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# Structural Characterization and Biological Activities of an Exopolysaccharide Kefiran Produced by *Lactobacillus kefiranofaciens* WT-2B$^T$

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*Lactobacillus kefiranofaciens*, isolated from kefir grains, produces an extracellular polysaccharide when cultured, not only in PYG10 medium but also in a liquid medium containing a rice hydrolysate that had been previously degraded by treatment with a glucoamylase. The maximum yield of the polysaccharide, using the rice hydrolysate as the medium, was 2.5 g/L after a 7-day culture period at pH 5.0 and 33 °C. Compositional analysis, methylation analysis, specific rotation, and $^1$H and $^{13}$C NMR spectroscopy revealed that the structures of polysaccharides obtained from these two different culture media are essentially identical. The polysaccharide is composed of a hexasaccharide repeating unit and, thus, is known as kefiran. The weight-average molecular weight and the *z*-average radius of gyration of a sample, purified from the rice hydrolysate medium, were determined to be $7.6 \times 10^5$ g/mol and 39.9 nm, respectively, by gel permeation chromatography equipped with a multiangle laser-light-scattering photometer. Changes in blood pressure and serum components were examined in SHRSP/Hos rats, using doses of 100 and 300 mg of kefiran/kg of rat. A suppression in the increase in blood pressure was observed in these rats after 30 days. This activity is discussed in terms of the concentration of serum components of the rat, with emphasis on lipid components such as cholesterols, triglycerides, and free fatty acids.

**KEYWORDS:** ***Lactobacillus kefiranofaciens*; kefiran; NMR; biological activities**

## INTRODUCTION

It has been reported that some lactic acid bacteria produce extracellular polysaccharides (EPS). These polysaccharides have variable uses that include improvement in the rheology and mouth feel of fermented foods, such as yogurt, but also have been reported to confer beneficial health effects (*1*, *2*). The EPS produced by *Lactobacillus kefiranofaciens*, which is known as kefiran, contains approximately equal amounts of glucose and galactose residues in the chain sequence (*3*). The structure of the repeating unit has been elucidated mainly by methylation analysis and NMR data (*4*−*6*). However, complete NMR assignments have not yet been reported. This polysaccharide has attracted considerable interest, mainly because of its biological activities. The oral administration of kefiran to mice indicates that it has antitumor activities and a delayed-type hypersensitivity induced by picryl chloride (*7*−*9*). In addition, kefiran shows an enhancing effect on the production of interferon $\beta$ from animal cells, which is suppressed by the stress hormones cortisol and noradrenaline (*10*).

In terms of industrial applications, procedures for the mass production of kefiran from this bacterium have been extensively studied (*11*−*13*). We have succeeded in producing EPS at a level required for large-scale industrial production through the cultivation of *L. kefiranofaciens* using a new rice hydrolysate medium that contains rice starch hydrolysates as the carbon source and a rice protein hyrolysate as the nitrogen source (*13*). The goal of this study was clarify the structural characteristics of the EPS from *L. kefiranofaciens* produced in a rice hydrolysate medium and to examine the effect of the EPS on the blood pressure and serum components of the rat, with emphasis on lipid components. It has been reported that the EPS from a ropy fermented milk has cholesterol-lowering activity (*14*).

## MATERIALS AND METHODS

**Bacterial Strain and Culture Conditions.** The strain of *L. kefiranofaciens* WT-2B$^T$ (JCM 6985 = ATCC 43761) was obtained from Dr. Tomohiko Fujisawa. This strain was separately cultured in rice hydrolysate medium and PYG10 medium. In the rice hydrolysate medium, 10% rice starch hydrolysate and a 0.35% rice protein hydrolysate were the main components, whereas the carbon and peptone sources were 10% glucose and 1.5% polypeptone in PYG10 medium. The other components of compositions of these media were as

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follows: 1% yeast extract, 0.1% Tween 80, 0.2% $K_2HPO_4$, 0.5% sodium acetate, 0.2% triammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnSO_4 \cdot 5H_2O$. The cultivation of *L. kefiranofaciens* and the isolation of the crude EPS were performed as described in a previous paper (*13*). The maximum yield of crude polysaccharide from the rice hydrolysate medium was 2.5 g/L after a 7-day culture period at pH 5.0 and 33 °C. These two samples are used for the examination of the biological activities. The crude polysaccharide was then purified further for the structural characterization of this polysaccharide.

**Purification of the Polysaccharide.** For deproteinization, an aqueous solution of the crude polysaccharide (500 mg/4 mL) was loaded onto a 750 × 25 mm i.d. DEAE-Sephadex A-50 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), eluted with 5 mM Tris-HCl buffer (pH7.5) at a flow rate of 0.8 mL/min. One peak corresponding to carbohydrate, as detected by the phenol−sulfuric acid method, was collected. The polysaccharide was precipitated with an equal volume of ethanol and then lyophilized. This polysaccharide sample was dissolved in water that had been previously purified using a Milli-Q system (Millipore, Bedford, MA) and size-fractionated by gel filtration chromatography using a 750 × 25 mm i.d. Toyopearl HW-65 column (Tosoh Corp., Tokyo, Japan) at a flow rate of 0.8 mL/min. One peak was observed, and no protein was detected, on the basis of the absorbance at 280 nm. Experimental data presented here are obtained for the purified kefiran sample from rice hydrolysate medium.

**Gel Permeation Chromatography−Multiangle Laser Light Scattering.** The molecular weight of the polysaccharide was estimated by gel permeation chromatography (GPC) using a DAWN-E multiangle laser-light-scattering (MALLS) photometer (Wyatt Technology Corp., Santa Barbara, CA). The purified sample (1 mg/mL, 100 $\mu$L) dissolved in purified water was injected on the G6000PWXL column (Tosoh Corp.) and eluted with water at a flow rate of 0.75 mL/min. The value of refractive index increments, d*n*/d*c*, was assumed to be 0.154.

**Sugar Analysis.** The polysaccharide was hydrolyzed in 90% formic acid (100 °C, 6 h) and then in 2.0 M $CF_3CO_2H$ (100 °C, 6 h). The sugars were converted to the alditol acetates by reduction, followed by treatment with acetic anhydride in an equal volume of pyridine (120 °C, 2 h), and then analyzed by GC-MS using a GC-MS-QP5050A instrument (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 25 m × 0.22 mm i.d. HiCap-CBP5 capillary column (Shimadzu).

**Methylation Analysis.** The polysaccharide was methylated twice according to the method reported by Ciucanu et al. (*15*). The permethylated polysaccharide was hydrolyzed and converted to the partially methylated alditol acetates (PMAA) using the same procedures as described for the sugar analysis of the polysaccharide. The PMAA were analyzed and quantified by GC-MS. The instrument used was the same as that used for the sugar analysis. Identification of the PMAA was made by comparison of the mass spectra obtained with those of known standards reported by Jansson et al. (*16*).

**NMR Spectroscopy.** $^1$H and $^{13}$C NMR spectra were recorded on JEOL 600 MHz (JNM-ECA600) and 400 MHz (JNM-AL400) spectrometers at 80 °C. All exchangeable H in the samples were substituted by D before the spectra were obtained in deuterium oxide. The sample concentration was 10 mg/mL. NMR chemical shifts were measured in reference to external sodium 4,4-dimethyl-4-sila[2,2,3,3-$D_4$]pentanoate (TSP-$d_4$), $\delta_H$ 0.00, or internal acetone, $\delta_C$ 30.8. The resolution-enhanced 1D $^1$H NMR spectra were recorded with a width of 11000 Hz (at 600 MHz) in 16000 complex data points. Proton-decoupled 99.45 MHz $^{13}$C NMR spectra were recorded. The data were collected in 32000 complex data points. Prior to Fourier transformation, noise was reduced using exponential multiplication. $^{13}$C distortionless enhancement by polarization transfer (DEPT) 135 NMR spectra were recorded. The data were collected in 4000 complex data points. Two-dimensional $^1$H−$^1$H correlated spectroscopy (COSY) were recorded with a total of 512 spectra of 512 data points with 112 scans per $t_1$ increment. The spectral widths were 1700 Hz in each dimension (at 400 MHz). A 2D total correlation spectroscopy (TCOSY) spectrum was recorded with a mixing time of 200 ms. The spectral width was 18000 Hz at 600 MHz in each dimension. A total of 128 spectra of 256 data points with 8 scans per $t_1$ increment were recorded. A 2D $^{13}$C−$^1$H heteronuclear multiple quantum coherence (HMQC) experiment was carried out at a $^1$H frequency of 395.75 MHz (99.5 MHz for $^{13}$C) with spectral widths of 1400 Hz for $t_2$ and 18000 Hz for $t_1$. A total of 256 spectra of 256 data points with 720 scans per $t_1$ increment were recorded. A 2D $^{13}$C−$^1$H heteronuclear multiple-bond correlation (HMBC) experiment was carried out at a $^1$H frequency of 600.17 MHz (150.91 MHz for $^{13}$C) with spectral widths of 5400 Hz for $t_2$ and 38000 Hz for $t_1$. A total of 256 spectra of 2000 data points with 32 scans per $t_1$ increment were recorded.

**Table 1.** Composition of the High-Fat Diet

| ingredient | g/100 g of diet | ingredient | g/100 g of diet |
|---|---|---|---|
| milk casein | 20.00 | cholesterol | 1.00 |
| sucrose | 56.75 | cholic acid | 0.25 |
| beef tallow | 10.00 | vitamin mixture | 1.00 |
| cellulose | 4.00 | mineral mixture | 7.00 |

**Optical Rotation Measurements.** Measurements of optical rotation at 589 nm and at 20 °C were made using a SEPA-300 spectropolarimeter (Horiba, Ltd., Kyoto, Japan) equipped with a cell with a 10-cm optical path length.

**Animals, Diets, and Experimental Procedures.** Six-week-old male SHRSP/Hos rats were purchased from Sankyo Lab Service, Japan. All animals were housed individually in cages in a controlled environment at 24 ± 1 °C and 55 ± 5% humidity with a 12-h light cycle. They were allowed free access to one of the diets and water throughout the experimental period. To allow adaptation to the environment, each animal was fed a CE-2 commercial diet (Clea Japan, Tokyo, Japan) and water during the period of acclimatization. After a 7-day acclimatization, the rats were randomly divided into five groups of 10 animals each, and each group was fed a high-fat diet for a 30-day period. The composition of the diet is summarized in **Table 1**.

Two kefiran sources were used as test materials: (1) K-R, kefiran isolated from the culture of *L. kefiranofaciens* in rice hydrolysate medium; (2) K-G, kefiran isolated from the culture of *L. kefiranofaciens* in PYG10 medium. The test materials were dried by lyophilization and powdered before use in the experiments. Each kefiran sample was dissolved in distilled water. The three groups were given kefiran at a dose of K-R100 (100 mg/kg of body weight), K-R300 (300 mg/kg), and K-G300 (300 mg/kg), respectively, by gastric intubation every day, whereas the unadministered group was given distilled water (control).

On days 0, 15, and 30, the systolic blood pressure and diastolic blood pressure were measured by the tail-cuff method using a BP-98A programmed electrosphygmomanometer (Softron, Tokyo, Japan). At the end of the experimental period, the rats were deprived of food for 18 h and then anesthetized with sodium pentobarbital (40 mg/kg of body weight) and decapitated. Blood was collected from the abdominal aorta. The chemical components in blood serum obtained after centrifugation were determined. The liver, thoracic aorta, and mesenteric artery were excised, weighed, and frozen at −20 °C before the analyses.

Serum lipid levels were determined using the commercially available Cholesterol E-test, Triglyceride E-test, and phospholipid test kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Lipids were extracted from liver tissue according to the method of Folch et al. (*17*). Cholesterol, phospholipids, and triglycerides in the lipid extract were determined by using the same diagnostic kits as were used for the serum analyses.

Enzyme extracts of thoracic aorta and mesenteric artery were prepared according to the method of Das and Soffers (*18*). Angiotensin I-converting enzyme (ACE) activity was determined according to the method of Cushman and Cheung (*19*). The protein concentration was measured by using the method of Lowry et al. (*20*) with bovine serum albumin as a standard. The excised thoracic aorta and coronary artery were fixed in a 10% neutral formalin buffered solution. The sections obtained from them were mounted on glass slides and stained with hematoxylin and eosin.

The data collected in this study are expressed as the mean value ± standard deviation. The statistical significance was evaluated by using Student's *t* test at the levels of $p < 0.05$ and $p < 0.01$.

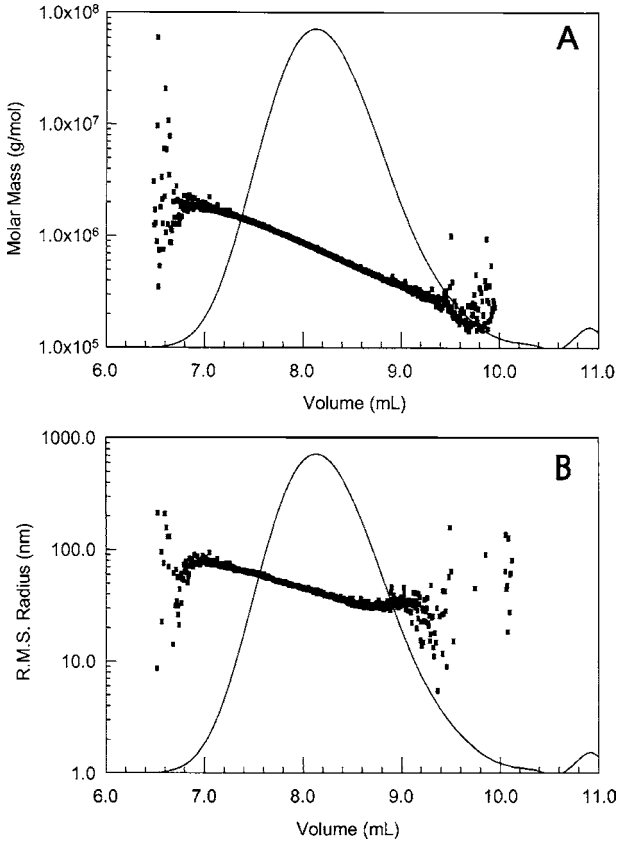


**Figure 1.** GPC-MALLS for kefiran: (**A**) molecular mass versus volume; (**B**) RMS radius versus volume.

**Table 2.** Methylation Analysis of the Polysaccharide by GC-MS

| methylated sugar (as alditol acetate) | $t_R$[a] | molar ratio | major mass fragments (*m/z*) |
|---|---|---|---|
| 2,3,4,6-$Me_4$-Glc | 1.00 | 1.00 | 43, 45, 71, 87, 101, 117, 129, 145, 161, 205 |
| 2,3,6-$Me_3$-Gal | 1.37 | 1.05 | 43, 45, 71, 87, 101, 117, 129, 161, 173, 233 |
| 2,3,6-$Me_3$-Glc | 1.41 | 1.02 | 43, 45, 71, 87, 101, 117, 129, 161, 173, 233 |
| 2,4,6-$Me_3$-Gal | 1.45 | 1.00 | 43, 45, 87, 101, 117, 129, 161, 233 |
| 2,3,4-$Me_3$-Glc | 1.49 | 1.05 | 43, 71, 87, 101, 117, 129, 161, 189, 233 |
| 2,3,4-$Me_3$-Gal | 1.63 | 0.17 | 43, 71, 87, 101, 117, 129, 161, 189, 233 |
| 3,4-$Me_2$-Gal | 2.14 | 0.88 | 43, 87, 99, 129, 189, 233 |

[a] Relative retention time relative to that of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl D-glucitol.

## RESULTS AND DISCUSSION

The polysaccharide had a positive specific rotation $[\alpha]^{20}_D$ +64.5° (*c* 1.0, $H_2O$), similar to reported values (*3*, *21*). **Figure 1** shows a MALLS chromatogram of the purified polysaccharide. On the basis of the chromatographic data, the weight-average molecular weight was determined to be $7.6 \times 10^5$ g/mol and the *z*-average radius of gyration was estimated to be 39.9 nm.

A sugar composition analysis showed that the polysaccharide was composed of glucose and galactose in a molar ratio of 1.00:1.05, which is in agreement with the reported values (*4*−*6*). The GC-MS of the alditol acetates from fully methylated sugars showed seven peaks (**Table 2**). Six major peaks and one minor peak were identified as alditol acetates derived from 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3,4-tri-*O*-methyl D-glucose and 2,3,6-tri-, 2,3,4-tri-, 2,4,6-tri-, and 3,4-di-*O*-methyl D-galactose (molar proportions 1.00:1.02:1.05:1.05:0.17:1.00:0.88 based on peak areas). **Figure 2** shows a $^1$H NMR spectrum of the polysaccharide in $D_2O$ at 80 °C. The anomeric region (δ 4.4−5.5) of the spectrum contained seven signals, one of which was a minor peak at δ 4.61 ($J_{1,2}$ = 7.92 Hz) assigned to (1→6)-β-D-Gal*p* corresponding to a small proportion (∼0.2 equiv per repeating unit) of 2,3,4-tri-*O*-methyl D-galactose. The other six peaks contained three well-resolved signals and three overlapping signals corresponding to a hexasaccharide repeating unit. The overlap of the anomeric doublets was confirmed in a COSY experiment. The six sugar residues were arbitrarily labeled **a**−**f**. On the basis of their observed chemical shifts and $J_{1,2}$ values, residue **c** (δ 5.14) was assigned to an α-hexapyranosyl residue and residues **f** (δ 4.82, $J_{1,2}$ = 7.92 Hz), **b** (δ 4.68), **e** (δ 4.53, $J_{1,2}$ = 7.52 Hz), **d** (δ 4.53, $J_{1,2}$ = 7.52 Hz), **a** (δ 4.49, $J_{1,2}$ = 7.92 Hz) were assigned to pyranose ring forms in a β anomeric configuration. The $^{13}$C NMR spectrum in **Figure 3** showed six signals in the anomeric region (δ 95−110) and are assigned to one α-hexapyranosyl residue **c** (δ 98.5) and five β-hexapyranosyl residues **a**, **b**, **d**, **e**, and **f** (δ 105.7), which were confirmed by cross-peaks in the HMQC spectrum.

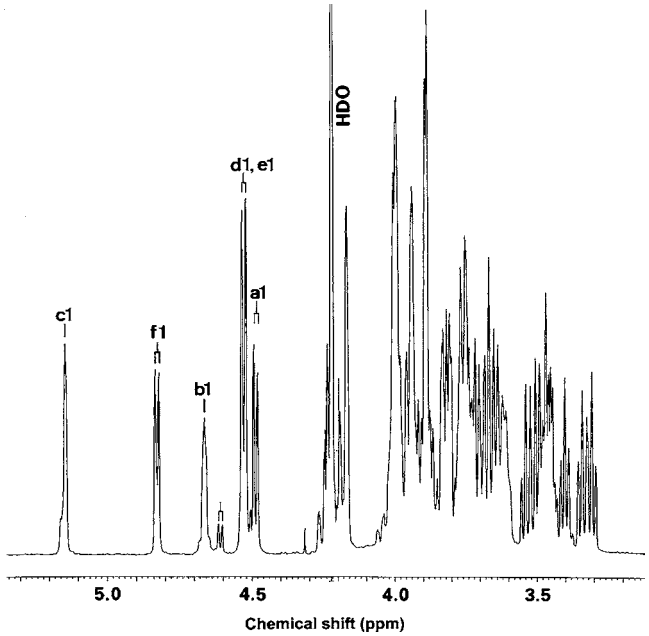


**Figure 2.** $^1$H NMR spectrum of kefiran in deuterium oxide at 80 °C.

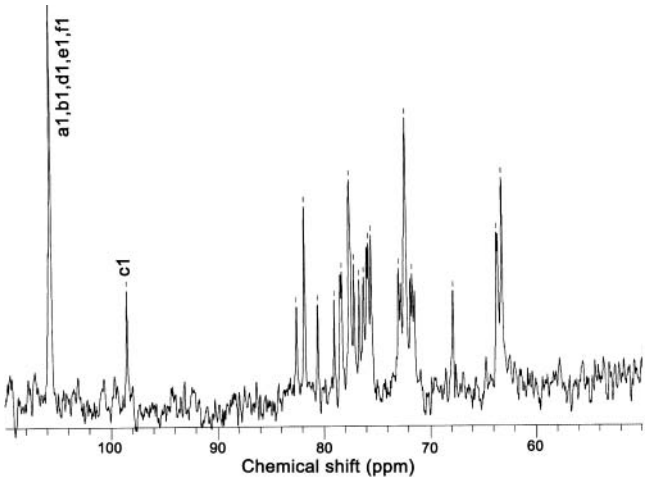


**Figure 3.** $^{13}$C NMR spectrum of kefiran in deuterium oxide at 80 °C.

The DEPT experiment showed that three carbon signals corresponding to the C-6 of residues **c**, **d**, **e**, and **f**, which appear at δ 63.7, 63.6, 63.2, and 63.2, and two carbon signals according

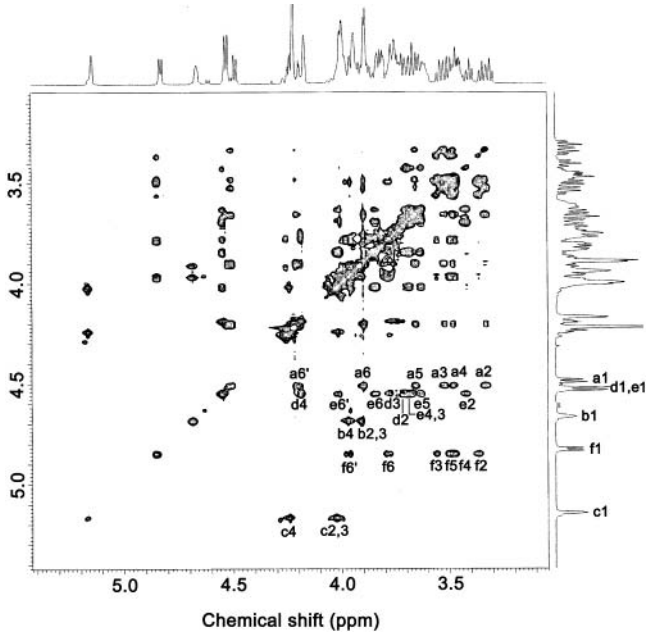


**Figure 4.** TCOSY spectrum of kefiran in deuterium oxide at 80 °C.

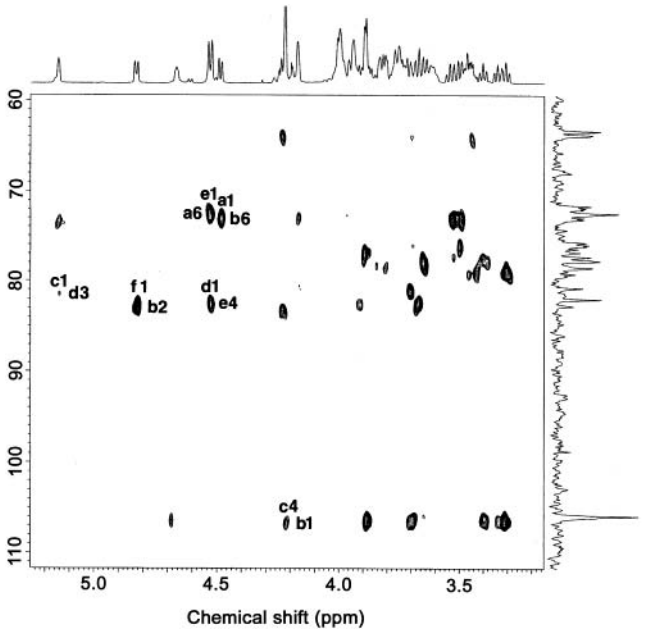


**Figure 5.** HMBC spectrum of kefiran in deuterium oxide at 80 °C.

**Table 3.** Chemical Shifts (Parts per Million) of $^1$H and $^{13}$C NMR Signals for Kefiran

| | $^1$H/$^{13}$C | | | | | | |
|---|---|---|---|---|---|---|---|
| sugar residue | 1 | 2 | 3 | 4 | 5 | 6 | |
| →6)-β-D-Glc*p*-(1→ | 4.49 (7.92[a]) | 3.31 | 3.51 | 3.46 | 3.65 | 3.88 | 4.18 |
| **a** | 105.7 | 75.8 | 78.4 | 72.3 | 77.6 | 71.6 | |
| →2,6)-β-D-Gal*p*-(1→ | 4.68 | 3.89 | 3.89 | 3.94 | 3.94 | ND[b] | 4.01 |
| **b** | 105.7 | 81.8 | 75.9 | 71.4 | 76.7 | 72.5 | |
| →4)-α-D-Gal*p*-(1→ | 5.14 | 3.99 | 3.99 | 4.22 | 4.23 | 3.75 | 3.90 |
| **c** | 98.5 | 71.8 | 73.0 | 82.6 | 72.8 | 63.2 | |
| →3)-β-D-Gal*p*-(1→ | 4.53 (7.52[a]) | 3.69 | 3.75 | 4.17 | 3.71 | 3.74 | 3.81 |
| **d** | 105.7 | 72.3 | 80.6 | 67.8 | 77.6 | 63.7 | |
| →4)-β-D-Glc*p*-(1→ | 4.53 (7.52[a]) | 3.40 | 3.65 | 3.66 | 3.63 | 3.82 | 4.00 |
| **e** | 105.7 | 75.6 | 77.2 | 81.8 | 77.6 | 63.2 | |
| β-D-Glc*p*-(1→ | 4.82 (7.92[a]) | 3.33 | 3.54 | 3.45 | 3.48 | 3.76 | 3.97 |
| **f** | 105.7 | 76.3 | 78.3 | 72.3 | 79.0 | 63.6 | |

[a] $J_{1,2}$ values are given in hertz in parentheses. [b] Not determined.

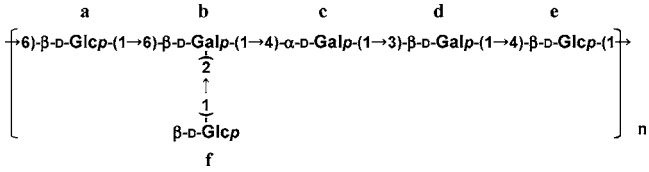


**Figure 6.** Repeating unit of kefiran. Letters denoting each sugar residue refer to the NMR assignments in **Table 2**.

to C-6 of residues **a** and **b**, which appear at $\delta$ 71.6 and 72.5, shifted to high field, indicating six substituted sugar residues.

All $^1$H resonances in the $^1$H NMR spectrum were assigned by means of COSY and TCOSY experiments. In the TCOSY spectrum (**Figure 4**) a complete series of cross-peaks between **a** H-1 ($\delta$ 4.49) and **a** H-2,3,4,5,6,6′ was observed, as well as between **e** H-1 ($\delta$ 4.53) and **e** H-2,3,4,5,6,6′ and **f** H-1 ($\delta$ 4.82) and **f** H-2,3,4,5,6,6′, indicating glucopyranosyl residues. In the TCOSY spectrum some cross-peaks were observed between H-1 in residues **c**, **d**, and **b**, indicating galactopyranosyl residues. The signal of **c** H-1 ($\delta$ 5.14) showed cross-peaks between **c** H-2,3,4, indicating a galactopyranosyl residue, due to the small $J_{4,5}$ value. The chemical shifts of **c** H-4,5,6,6′ were determined by the cross-peaks between **c** H-4 and **c** H-2,3 and between **c** H-5 and **c** H-6,6′, in a COSY spectrum. Cross-peaks were observed between **d** H-1 ($\delta$ 4.53) and **d** H-2,3,4 and between **d** H-4 and **d** H-5,6,6′ in the TCOSY spectrum. Cross-peaks were observed between **b** H-1 ($\delta$ 4.68) and **b** H-2,3,4 in the TCOSY spectrum. The HMQC spectrum allowed the complete assignment of the $^{13}$C spectrum. **Table 3** summarizes the $^1$H and $^{13}$C NMR spectral assignments together with the coupling constants for the anomeric protons. These assignments are based on COSY, TCOSY, HMQC, and HMBC spectra and are in agreement with reported values obtained for polysaccharides produced from *Lactobacillus helveticus* (*22*, *23*) and from *Lactococcus lactis* (*24*).

The six cross-peaks (between **a** H-1 and **b** C-6, between **c** H-1and **d** C-3, between **d** H-1and **e** C-4, between **e** H-1and **a** C-6, between **f** H-1and **b** C-2, and between **b** C-1and **c** H-4), which are shown in an HMBC spectrum (**Figure 5**), are assigned to six glycosidic linkages, as indicated by **a**(1→6)**b**, **c**(1→3)**d**, **d**(1→4)**e**, **e**(1→6)**a**, **f**(1→2)**b**, and **b**(1→4)**c**, respectively. The above results indicate that the polysaccharide produced by *L. kefiranofaciens* from rice hydrolysate medium basically contains a hexasaccharide repeating unit, as shown in **Figure 6**. This structure is essentially identical to that of kefiran from the PYG medium, because the $^{13}$C and $^1$H NMR spectra of kefiran from the PYG medium is identical to that from the rice hydrolysate medium (data not shown). The EPS composition may vary depending on carbon source (*1*, *2*). The rice starch hydrolysate consists of glucose identical to the carbon source in the PYG medium. Thus, it is normal that EPS produced in these two media are almost identical.

The branching structure of kefiran has been ambiguous in terms of whether it contains a hexasaccharide or heptasaccharide repeating unit, and the issue of whether, or at what position (2 or 6), a side chain is linked to a galactosyl residue at the branching point is unclear (*4*, *5*, *25*). Our data clearly show that the branching structure is a hexasaccharide repeating unit and that a single glucose residue is attached to the branch point at the *O*-2 of Gal*p*(**b**) of the main chain. However, not all of the **b** units contain a branched chain **f**. The small doublet peak at $\delta_H$ 4.61 ($J_{1,2}$ = 7.92 Hz) in the anomeric region in the $^1$H NMR spectrum was assigned to a (1→6)-β-D-galactopyranosyl residue, which appears to be an unbranched main chain of **b**. The smaller ratio of 2,3,4-tri-*O*-methyl D-galactose from the

**Table 4.** Changes in Blood Pressure in SHRSP/Hos Rats after Feeding on Kefiran for 30 Days[a]

| | group | | | |
|---|---|---|---|---|
| | control ($n = 10$) | K-R100 ($n = 10$) | K-R300 ($n = 10$) | K-G300 ($n = 10$) |
| systolic blood pressure (mmHg) | | | | |
| initial | 163.5 ± 3.4 | 169.8 ± 5.2 | 156.6 ± 2.4 | 160.3 ± 3.5 |
| 15th day | 186.5 ± 6.4 | 179.7 ± 2.4 | 168.6 ± 4.5a,b | 171.6 ± 3.0 |
| 30th day | 192.1 ± 3.0 | 181.9 ± 4.6aa | 173.1 ± 4.0aa, b | 178.3 ± 4.3aa |
| diastolic blood pressure (mmHg) | | | | |
| initial | 124.2 ± 4.2 | 122.1 ± 2.3 | 136.4 ± 6.8 | 127.4 ± 3.7 |
| 15th day | 139.2 ± 5.5 | 139.8 ± 2.4 | 136.5 ± 2.3 | 131.2 ± 4.3 |
| 30th day | 158.9 ± 4.0 | 147.8 ± 3.4a | 135.2 ± 3.9aa, bb | 136.0 ± 5.0aa, b |
| mean blood pressure (mmHg) | | | | |
| initial | 137.2 ± 3.8 | 133.4 ± 2.2 | 136.0 ± 2.2 | 138.5 ± 3.4 |
| 15th day | 154.9 ± 5.5 | 152.9 ± 2.2 | 148.1 ± 2.2 | 143.6 ± 4.0 |
| 30th day | 171.5 ± 3.1 | 159.0 ± 3.6aa | 149.5 ± 3.4aa, b | 146.3 ± 5.1aa, b |

[a] Values are means ± SEM. a, $p < 0.05$, aa, $p < 0.01$: significantly different from the control group. b, $p < 0.05$, bb, $p < 0.01$: significantly different from the K-R100 group.

**Table 5.** ACE Activities in the Serum, Thoracic Aorta, and Mesenteric Artery of SHRSP/Hos Rats after Feeding on Kefiran for 30 Days[a]

| | control ($n = 10$) | K-R100 ($n = 10$) | K-R300 ($n = 10$) | K-G300 ($n = 10$) |
|---|---|---|---|---|
| serum (units/L) | 21.73 ± 0.29 | 20.28 ± 0.43aa | 19.80 ± 0.60a | 19.97 ± 0.71a |
| thoracic aorta (milliunits/mg of protein) | 23.2 ± 0.9 | 21.9 ± 0.8 | 19.9 ± 0.6aa,b | 20.1 ± 0.5a |
| mesenteric artery (milliunits/mg of protein) | 10.2 ± 0.5 | 9.7 ± 0.3 | 9.5 ± 0.3 | 9.3 ± 0.4 |

[a] Values are means ± SEM. a, $p < 0.05$, aa, $p < 0.01$: significantly different from the control group. b, $p < 0.05$: significantly different from the K-R100 group.

**Table 6.** Lipid Concentrations in the Serum and Liver of SHRSP/Hos Rats after Feeding on Kefiran for 30 Days[a]

| | control ($n = 10$) | K-R100 ($n = 10$) | K-R300 ($n = 10$) | K-G300 ($n = 10$) |
|---|---|---|---|---|
| serum lipids (mg/dL) | | | | |
| total-cholesterol | 495.60 ± 13.57 | 475.80 ± 16.77 | 450.30 ± 12.29aa | 440.20 ± 16.38a |
| VLDL-cholesterol | 174.03 ± 5.61 | 152.25 ± 5.37a | 150.52 ± 4.63aa | 156.48 ± 7.32 |
| LDL-cholesterol | 310.79 ± 9.57 | 294.99 ± 10.40 | 272.77 ± 8.79aa | 280.71 ± 13.12 |
| HDL-cholesterol | 24.30 ± 0.75 | 23.81 ± 0.84 | 23.54 ± 0.76 | 23.03 ± 1.07 |
| triglyceride | 46.30 ± 2.67 | 41.40 ± 4.17 | 28.40 ± 2.98aa,b | 28.70 ± 1.98aa, b |
| VLDL-triglyceride | 43.38 ± 3.28 | 34.38 ± 3.46 | 18.36 ± 1.78aa, bb | 23.52 ± 1.62aa,b |
| LDL-triglyceride | 4.56 ± 0.46 | 3.43 ± 0.24a | 2.70 ± 0.46aa | 1.96 ± 0.15aa, bb |
| HDL-triglyceride | 2.38 ± 0.13 | 2.31 ± 0.15 | 2.30 ± 0.17 | 2.44 ± 0.21 |
| phospholipids | 222.10 ± 6.18 | 221.20 ± 6.03 | 221.90 ± 5.62 | 217.80 ± 7.44 |
| liver lipids (mg/g of liver) | | | | |
| cholesterol | 55.5 ± 0.7 | 50.0 ± 2.4 | 44.9 ± 1.0aa | 48.2 ± 1.3aa |
| triglyceride | 15.7 ± 0.5 | 14.2 ± 0.6a | 11.3 ± 0.4aa,bb | 13.6 ± 0.2aa |
| phospholipids | 7.0 ± 0.2 | 5.5 ± 0.5a | 4.4 ± 0.6aa | 4.9 ± 0.2aa |

[a] Values are means ± SEM. a, $p < 0.05$, aa, $p < 0.01$: significantly different from the control group. b, $p < 0.05$, bb, $p < 0.01$: significantly different from the K-R100 group.

methylation analysis suggests that ~20% of the repeating units contain no glucopyranosyl residues attached as side chains.

**Effect of Kefiran on Blood Pressure and Lipids in SHRSP/Hos Rats. Table 4** shows the results of changes in blood pressure in SHRSP/Hos rats. Each group that was fed kefiran showed a significantly lower blood pressure than the control group. A dose-dependent effect was, separately, observed in the K-R100 and K-R300 groups. ACE catalyzes the formation of the potent vasopressor angiotensin II from angiotensin I, which plays an important role in increasing blood pressure (*26*). ACE activities in the serum and thoracic aorta of SHRSP/Hos rats in all of the kefiran-fed groups were significantly lower than those in the control group (**Table 5**). The inhibition of ACE in the serum and thoracic aorta in the kefiran-fed groups would result in resistance of the continuous increase in blood pressure in SHRSP rats.

It is known that consumption of a high-fat diet leads to hyperlipidemia and arterial lipid deposition in SHRSP rats (*27*−*29*). Hypertension and hypercholesterolemia are generally considered to be risk factors for atherosclerosis. Lipid levels in the serum and liver of SHRSP/Hos rats after the consumption of kefiran for the 30-day period are summarized in **Table 6**. The results showed that the groups fed kefiran had lower serum levels of total-cholesterol, LDL-cholesterol, and triglycerides in the serum in comparison with those of the control group. The levels of cholesterol, triglycerides, and phospholipids in the liver of the kefiran-fed groups were significantly lower than those in the control group. In addition, the levels of lipids in the serum and liver in the K-R300 and K-G300 groups were even more improved than those in the K-R100 groups.

Advanced plaque with lipid deposition in the intima of the aortic arch was not as evident in the K-R100 group (**Figure 7B**) in comparison with that in the control group (**Figure 7A**). Moreover, the lesions associated with lipid deposition observed in the K-R300 and K-G300 groups were the slightest (**Figure 7C**,**D**), and the number of rats with lesions was the lowest among all of the experimental groups. These results of metabolic and morphological changes in SHRSP rats suggest that the consumption of kefiran could have influenced lipid metabolism by several possible mechanisms.

No significant differences were observed in body weight among the experimental groups (data not shown). The physi-

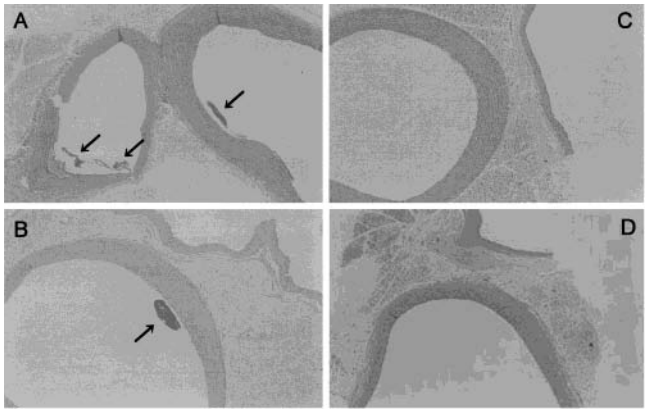


**Figure 7.** Photomicrographs of tissue cross sections from the aortic arch: (**A**) control group; (**B**) K-R100; (**C**) K-R300; (**D**) K-G300 groups, respectively. The lesions associated with lipid deposition are shown by arrows in panels **A** and **B**.

ological effects of kefiran could be, in part, interpreted in terms of its physicochemical properties such as water solubility, viscosity, large molecular weight, and considerable resistance to enzymatic hydrolysis. These physicochemical properties would cause delayed gastric emptying and a slower transit time through the small intestine, resulting in a reduced rate of nutrient digestion and absorption. In addition, increased defecation can be attributed to the water-holding capacity and swelling of kefiran in the large intestine. However, the mechanism of physiological activities of this polysaccharide is clearly a subject for further studies. The most valuable application of kefiran production in *L. kefiranofaciens* may involve its health-promoting characteristics in designer-type foods.

## LITERATURE CITED

(1) Welman, A. D.; Maddox, I. S. Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. *Trends Biotechnol.* **2003**, *21*, 269−274.

(2) De Vuyst, L.; Degeest, B. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol. Rev.* **1999**, *23*, 153−177.

(3) La Riviere, J. W.; Kooiman, P. Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis*. *Arch. Mikrobiol.* **1967**, *59*, 269−278.

(4) Mukai, T.; Toba, T.; Itoh, T.; Adachi, S. Structural investigation of the capsular polysaccharide from *Lactobacillus kefiranofaciens* $K_1$. *Carbohydr. Res.* **1990**, *204*, 227−232.

(5) Kooiman, P. Chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohydr. Res.* **1968**, *7*, 200−211.

(6) Yokoi, H.; Watanabe, T.; Fujii, Y.; Mukai, T.; Toba, T.; Adachi, S. Some taxonomical characteristics of encapsulated *Lactobacillus* sp. KPB-167B isolated from kefir grains and characterization of its extracellular polysaccharide. *Int. J. Food Microbiol.* **1991**, *13*, 257−264.

(7) Murofushi, M.; Mizuguchi, J.; Aibara, K.; Matuhasi, T. Immunopotentiative effect of polysaccharide from kefir grain, KGF-C, administered orally in mice. *Immunopharmacology* **1986**, *12*, 29−35.

(8) Murofushi, M.; Shiomi, M.; Aibara, K. Effect of orally administered polysaccharide from kefir grain on delayed-type hypersensitivity and tumor growth in mice. *Jpn. J. Med. Sci. Biol.* **1983**, *36*, 49−53.

(9) Shiomi, M.; Sasaki, K.; Murofushi, M.; Aibara, K. Antitumor activity in mice of orally administered polysaccharide from kefir grain. *Jpn. J. Med. Sci. Biol.* **1982**, *35*, 75−80.

(10) Kabayama, S.; Osada, K.; Tachibana, H.; Katakura, Y.; Shirahata, S. Enhancing effects of food components on the production of interferon $\beta$ from animal cells suppressed by stress hormones. *Cytotechnology* **1997**, *23*, 119−125.

(11) Yokoi, H.; Watanabe, T. Optimum culture conditions for production of kefiran by *Lactobacillus* sp. KPB-167B isolated from kefir grains. *J. Ferment. Bioeng.* **1992**, *74*, 327−329.

(12) Cheirsilp, B.; Shimizu, H.; Shioya, S. Modelling and optimization of environmental conditions for kefiran production by *Lactobacillus kefiranofaciens*. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 639−646.

(13) Maeda, H.; Zhu, X.; Mitsuoka, T. New medium for the production of exopolysaccharide (OSKC) by *Lactobacillus kefiranofaciens*. *Biosci. Microflora* **2003**, *22*, 45−50.

(14) Nakajima, H.; Suzuki, Y.; Kaizu, H.; Hirota, T. Cholesterol lowering activity of ropy fermented milk. *J. Food Sci.* **1992**, *57*, 1327−1329.

(15) Ciucanu, I.; Kerek, F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **1984**, *131*, 209−217.

(16) Jansson, P. E.; Kenne, L.; Liedgren, H.; Lindberg, B.; Lonngren, J. A practical guide to the methylation analysis of carbohydrates. *Chem. Commun., Univ. Stockholm* **1976**, *8*, 1−75.

(17) Folch-Pi, J.; Lees, M.; Stanley, G. H. S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−509.

(18) Das, M.; Soffer, R. L. Pulmonary angiotensin-converting enzyme. Structural and catalytic properties. *J. Biol. Chem.* **1975**, *250*, 6762−6768.

(19) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637−1648.

(20) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(21) Micheli, L.; Uccelletti, D.; Palleschi, C.; Crescenzi, V. Isolation and characterization of a ropy *Lactobacillus* strain producing the exopolysaccharide kefiran. *Appl. Microbiol. Biotechnol.* **1999**, *53*, 69−74.

(22) Staaf, M.; Yang, Z.; Huttunen, E.; Widmalm, G. Structural elucidation of the viscous exopolysaccharide produced by *Lactobacillus helveticus* Lb161. *Carbohydr. Res.* **2000**, *326*, 113−119.

(23) Staaf, M.; Widmalm, G.; Yang, Z.; Huttunen, E. Structural elucidation of an extracellular polysaccharide produced by *Lactobacillus helveticus*. *Carbohydr. Res.* **1996**, *291*, 155−164.

(24) Stingele, F.; Vincent, S. J.; Faber, E. J.; Newell, J. W.; Kamerling, J. P.; Neeser, J. R. Introduction of the exopolysaccharide gene cluster from *Streptococcus thermophilus* Sfi6 into *Lactococcus lactis* MG1363: Production and characterization of an altered polysaccharide. *Mol. Microbiol.* **1999**, *32*, 1287−1295.

(25) Mukai, T.; Toba, T.; Itoh, T.; Adachi, S. Structural microheterogeneity of kefiran from kefir grains. *Nippon Chikusan Gakkaiho* **1988**, *59*, 167−176.

(26) Skeggs, L. T.; Kahn, J. R.; Shumway, N. P. The preparation and function of the hypertension-converting enzyme. *J. Exp. Med.* **1956**, *103*, 295.

(27) Mori, H.; Ishiguro, K.; Okuyama, H. Hypertension in rats does not potentiate hypercholesterolemia and aortic cholesterol deposition induced by a hypercholesterolemic diet. *Lipids* **1993**, *28*, 109−113.

(28) Ogawa, H.; Shiota, C.; Nishikawa, T.; Fukushima, S.; Sasagawa, S. Studies on stroke-prone spontaneously hypertensive rats (SHRSP) fed on high-fat and -cholesterol diets-alterations in lipo- and apo-proteins. *Nippon Eiseigaku Zasshi* **1985**, *40*, 627−635.

(29) Yamori, Y. Metabolic pathology of vasculatures in hypertension and vascular lesions in spontaneously hypertensive rats. *Trans. Soc. Pathol. Jpn.* **1974**, *63*, 226−227.

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