grains were washed three times with distilled water, followed by lyophilization. The lyophilized kefir grains were crushed in a mortar and suspended in water. The suspension was heated at 80°C for 30 min, then centrifuged at $7000 \times g$ for 20 min. Extraction of the grains was repeated two times and the supernatants were collected.

Kefiran was precipitated from the supernatant by mixing it with an equal volume of ethanol and dispersing it in water. It was reprecipitated with an equal volume of ethanol, and this procedure was repeated three times. Kefiran was finally dissolved in water, dialysed against distilled water and then lyophilized.

Enzymatic hydrolysis of kefiran

Kefiran (100 mg) was incubated with cellulase from *Trichoderma reesei* (70 units/mg dry wt., Cooper Biochem, Inc., Malvern, U.S.A.) in 100 ml of 0.05 M citrate buffer (pH 5.0). The mixture was kept at 37°C for 6 days with a few drops of toluene. The reaction was stopped by heating the solution in a boiling water bath for 10 min. The residual polysaccharide and enzyme were removed through an ultrafiltration unit (Bio Engineering Co., Ltd. Tokyo, Japan) with a membrane of an exclusion molecular weight of 2000 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The retentate was discarded and the filtrate was applied to carbon-celite (1 : 1) column (2 × 30 cm). After washing

the column with water (500 mL) to remove monosaccharide, the retained oligosaccharide was eluted with 50% ethanol (500 mL) and lyophilized.

Partial acid hydrolysis of oligosaccharide

Partial acid hydrolysis of the oligosaccharide was performed by heating it with 0.5 N trifluoroacetic acid at 80°C for 3 h.

Analysis of monosaccharide composition

The monosaccharide composition was determined by gas-liquid chromatography (g.l.c) after trimethylsilylation in accordance with the method of BROBST and LOTT⁷⁾. G.l.c was carried out on a Hitachi 163 gas chromatograph equipped with a flame ionization detector. The instrument was fitted with a stainless-steel column (3 mm × 50 cm) packed with 2% Dexsil 300 GC on Uniport HP, 60-80 mesh (Gasukuro Kogyo Inc., Tokyo, Japan) and programmed to rise from 120°C to 340°C at 10°C/min.

After reduction of the oligosaccharide with sodium borohydride and hydrolysis with trifluoroacetic acid (TFA) (2 N, 105°C, 6 h), the reducing end residue was identified as its alditol trifluoroacetate. Trifluoroacetyl derivatives were prepared as per IMANARI *et al.*⁸⁾ and were applied to a Hitachi 163 gas chromatograph equipped with a hydrogen flame detector. The instrument was fitted with a stainless-steel column (3 mm × 1 m) packed with 10% SE-30 on Chromosorb W AW-DMCS, 80-100 mesh, and operated at 105°C or 110°C.

Methylation analysis

The polysaccharide and oligosacchariditol were methylated via the method of HAKOMORI⁹⁾. The oligosacchariditol was obtained by reducing the oligosaccharide with sodium borodeuteride. The permethylated polysaccharide and oligosacchariditol were hydrolysed at 105°C for 5 h in 2 N TFA. The resulting methylated monosaccharides were converted into their alditol acetates as described by LINDBERG¹⁰⁾. The partially methylated alditol acetates were injected into a JEOL DX-300 gas chromatograph-mass spectrometer fitted with a fused-silica capillary column (0.32 mm × 60 m) of SPB-1 (Spelco, INC., Bellefonte, U.S.A.) and programmed to rise from 200°C to 300°C at 4°C/min. Mass spectra were recorded at 25 eV.

Fast-atom-bombardment mass-spectrometry

F.a.b-m.s. was performed on a JEOL DX-300 mass-spectrometer fitted with an f.a.b. source. The sample was dissolved in water and added to a drop of glycerol on the stainless-steel target previously coated with sodium acetate. The target was bombarded with argon atoms having a kinetic energy equivalent to 3 KeV. The spectra were recorded in a positive ion mode.

¹³C-nuclear magnetic resonance (¹³C-n.m.r.)

¹³C-n.m.r. spectra were obtained in D₂O with a JEOL FX-100 spectrometer operating at 25 MHz, and chemical shifts were recorded in ppm downfield to 3-(trimethylsilyl)-propionic acid sodium salt (TSP-d₄).

Results and Discussion

Composition and structural analysis of kefiran

Kefiran obtained from kefir grains by reprecipitation with ethanol gave a single peak via gel filtration (data not shown) and zone electrophoresis (Fig. 1). The $[\alpha]_D$ (c 1, water) was $+50.5^\circ\text{C}$. Elementary analysis revealed the composition: C, 41.8; H, 6.43; O, 51.8%. These results indicated that kefiran had adequate purity for structural analysis.

This kefiran was composed of glucose and galactose by g.l.c analysis in a molar

Fig. 1. Zone electrophoresis of kefiran.

Fig. 2. ^{13}C -n.m.r. spectrum of kefiran.

Table 1. Methylation analysis of pentasaccharide from kefiran

Identified O-methylsugar	Mode of linkage	Molar ratio ^a	
		kefiran	kefirose
1,2,3,5,6-Me ₅ -Glc	→4) -Glc	ND ^b	—
2,3,4,6-Me ₄ -Glc	Glc- (1→	1.00	1.00
2,3,4-Me ₃ -Glc	→6) -Glc- (1→	1.26	ND
2,3,6-Me ₃ -Glc	→4) -Glc- (1→	1.21	ND
2,3,6-Me ₃ -Gal	→4) -Gal- (1→	1.23	1.03
3,4,6-Me ₃ -Gal	→2) -Gal- (1→	ND	1.65 ^c
2,4,6-Me ₃ -Gal	→3) -Gal- (1→	1.31	1.65 ^c
2,3,4-Me ₃ -Gal	→6) -Gal- (1→	0.20	ND
3,4-Me ₂ -Gal	→2,6) -Gal- (1→	1.15	ND

a: Calculated from peak area in g. l. c..

b: Not detected.

c: Not separated.

Me₅: penta-O-methyl, Me₄: tetra-O-methyl, Me₃: tri-O-methyl, Glc: glucose, Gal: galactose

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ratio of 1 : 0.92 and gave 2,3,4,6-tetra-*O*-methylglucose, 3,4-di-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylgalactose by methylation analysis in molar ratios of 1.00 : 1.15 : 1.26 : 1.21 : 1.23 : 1.31 : 0.20 (Table 1). The presence of 2,3,4-tri-*O*-methylgalactose implied the presence of 6-*O*-substituted galactose in the structure of kefiran which has not found by KOOIMAN³⁾.

Fig. 2 shows the ¹³C-n.m.r. spectrum of kefiran. The signals at 105.7 ppm and 98.6 ppm in the anomeric carbon region indicated that β - and α - anomeric forms can

Fig. 3. Thin layer chromatogram of oligosaccharide released from kefiran with *Trichoderma reesei* cellulase.

β -CD, β -cyclodextrin derived oligosaccharides (G_1 , glucose; G_2 , maltose; G_3 , maltotriose; G_4 , maltotetraose; G_5 , maltopentaose; G_6 , maltohexaose), K, enzymatic hydrolysate of kefiran.

Fig. 4. Fast-atom-bombardment mass spectrum of the pseudomolecular ion region of oligosaccharide from kefiran.

kefiran. The two signals at 71.7 ppm and 71.1 ppm suggested the presence of C-6 of 6-*O*-substituted hexose when the signals were compared with those of reference sugar¹¹⁾. Therefore, the presence of 6-*O*-substituted galactose has also suggested from the results of ¹³C-n.m.r..

Enzymatic hydrolysis of kefiran

As shown in Fig. 3, *T. reesei* cellulase can liberate glucose and an oligosaccharide from kefiran. Approximately 75% of kefiran was hydrolysed by cellulase after incubation for 6 days, but about 25% of kefiran remained intact even after increasing the enzyme level even over ten times. This result suggests that the resistant part to

Table 2. ¹³C-n.m.r. data and assignments of pentasaccharide from kefiran

Chemical shift ^a	Assignments ^b				
	Reducing end residue 4)- α , β -Gal	Non-reducing end residue β -Glc-(1	Internal residue		
			3)- β -Gal-(1	4)- α -Gal-(1	2)- β -Gal-(1
105.9					1'
105.6			1'''		
105.4		1'''			
98.3	1 β			1''	
94.4	1 α				
82.8					2'
81.7				4''	
81.4	4 β				
81.2	4 α				
80.3			3''		
79.0		5'''			
78.0		3'''			
77.8					
77.6	5 β				5'
77.3	3 β				
77.0			5''		
76.5	2 β				
75.8		2'''			3'
74.1	2 α , 3 α				
73.7				3''	
72.8	5 α				
72.7			2''		
72.4		4'''			
72.1				5''	
71.7				2''	
71.3					4'
67.7			4'''		
63.5		6'''	6''	6''	6'
63.0	6 α , 6 β				

a : Chemical shifts (ppm) from TSP-d₄

b : Unprimed numbers refer to reducing residue, primed number to next residue, etc.

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the enzyme reaction exists in the kefiran molecule. The oligosaccharide separated from hydrolysate by the carbon-celite column gave a single spot on t.l.c. and electrophoresis, and had $[\alpha]_D + 70.8^\circ$ (c 1, water). F.a.b.-m.s. of the oligosaccharide (Fig. 4) gave $M + Na^+$ ions at 851 corresponding to pentasaccharide. Gas chromatographic analysis showed that the oligosaccharide was composed of D-glucose and D-galactose in a molar ratio of 2.00 : 3.07 and that glucose was in the reducing end residue. The t.l.c. and g.l.c. of partial acid hydrolysate indicated the presence of lactose incorporated into the structure. Electron impact-mass spectrometry (e.i.-m.s.) of the permethylated oligosacchariditol involved m/z 134 fragment (data not shown), which indicates that the reducing end glucose was substituted at the 4-O position. The gas chromatography-mass spectrometry (g.c.-m.s.) of partially methylated alditol acetates obtained from the oligosacchariditol is summarized in Table 1. The presence of 1,2,3,4,6-penta-O- and 2,3,4,6-tetra-O-methylglucose indicated that glucose locates at both non-reducing and reducing terminals. Three D-galactose residues are all in the internal residues and linked by (1 $\rightarrow$ 2), (1 $\rightarrow$ 3), and (1 $\rightarrow$ 4) linkages, as indicated by the presence of 3,4,6-, 2,4,6-, and 2,3,6-tri-O- methylgalactose.

¹³C-n.m.r. of pentasaccharide

¹³C-n.m.r. was used to determine the monosaccharide sequence and configuration of glycosidic linkages of the pentasaccharide. Assignments of the resonances of the pentasaccharide were performed on the basis of comparison to the published chemical shifts of compounds and empirical rules proposed by BRADBURY and JENKINS¹²⁾.

Table 2 and Table 3 list all of the chemical shifts obtained from the pentasaccharide and the model compounds already reported, respectively. ¹³C-n.m.r. spectrum of the pentasaccharide showed twenty-nine signals out of thirty-six theoretically possible signals (Table 2). The three signals at 105.9 ppm, 105.6 ppm, 105.4 ppm

Table 3. ¹³C-NMR data for compounds related to pentasaccharide from kefiran^a

Compound	C-1	C-2	C-3	C-4	C-5	C-6	ref.
β -Gal- (1 $\rightarrow$ 4) -	105.5	73.5	75.0	71.0	77.8	63.5	(13)
α -Glc	94.3	73.6	73.8	81.0	72.5	62.5	
β -Gal- (1 $\rightarrow$ 4) -	105.5	73.5	75.0	71.7	77.8	63.5	(13)
β -Glc	98.2	76.3	76.8	80.9	77.2	62.6	
α -Gal- (1 $\rightarrow$ 3) -	97.9	70.8	71.8	71.7	73.4	63.5	(14)
β -Gal-O-R ^b	105.1	71.9	80.0	67.4	77.3	63.5	
β -Gal- (1 $\rightarrow$ 2) -	106.5	74.1	75.4	70.9	78.1	63.5	
β -Gal- (1 $\rightarrow$ 2) -	105.0	82.7	75.2	71.3	77.7	63.5	(15)
β -Gal-O-Me	105.1	82.8	75.2	71.3	77.7	63.5	
α -Gal- (1 $\rightarrow$ 4) -	103.4	71.3	72.0	71.9	73.9	63.5	(16)
α -Gal-O-Me	102.4	71.5	73.9	81.8	72.1	63.5	

a : The published chemical shift values have been corrected to give the terminal C-6 atom a value of 63.5

b : R= (CH₂)₈CO₂Me

obviously come from the resonances of glucose C-1 β of non-reducing end residue and two galactose C-1 β of internal residues. The signals at 98.3 ppm and 94.4 ppm were assigned glucose C-1 β and C-1 α of reducing end residue, respectively. The peak (98.3 ppm) was of nearly double intensity and these constituted coincidental resonances of C-1 β of reducing end residue and galactose C-1 α of internal residue. The signals at 82.8 ppm, 81.7 ppm, 81.4 ppm, 81.2 ppm and 80.3 ppm are considered the resonances of carbon atoms involved in the linkages according to the empirical rule that the resonance of a carbon atom involved in a linkage shifts downfield by 4–10 ppm¹²⁾. The signals at 81.4 ppm and 81.2 ppm were assigned to C-4 β and C-4 α of reducing end residue based upon lactose data¹³⁾ and because of low-intensity. The signals at 80.3 ppm and 67.7 ppm were assigned to C-3 and C-4 of 3-*O*-substituted β -galactose, respectively, based upon the data of a model compound α -D-Gal-(1 $\rightarrow$ 3)- β -D-Gal-OR¹⁴⁾. Furthermore, the adjacent α -D-galactosyl residue at the 3-*O* position was deduced from a characteristic chemical shift at 67.7 ppm¹⁴⁾. The signal at 82.8 ppm was assigned to C-2 of the 2-*O* substituted β -galactose according to the literature value¹⁵⁾. Consequently, the signal at 81.7 ppm was assigned to C-4 of the 4-*O*-substituted α -D-galactose and its value was highly consistent with literature value¹⁶⁾.

Based on mass spectral and n.m.r. data, there are two possibilities for the structure of the pentasaccharides (1 or 2).

The cellulase of *T. reesei* hydrolyses cellobiose and cellotriose to glucose but not sophorose. If the pentasaccharide is 2, digestion of the pentasaccharide with *T. reesei* cellulase should yield free glucose and tetrasaccharide. However, treatment of the pentasaccharide with *T. reesei* cellulase did not release such components. Therefore, the structure of the pentasaccharide was finally determined to be 1 which is identical with the kefirese structure presented by KOOIMAN³⁾.

Structure of kefiran

From the results so far described, we can draw the following conclusion : Release of glucose and pentasaccharide (kefirese) from kefiran by digestion with *T. reesei* cellulase and the generation of 2,3,4,6-tetra-*O*-methylglucose and 3,4-di-*O*-methylgalactose by methylation analysis support the structure of kefiran proposed by KOOIMAN³⁾. I.e., kefiran was composed of a hexasaccharide repeating unit in which β -glucosyl residue is linked at the C-6 position of the C-2 linked β -galactosyl residue in a pentasaccharide core (kefirese part).

However, the presence of 2,3,4-tri-*O*-methylgalactose detected in methylated derivatives from kefiran (Table 1) and the signals in the ¹³C-n.m.r. analysis of kefiran strongly suggested the presence of 6-*O*-substituted galactose in the structure of kefiran as an additional linkage.

The position of a such linkage is still obscure, but it is probably located in that part of kefiran which was not hydrolysed by cellulase (25%).

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Kefiran was partially hydrolysed by cellulase from *T. reesei*, but it was not hydrolysed by β -galactosidase (*Escherichia coli*, Boehringer Mannheim Yamanouchi, Tokyo, Japan) and α -galactosidase (*Mortierella vinacea*, Seikagaku Kogyo Co., Ltd. Tokyo, Japan) (data not shown). To make progress in structural analysis, a more effective enzyme is required in order to hydrolyse kefiran.

The variability of the structure of kefiran from different sources of kefir grain remains unidentified. However, the polysaccharides which were isolated from kefir grains collected from Copenhagen, Delft, Kiel, Moscow and Zürich by LA RIVIÈRE *et al.*¹⁾ and our kefiran collected from Chr. Hansen's Lab. were composed of glucose and galactose in the same molar ratio of 1 : 1. Therefore, the polysaccharide of kefir grains from different origins is considered to be composed of the same component.

Microheterogeneity in the structure including 6-*O*-substituted galactose in kefiran can, however, occur while the grain is growing.

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ケフィール粒由来, ケフィランの構造に おける微視的不均一性

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ケフィール粒由来, ケフィランの構造は, 既に, KOOIMAN により提示されている. しかし, メチル化分析の結果, 彼の示した構造には, みられない 6-O 置換ガラクトースの存在が明らかとなったため, ケフィランの構造を再検討した. ケフィランを *Trichoderma reesei* 由来セルラーゼで, 加水分解すると, グルコースと五糖のみが遊離した. その五糖の構造は, メチル化分析および ^{13}C -n.m.r. 分析によって, β -D-Glc-(1

$\rightarrow 2)$ - β -D-Gal-(1 $\rightarrow 4)$ - α -D-Gal-(1 $\rightarrow 3)$ - β -D-Gal-(1 $\rightarrow 4)$ -D-Glc と解明され, これは, KOOIMAN³⁾ の提示したケフィロースの構造と一致した. しかしながら, ケフィランのメチル化分析と, ^{13}C -n.m.r. 分析は, 6-O 置換ガラクトースの存在を示唆しており, ケフィランは, KOOIMAN³⁾ の提示した構造のほかに, 6-O 置換ガラクトースを含む繰り返し単位を有すると推定された.

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