Structural and quantitative analysis of exopolisaccharides and oligosaccharides produced by lactobacillus. II. The connection status-, configuration-, phosphorous content-, modification-, structure of monosaccharides, exopolysaccharides in yogurt, galacto-oligosaccharides

J. Csapó
email: csapojanos@sapientia.siculorum.ro,
   csapo.janos@gmail.hu

R. V. Salamon
email: salamonrozalia@sapientia.siculorum.ro

Sz. Salamon
email: salamonszidonia@sapientia.siculorum.ro
Sapientia Hungarian University of Transylvania, Faculty of Technical and Social Sciences, Department of Food Science, Piața Libertății 1, 530104 Miercurea Ciuc, Romania

Sz. Toró
email: salamonszidonia@sapientia.siculorum.ro
Babeș-Bolyai University Cluj-Napoca, Nutritional Science, MSc. student

Keywords and phrases: exopolysaccharides (EPS), galacto-oligosaccharides (GalOS), isolation, quantitative determination, molecular mass, monosaccharide composition, analytical methods.
Abstract. After derivatization and hydrolysis, it was possible to determine the amount and proportion of the various forms of chemical bonds. The configuration and phosphor content was also examined, and in order to determine the structure of the EPS as well as its physical and chemical properties, there have been carried out chemical modifications, too. By using nuclear magnetic resonance spectroscopy, it was also possible to study the fine structure of the constituent atomic groups of the EPS, elucidating this way the structure of the repeating units and the monomers of these units, the isomerism of the anomic carbon atoms and the frequency of occurrence of some atom groups. In the second part of the presentation, the glucose, galactose and lactose content of yogurt were determined by the following steps: extraction of the saccharides from yogurt, determination of the quality and quantity of the saccharides by HPLC, and the evaluation of the methods. At the end of the article, different methods are reviewed for the determination of the composition and quantity of the galacto-oligosaccharides.

1.1 The connection status between monosaccharides

All the authors who dealt with the analysis of the binding sites marked the carbon atoms involved in the glycosidic bond, with an acetate group (Dueñas-Chasco et al., 1998; Urashima et al., 1999; Uemura et al., 1998; Casteren et al., 1998). Polysaccharides were first methylated in the free hydroxyl group, and then hydrolysed. After the hydrolysis of the partially methylated sugars, the liberated glycosidic and non-glycosidic hydroxyl groups were converted into acetate, whereby partially methylated alditol acetates were obtained, for which the location of the acetate groups was identical with the binding sites of the monomers before the hydrolysis. Knowing the quantity of derivatives, it is also possible to determine the ratio of the various bonding forms. The main steps were as follows:

• methylation
• purification
• hydrolysis
• acetylation
Dueñas-Chasco et al. (1998) methylated the polysaccharides two times according to the method of Ciucanu & Kerek (1984). The product was purified by reverse-phase chromatography using Sep-pak C18 column (Waeghe et al., 1983), and then hydrolysed with 2 M trifluoroacetic acid. The products were reduced according to the method of Blankeney et al. (1983), and then acetylated. The partially methylated alditol acetates were analysed by GLC-MS, whereby the temperature programme began with a one-minute heat isotherm phase at 100°C, then increasing the temperature to 250°C with a speed of 5°C/min.

Urashima et al. (1999), Uemura et al. (1998) and Casteren et al. (1998) used the Hakomori method for the methylation of the purified EPS. Urashima et al. (1999) purified the methylated polysaccharide further on a silica gel column (Wakogel S-1). The partially methylated alditol acetates were produced from the per-methylated polysaccharides according to the method of Stellner et al. (1973). The analysis of the derivatives was performed on a Hitachi 163 GC, equipped with a FID detector and a 0.2 × 200-cm-sized glass column filled with chromosolv W and moistened with 2% OV-17. During the analysis, a temperature gradient of 3°C/min ranging from 150°C to 250°C was applied. The GC-MS analysis was performed on a Jeol HX-105 mass spectrometer, in which the ionization power was 100 µA, the voltage was of 70 eV, the column linked to the MS was of type MXT-5, with an internal diameter of 0.25 mm and a length of 60 m. The temperature of the column space increased during the measurements from 150°C to 250°C with a speed of 3°C/min. The determination of the amount of alditol acetates was on the basis of the area under the GC peak.

Uemura et al. (1998) purified the per-methylated carbohydrates on a silica gel column chromatography (Wakogel S-1: 0.3 × 3 cm, Wako Pure Chemicals, Osaka, Japan). The sample was hydrolysed with 90% formic acid at 100°C for one hour (formolysis), and then hydrolysed with 2 M trifluoroacetic acid at 100°C for 5 hours. The partially methylated monosaccharides were converted into alditol acetates derivatives. The derivatives were separated on a Hitachi 163 GC 0.8 mm × 30 m UA-1 capillary column (Hitachi Co. Ltd., Japan). During the analysis, the temperature was raised from 150°C to 240°C with a speed of 1°C/min. The identification was carried out by a GC HP 5890A/Jeol JMS HX-105 GC-MS system, the electron acceleration energy was of 70 eV. During the separation, a 0.28 mm × 60-m-sized MXT-5 capillary column (Restek, Bellfoute, USA) was used, the determination of the molar ratios was carried out by measuring the areas under the peak. The identification of the mass spectra was based on the comparison of the obtained mass spectra.
with an already known, partially methylated alditol acetate spectrum.

*Casteren et al.* (1998) dialysed the methylated EPS with water, and then lyophilized them. The hydrolysis was carried out with a 2 M trifluoroacetic acid for 1 hour at 121°C. After the solution was concentrated under a stream of air at a temperature lower than 20°C, the partially methylated sugars were converted into alditol acetates based on the method of Englyst & Cummings (1984). The analysis was carried out according to the method described by *Vincken et al.* (1994) on a Carlo Erba HRGC 5160 Series GC with flame ionization detector. The amount of partially methylated alditol acetates were determined based on the effective carbon number (Sweet et al., 1975). The identification of the components was performed on HP 5973 mass selective detector to which an HP-6890 GC was attached, equipped with a 25 m × 0.25 mm, 0.2 µm thick, CP Sil 19 CB quartz capillary (Chrompack). During the temperature programme, the first heating range was carried out from 160°C to 185°C with a speed of 0.5°C/min, while the second one from 185°C to 230°C with a speed of 10°C/min, and then held the column at 230°C for 5.5 minutes. For the data analysis, a Hewlett-Packard ChemStation software was used.

*Casteren et al.* (1998) have shown that, when determining the binding sites, in the case of phosphorus-containing polysaccharides, the incomplete hydrolysis of the phosphate ester bonds could pose a problem: it may result in the underestimation of the monomers involved in the phosphate binding.

### 1.2 Configuration of the monosaccharides

Dueñas-Chasco et al. (1998) determined the optical isomers by creating enantiomeric pairs and then analysing them with gas chromatography. After the methanolysis with 0.625 M HCl methanol of the polysaccharides, these were treated with 0.625 M (+)-2-butanol HCl solution under the same conditions, such as in the case of sugar composition analysis, and then trimethylsilylated. The trimethylsilylated 2-butyl glycosides were analysed by GLC-MS, under conditions identical to the monosaccharide composition determination assay, being the only difference in the temperature gradient, which was isothermal at the beginning of the measurement at 130°C, and then raised to 250°C with a speed of 2°C/min.
1.3 Examination of the phosphorous content of the exopolysaccharides

The phosphorus content of NPS and APS was determined by Casteren et al. (1998) based on the methods defined by Dittmer & Wells (1969). After removing the phosphorus from the APS and NPS by perchloric acid hydrolysis, the resulting derivatives were treated with ammonium molybdate to result in a colour reaction. Measuring the resulting colourful derivative with photometric methods, it was found that the NPS did not contain phosphorus, while the APS contained 0.1% of phosphorus. This was in accordance with the acidic character of the latter one, which came to light during the ion-exchange column chromatography.

Casteren et al. (1998) determined the total phosphorus content of the samples with a colour reaction by treating them with 72% perchloric acid at 180 °C for 20 minutes and the free phosphorus content with colour reaction without any treatment. After the dilution of the treated and the untreated samples, these were analysed based on the methods of Chen et al. (1956). To find out whether the hydrolysis carried out during the sugar composition analysis broke all the sugar – phosphate bonds down, the free phosphorus content of the hydrolysed samples was also determined.

1.4 Chemical modification of the exopolysaccharides

Chemical modifications of the EPS were carried out by Casteren et al. (1998) in order to examine the structure of the EPS and the physical and chemical properties of the modified EPS. During the treatment with sulphuric acid, 25 mg of EPS was dissolved in 6.25 cm$^3$ of distilled water, and then 6.25 cm$^3$ of 0.6 M sulphuric acid was added to the solution. The solution was incubated for 2 hours at 37 °C, and then cooled on ice and neutralized with 2 M NaOH. During the treatment with hydrogen fluoride, to 50 mg EPS 2.5 cm$^3$ of 28 M (approximately 48%) HF was added at 0 °C, stirred for 48 hours, followed by neutralization with 6 M NaOH under ice-water cooling. During the treatment with NaOH, 25 mg EPS was dissolved in 12.5 cm$^3$ of distilled water, and then 12.5 cm$^3$ of 4 M NaOH solution containing 1 mg/cm$^3$ NaBH$_4$ was added; it was left at room temperature for 3 minutes, then heated to 80 °C for 4 hours, then cooled with ice water and neutralized with 2 M acetic acid.

After the chemical modification, the samples were analysed by HPAEC. The remaining samples were dialysed and divided into two parts: one part was concentrated and then analysed by HPSEC and with “light scattering”;
the other part was lyophilized and used for the monosaccharide composition analysis and the phosphorus analysis.

The measurements of the monosaccharides released as a result of the chemical treatments were performed with high-performance anion exchange chromatography (HPAEC) as follows. The Dionex system device consisted of the following modules: a gradient pump, eluent degassing unit (He), 4 × 250 mm CarboPac PA1 column with CarboPac PA100 column, a pulsed electrochemical detector (PED-2) in pulsed amperometric detection (PAD) mode and a Spectra Physics AS3000 sampler. The chromatograms were recorded using PC1000 software. The eluent was measured with gold electrode containing PED-2 detector; the reference electrode was Ag/AgCl. For the T1 0.4 s, T2 0.2 s and T3 0.4 s retention times, the following potentials were applied: E1 0.1; E2 0.7; E3 0.1. The gradient elution was created by mixing Millipore distilled water and 0.1 M NaOH, 1 M NaOAc and 0.1 M NaOH. After equilibration with 16 mM NaOH, 20 µL of sample were injected onto the column. The eluent programme was as follows: 0 → 20 minutes 16 mM NaOH; 20 → 25 minutes, 0 → 1 M NaOAc; 25 → 30 minutes, 1 M NaOAc; 30 → 35 minutes, 0.1 M NaOH; 35 → 40 minutes, 0.1 M → 16m M NaOH; 40 → 55 minutes, re-equilibration with 16 mM NaOH.

The amount of the released galactose-1-phosphate was determined with HPAEC, with the already presented method. The eluent composition (flow rate 1 mL/min) has been changed as the following: 0.1 M NaOH solution and 1 M NaOAc containing 0.1 M NaOH solution was mixed. The eluent programme was as follows: 0 → 5 minutes, 0.1 M NaOH isocratic; 5 → 72 minutes, linear gradient 0 → 0.6 M NaOAc; 72 → 77 minutes, 0.6 M NaOAc isocratic; followed by the washing step, 77 → 82 minutes 0.6 → 1 M NaOAc; 82 → 87 minutes, 1 M NaOAc; 87 → 102 minutes; re-equilibration with 0.1 M NaOH. α-D-galactose-1-phosphate (Sigma) was used as a standard.

After the chemical treatments, the sugar composition and the phosphorus contents of the resulting polymers were determined. After sulphuric acid treatment, the monosaccharide content of the samples was analysed by HPAEC, and there have been found only galactose monomers. As the sugar-1-phosphate bonds are sensitive to diluted acids, the galactose has probably bounded terminal to the phosphate, although the amount of the released galactose was less than it could have been expected on the basis of the assumed structure of the EPS. This may be either owing to the fact that only a part of the terminal galactose molecules have been released due to the sulphuric acid treatment, or owing to the fact that not every repeating unit contained terminally bounded galactose in this structure. The later NMR studies revealed that
the sulphuric-acid-treated EPS contained only phosphomonoester bonds in a
significant amount; thus, almost all of the terminally linked galactose could
be removed with the treatment. Thus, the amount of the measured galactose
was less than expected because the EPS chain structure was different from the
presumed one.

The treatment with HF removed the total amount of phosphorus contained
in the EPS; thus, the treated polysaccharide contained less galactose and
rhamnose, while the glucose content remained unchanged. During the treat-
ment with HF, the sugar phosphate bonds cleaved while the sugar-sugar bonds
were unchanged (Hancock & Pozton, 1980; Nakajima et al., 1992). In addi-
tion, several authors have found that the rhamnose bonds could also cleave as
a result of HF treatment (Lugowski & Jennings, 1984; Janssen et al., 1998;
Robijn et al., 1995). The hydrodynamic volume of the polymer was mea-
sured by HPSEC and with static “light scattering” detector, according to the
method described by Tuinier et al. (1998). As the hydrodynamic volume of
the molecule did not change greatly after the removal of phosphate and rham-
nose, these have to be present in the side groups of the EPS molecule and not
in the main chain. The appearance of the galactose after treatment indicated
that it was linked to the main chain via the phosphate group.

The treatment with HF removed all the phosphorus as well as all the termi-
nally linked galactose while the sulphuric acid treatment released most of the
terminally linked galactose, but left the phosphorus almost unchanged. The
polymer galactose content subjected to the sulphuric treatment was found to
be relatively less than in the case of the HF treatment, from which it was con-
cluded that in the presence of phosphorus only a small part of the galactose
contained in the main chain could be analysed, i.e. only a part of the galactose
phosphate bonds have been degraded during the hydrolysis and thus the free
galactose, which could convert to alditol-acetate, was less. The correctness of
the assumption was examined by measuring the total and free phosphorus con-
tent after each step of the monosaccharide composition determination assay.
The free phosphorus content was almost negligible in the raw EPS; however,
after the hydrolysis, 30-40% of the total phosphorus amount was free phos-
phorus. This meant that only a part of the galactose-phosphate bonds of the
main chain were hydrolysed, which resulted in the fact that only 30-40% of
the galactose contained in the main chain could be detected; however, in the
case of terminal galactose, the total quantity of galactose could be detected,
as it was fully released during the hydrolysis.

The NaOH treatment resulted in polymers with lower phosphorus and galac-
tose content as compared to the HF treatment; the NaOH treatment did not
remove all of the phosphorus from the EPS and the rhamnose bonds remained unbroken.

From the effects of chemical modifications, it can be concluded that the amount of galactose in the main chain of the purified EPS was underestimated due to incomplete hydrolysis of galactose-phosphate esters in the main chain.

In the case of the analysis (methylation, hydrolysis, acetylation) dedicated to the exploration of the monosaccharides binding sites, the phosphorus content was measured as well. The samples were divided into three parts: one of them was subjected to treatment with HF before methylation; another part of the samples was treated prior to alditol acetate derivatization after methylation; in the case of the third part, no HF treatment was applied. The untreated EPS contained, according to the results, 1,4-linked glucose and rhamnose (1,4-di-O-acetyl derivatives), and traces of 1,2,4-linked and 1,2,3,4-linked galactose.

Several authors mentioned that in the case of phosphorylated polysaccharides, during the methylation, a partial dephosphorylation occurs and the hydrolysis of phosphate esters is also not complete (Janssen et al., 1998; Nakajima et al., 1992; Robijn et al., 1995; Robijn et al., 1996). As an effect of the treatment with HF after the methylation, the recovery of the 1,2,3,4-linked galactose increased as compared to untreated EPS, which was explained with the following: as all the sugar-phosphate bonds broke up during treatment with HF, the phosphorus did not alter the hydrolysis anymore and therefore all the galactose contained in the main chain could be analysed. Due to the effects of the treatment with HF before methylation, instead of 1,2,3,4-linked galactose, there has been found 1,2,4-linked galactose in nearly identical quantity. As the binding site of the removed phosphate group was constituted from the point of view of methylation to be a free group, this site was methylated; therefore, no acetylation could occur. Thus, the 1,2,3,4-linked galactose in the EPS chain is substituted on the third carbon atom with phosphate.

During the methylation analysis, “under-methylation” occurred, probably because of the poor dissolution of the EPS in the methylating agent. However, the composition of the non-methylated substances was similar to that of the EPS sugar composition. The results were well reproducible, and in the case of the terminally linked rhamnose and 1,4-linked glucose no di-O-methyl and mono-O-methyl sugars have been observed; therefore, it was assumed that the methylated part of the EPS represents the total EPS.

Regarding the structure of the EPS, the results obtained by Casteren et al. (1998), except for one, were identical with the results obtained by other authors. Casteren et al. (1998) did not detect terminally linked galactose, but this was expected in the case of HF treated samples as the terminally linked
galactose linked to the main chain by phosphate was cleaved because of the treatment, and the subsequent dialysis removed it. In contrast, in the case of untreated EPS, the absence of the terminally linked galactose was unexpected. As the pH was strongly alkaline during the structure exploratory analysis, it seemed likely that the galactose-3-phosphate had cleaved, after which the resulted galactose-1-phosphate was removed during the dialysis. This contradicts the fact that the HF treatment occurred after the methylation had resulted in the formation of 1,2,3,4-linked galactose derivatives, which means that the phosphate group was still present during the methylation. Nakajima et al. (1992) found some amount of terminally linked galactose after the purification of the methylated, HF treated and re-methylated polysaccharide – but less than expected. As the surplus HF was removed with vacuum using a KOH desiccator, the terminally linked galactose was retained which was detached during the HF treatment. If the terminally linked galactose would have been released during the methylation, this would have been also retained. Thus, the differences appearing regarding the recovery amount of the terminally linked galactose may be due to the differences in the purification process.

In summary, it can be concluded that after the chemical treatments the released sugars and the composition of the residual polymer were also analysed. During the sulphuric acid treatment, units linked to the phosphate through 1-glicosidic bonds were released; thus, their structure and quantity could also be determined. The HF treatment removed all the phosphate and a part of the rhamnose, and with the determination of the hydrodynamic volume of the molecule it was possible to realize whether these components are situated mainly in the main chain or in the side chain. The analysis of the components of the residual polymer gave rise to the suspicion that in the presence of phosphate, the hydrolysis of the polymer is not perfect, which was confirmed during the determination of the total and free phosphate content after hydrolysis. As an effect of the HF treatment, the recovery amount of the phosphate-linked monosaccharide increased as the hydrolysis inhibitor phosphate-ester bond was cleaved prior to hydrolysis. The combination of the methylation analysis with the HF treatment allowed the identification of the binding site of the phosphorus-containing substituent (HF treatment prior to methylation, then after methylation).

With the help of chemical treatments, the following results have been achieved: the determination of the structure and quantity of the units bond through 1-glicosidic linkage to the phosphate, the determination of the location of phosphate and rhamnose in the molecule (main or side chain) with the measurement of the hydrodynamic volume, the determination of the binding
sites of the phosphorus-containing substituents (combination of methylation with HF treatment), and finally it was determined that the recovery amount of the monosaccharides linked to phosphate increased due to the HF treatment.

1.5 Examination of the structure of monosaccharides by nuclear magnetic resonance spectroscopy

These kinds of examinations allow an extensive exploration of the structure, suitable not only for the determination of atomic groups, but also for the fine-structure studies. With the study of the signals generated by the chemical shifts (the electron cloud alters the local magnetic field acting on the nucleus, and thereby the frequency of the nucleus resonance) and the spin-spin interactions (the spin orientation of the protons acts upon the neighbouring groups; the effect is mediated by the binding electron pairs), even low environmental differences can be detected. The applied analyses in the case of EPS were as follows: the detection of the repeating units in the structure, identification of the monomers forming the repeating units, the analysis of the isomerism of the anomeric carbon atoms ($\alpha$ or $\beta$), determination of the linkage mode of monomers and the occurrence and frequency of certain atomic groups.

Dueñas-Chasco et al. (1998) treated the samples with deuterium for several times; then removed the deuterium with freeze-drying and analysed the solution (3 mg/cm$^3$), solving it in 99.98% D$_2$O. The spectrums were collected at 303 or 333 K with a Bruker AMX 500 spectrometer, where the frequency of the electromagnetic radiation was 500.13 MHz ($^1$H) and 125.75 MHz ($^{13}$C), respectively. The chemical shift was given in ppm, the signal of HDO (4.75 ppm, 303 K or 4.33 ppm, 333 K) ($^1$H), or using the signal of an external standard dimethyl-sulfoxide (39.5 ppm) ($^{13}$C) as a reference.

In the $^1$H-NMR spectrum, the following was determined: the number of different resonant frequency shift indicating anomeric protons (i.e. the hydrogen atoms linked to anomeric carbon atoms) in different environments. By determining the configuration of the anomers ($\alpha$ or $\beta$) and their ratio, it was revealed how many different $\alpha$-glucopyranose and different $\beta$-glucopyranose units are there in the EPS and what their ratio is. Based on the initial NMR examination and on the studies regarding the linkage position of the monomers, it was likely that the EPS is a mixture of two kinds of polysaccharides: one part consists of $\alpha$ configuration unit ($\alpha$ PS), the other part of $\beta$-configuration unit (PS $\beta$). This assumption was confirmed by $^1$H-NMR analysis of the fractions of EPS precipitated with alcohol, where the $\alpha$/\$\beta$ polysaccharide ratio was measured in the anomeric region of the spectrum of the fractions.
The PSa unit was analysed in detail with NMR spectroscopy. The NMR signals of the $^1$H and $^{13}$C fractions were analysed for their chemical shift, whereby the signals of three different resonant frequency transmitter anomeric protons were observed on the one-dimensional $^1$H-NMR spectrum: PSα was constituted of three type of α-D glucopyranoses (A, B, C). The resonant signals of the protons belonging to carbon atoms (1, 2, . . . 6) of individual subunits (A, B, C) were also observed and the spin-spin interactions were also analysed, specifically the interaction between the three different spin systems (A, B, C). The C-2 (the second carbon atom of the monomer) and the associated H-2 resonant signals showed chemical shifting towards the lower magnetic field strength in the A region compared to the identical nucleus of B and C units. It followed that the second carbon atom of unit A, unlike the other units, was linked with some type of substituent; thus, the unit containing 1,2,6 bonds is the side ramification point of the main chain. The C-6 resonant signal of the B unit showed chemical shifting towards the higher magnetic field strength as compared to units A and C, which means that the 6 carbon atom is unsubstituted in unit B, i.e. the B terminal unit is nothing else than the α-D-glucopyranose linked with its anomer carbon atom to the chain. The C unit is unsubstituted and forms the α-D-glucopyranose main chain with its 1,6 bonds.

Urashima et al. (1999) dissolved the polysaccharide in 1 cm$^3$ heavy water (99.96 D atomic%, Aldrich, USA) and then placed it into the NMR sample holder. The chemical shift value was given relatively compared to the 2,2-dimethyl-2-silapentan-5-sulphonic acid sodium salt (DSS), but during the analysis the chemical shift was measured compared to the acetone. The $^1$H-NMR (proton NMR) spectrum was determined in heavy water (99.96 D atomic%, Aldrich, USA), at a frequency of 270 MHz, using a Jeol JNM-GSX-270 spectrometer, in which the sample temperature was 300K. Based on the different chemical shift values showing anomeric resonant signals of the $^1$H-NMR spectrum, the structure of the polysaccharide was formed by repeating rings consisting of seven units. The rings were formed by five α-glycosides and two β-glycosides. The rhamnose (H-6) resonant signal was also observed on the spectrum.

Uemura et al. (1998) determined the $^1$H-NMR spectrum also in D$_2$O (Merck, 99.75%, Darmstadt, Germany), at the temperature of 300 of 354 K, using acetone as an internal standard. The NMR apparatus was a Varian Unity INOVA 600 model, which operated in Pulse Fourier Transform mode. There were found five, H-1 (the hydrogen atom belonging to the first carbon atom of the monomer) resonant signals with nearly identical intensity on the $^1$H-
NMR spectrum determined in a NPS at 50°C and D₂O solution at 600 MHz. Based on the chemical shift, the five signals formed two different groups: the first group was formed by three nearly identical signals, while the second group was formed by two nearly identical signals. From this, it was concluded that a pentasaccharide unit repeated in the chain (five H-1 with different environment), in which three members were of α spatial orientation and two members of β spatial orientation. The NMR spectrum of the APS included also five H-1 signals with similar chemical shift values as in the case of NPS. This indicated that the same pentasaccharide repeating unit was present in the APS as well as in the NPS. None of the following was found on the spectrum: methyl group, 6-deoxi-saccharide, amino group of the hexosamine and resonant signal of sialic acid (H-3). Based on the knowledge of the NMR spectrum and the chemical composition, the carbohydrate structure was identical in the case of the APS and NPS; there were differences only in the phosphorus content.

_Casteren et al. (1998)_ analysed the resonance of the nucleus different than protons. During the $^{31}$P NMR analysis, the spectrum of the sample was determined in 5-10% D₂O containing water at 121.500 MHz using a Bruker AMX-300 type spectrometer. The sample holder was of 10 mm, the temperature was 27°C. The chemical shift was determined compared to the internal standard L-α-glycero-phosphoryl choline (GPC, Sigma). Chemical shifts caused by changes in pH were measured to determine whether there are phosphomonoesters or/and phosphodiesters in the sample. The pH of the sample was adjusted with 25 mM HCl or 25 mM NaOH solution.

The 1D, 400, 13 MHz $^1$H-NMR spectrum of the HF treated EPS and the spin decoupled 100.63 MHz $^{13}$C NMR spectrum of the proton was determined in 8 mg/cm$^3$ D₂O at 60°C with a Bruker DPX-400 spectrometer, where the sample holder was of 5 mm. The $^{31}$P NMR spectrum of the purified EPS included one resonance signal at neutral pH, which confirmed the existence of a single type of phosphorus-containing group in a repeating unit (Moreau et al., 1998). During the titration experiments, the resonance frequency was not shifted substantially at the range of pH 6.1 to 10.0, which indicates the presence of phosphodiesters (Ilg et al., 1996). After the purification, there were no phosphate monoesters found, which indicates that the galactose-1-phosphate bond – sensitive to acids – did not become cleaved during the trichloroacetic acid purification and that all the phosphate groups were present in the form of diester. However, in the case of EPS exposed to the effect of trichloroacetic acid for a longer time, the breakdown of phosphodiesters could also appear. In such a case, in addition to the phosphodiester resonance signal, the phosphomonoester resonance signal is also observable in the
$^{31}$P NMR spectrum.

At pH 4.0, the sulphuric-acid-treated samples gave a resonance line, which was shifted during the titration experiments. This phenomenon indicates the presence of titratable phosphomonoester. The signal of the phosphodiester was very low, from which it was concluded that almost all the galactose (linked terminally to the chain with phosphate-ester bond) was removed with the sulphuric acid treatment. In the case of EPS treated with HF, there were no resonance signals when the $^{31}$P NMR was measured, whereas the treatment resulted in the complete dephosphorylation of the EPS. The spectrums taken with $^1$H-NMR and $^{13}$C NMR techniques have been compared to EPS spectrums of other origins, analysed by other authors, and it was used for the study of repeating structural units.

1.6 Glucose, galactose and lactose content of yogurt

1.6.1 Extraction of the saccharides from yogurt

Richmond et al. (1987) measured 10 g of homogenized yogurt at room temperature into a centrifuge tube, and layered onto it an amount of absolute ethanol until the final ethanol concentration became 80% (v/v%). The slurry was mixed and left it to stand for 20 minutes at room temperature until the proteins had sedimented. Subsequently, 80% by volume of ethanol was added to the mixture so that the total volume of the solution became 50.0 cm$^3$, and the precipitate was centrifuged. The supernatant was discarded and the precipitate was washed with 25 cm$^3$ of 80% v/v of ethanol. The extracts and the washing liquid were pooled, and then the alcohol was removed with a rotary rapid evaporator at 25-27°C. The volume of the extracts was filled until 25 cm$^3$ with water and filtered over a Whatman No. 42 filter paper. The samples and the standard solutions were also filtered over a 0.45 µm Metricel membrane and were stored in vials frozen at -10°C until the start of the analysis.

1.6.2 Determination of the quality and quantity of the saccharides by HPLC

The HPLC system consisted of the following elements: Waters Assoc. (Milford, MA) M-45 solvent delivery unit, Waters U6K septum free sampler, Waters Model RI-401 differential refractometer, and a Linear Instruments Model 232 recorder. The column was a Bio-Rad Aminex HPX-87 carbohydrate column of the size of 300 mm $\times$ 7.8 mm. The column was held at the temperature of 80 °C by an Alltech Assoc. water jacket (catalogue Nr.: 9502) equipped with
a Precision Scientific 66600 water bath and a 62538 thermometer. The unwanted anions were removed with a Bio-Rad Aminex type A-25, 40 mm × 4.6 mm sized Microguard Anion/OH cartridge patron system column. The eluent was ammonia-free water deionized by reverse osmosis, which was degassed by vacuum and held at 50 °C until the start of the HPLC analysis to lower the re-dissolution of the gases. The samples and the standards were injected into the device with a 10 µL Hamilton syringe. Qualitative identification was made by retention time, quantitative identification was made by external standard calibration, based on the equation of the straight fitted onto six measurement points (lactose concentration range: 0.20-2.00 g/100 cm$^3$; glucose and galactose concentration range, one by one: 0.1-1.00 g/100 cm$^3$).

1.6.3 Examination of the methods

The recovery was analysed in the case of lactose and galactose. The extraction steps were applied first to standard solution, then to yogurt-sugar preparation. 10 g of standard solution (2.00 g/100 cm$^3$ lactose and 1.00 g/100 cm$^3$ galactose) and 10 g yogurt were mixed, the mixture was extracted, and then the sugar content of the extract was measured. 1 g of the standard sugar solution was mixed with 10.0 g of yogurt and was also extracted. The carbohydrate content of the following samples was determined repeatedly: untreated standard solution, extracted standard solution, yogurt, yogurt and added carbohydrates. The recovery of lactose was of 103.6% and that of the galactose was 103.4%. During the analysis of the heat-treated samples, a new peak appeared on the chromatogram, which was identified as lactulose based on the retention time of the standards.

2 Determination of the composition and quantity of the galacto-oligosacharides

Hyun-Jae Shin et al. (1998) determined the quantity of GalOS, while Yanahira et al. (1998), in addition to the quantity, determined their structure, too. Hyun-Jae Shin et al. (1998) analysed the total carbohydrate content (mono- and oligosaccharates) of the ferment, using high-performance liquid chromatography (HPLC). The Waters (USA) device was equipped with reflective index (RI) detector and Sugar Pak I column (Waters, USA). The mobile phase was 10 mM Ca-EDTA solution, the flow rate was 0.5 cm$^3$/min and the column space temperature was 90 °C. The authors did not report the chromatogram,
and they observed di- and a trisaccharides as oligosaccharides; as their identification was not carried out, the authors described their structure based on data from literature.

Rustom et al. (1998) determined the lactose, glucose+galactose and oligosaccharide content of the permeates with HPLC based on the method described by Jeon & Mantha (1985). The device was constituted of the followings: 20 µL manual sampler, pump (Waters, M-6000A), a pre-column (Nucleosil 120-7NH₂, 30 × 4 mm ID) and a 250 × 4-mm-sized column (Nucleosil 120-7NH₂), reflective index detector (Varian R1-A, 16, temperature at 35 °C) and a Crome Jet SP 4400 integrator (AT=8, C5=0.5 cm/min). The mobile phase was acetonitrile/water mixture (75%;25% v/v), which was filtered over a microfilter with the pore size of 0.22 µm and degassed in an ultrasonic bath for 30 minutes. The samples were diluted to the twenty-fold with bi-distilled water, filtered over a filter with the pore size of 0.22 µm, injected at room temperature, then eluted with a 2.0 cm³/min flow rate. Each measurement was repeated three times. The retention time of glucose and galactose was so close to each other that their quantity could be determined only together. Only the amount of the oligosaccharide concentration was reported, structural identification was not carried out.

Yanahira et al. (1998) studied the structure of oligosaccharides with a HPLC “LC module 1” device, “model 490” UV (208 nm) and “model 410” refractive index detector (Waters, Tokyo, Japan) under the following circumstances: “A” version: Bior-Rad HPX-87H 6×250-mm-sized ion exclusion column, 0.005 M sulphuric acid solution as eluent, flow rate of 1.0 cm³/min. “B” version: Shodex SH-1821 (Showa Denko Co.) column; 0.005 M sulphuric acid solution as eluent, flow rate of 0.75 cm³/min. The N1 signed acidic oligosaccharide could be separated into two substances (N1-1; N1-2) by carbon column chromatography with a 5.0 × 50-cm-sized column. During the measurement, applying a linear gradient, the quantity of ethanol varied between 0% → 15%. The FAB-MS spectrum was determined in negative ion operation mode using a Jeol JMS-700 mass spectrometer with 6 kV equivalent kinetic energy Xe atoms, using glycerin as matrix.

The ¹H-and ¹³C-NMR spectrums were determined in 2-3% D₂O containing solution at a temperature of 25°C with a Jeol Alpha-500 NMR spectrometer. The chemical shift was given in ppm with a magnetic field strength decrease orientation compared to the signal of 4,4-dimethyl-4-silapentanoate, related to 1,4-dioxane. The analysis of acidic oligosaccharides was based on NMR measurements. Acidic oligosaccharides were purified by anion exchange chromatography (5.0 × 50 cm, Dowex 1, acetate form). The column was washed
amply with water, removing the neutral sugars, and then the acidic oligosaccharides were eluted with the linear gradient (0 → 0.2 M) of sodium-acetate solution. Each fraction was desalted with a Micro Acilyzer S1 (Asahi Chemical Ind. Co Ltd.) device, and then concentrated in vacuum, and finally they were lyophilized. Four different acidic GalOS (N1, N2, N3, N4) could be separated with HPLC. Under the “A” analytical circumstances, N1 and N2 oligosaccharides were separated. Under the “B” analytical circumstances, G1 and G2 oligosaccharides were separated. The N1 signed oligosaccharide could be further separated into two different substances (N1-1; N1-2), using carbon column chromatography. For the means of oligosaccharide yield measurement, GalOS were extracted from the reaction mixture by anion-exchange chromatography, and the same preparations were used for the structure identification studies.

The detailed structure of acidic GalOS was determined by NMR spectroscopy. The H1-H6 resonance signals of the N1 acidic GalOS galactosyl residue were identified based on the spectrums, whereby the anomic proton was identified first (β-galactose H1). Based on the chemical shifts, there was no α-galactose (H1) signal visible on the N1-1 spectrum, from which it was concluded that the galactose unit contained in N1-1 was situated on the non-reducing chain end; thus, the glycosidic OH group of galactose was in bond. The signal of N-acetyl muramic acid (NeuAc) axially situated H3 proton and equatorially situated H3 proton were also observed in the spectrum, and with the help of these signals the sequence of protons H4, H5 and H6 could also be determined. In order to select the NeuAc H7, H8 and H9 signals, first, the signal of H9, then that of H7 and H8 were identified using the spectrum. Subsequently, it was controlled with the spectrum if the signals of N1-1 constituting carbon atoms were identified correctly. The C8 resonance signal of the NeuAc unit showed chemical shift towards lower field strength because of its involvement in the glycosidic bond. From these results, the GalOS structure was determinable: one β-galactose molecule was linked with 1→8 bonding to the NeuAc molecule.

Similarly to the previous ones, the 1H- and 13C-NMR data of the N1-2 revealed that the non-reducing galactose group has similar chemical shifts as the N1-1 galactose group. The C9 signal of the NeuAc unit showed chemical shift towards lower field strength on the N1-2 13C-NMR spectrum, which was also confirmed by the distortion-free increase of the polarization transfer. Based on these results, the N1-2 structure was the following: one β-galactose molecule linked with a 1→9 bonding to the NeuAc molecule.

Among the N2 GalOS 1H- and 13C-NMR data, chemical shift signals of the non-reducing galactosyl group and the NeuAc were similar to that of N1-1,
and the C’3 signal of the internal galactose unit showed chemical shift towards the lower field strength because of the glycosylation. The N2 structure was as follows: galactose β-(1 → 3)-galactose β-(1 → 8)-NeuAc. The structure identification of G1 and G2 GalOS was performed also with the study of 1H- and 13C-NMR spectrum according to the method described earlier.

References


Structural and quantitative analysis of exopolysaccharides...


