A two-dimensional mapping analysis was performed by HPLC for 4 kinds of standard galactosyllactoses (GLs, trisaccharide) which were assumed to be produced from lactose (galactopyranosylβ1→4 glucopyranose) in yogurt during the fermentation of lactic acid bacteria. After the pyridylation of GLs, they were analyzed by HPLC in the reverse-phase (RP) and anion-exchange (AE) modes. The retention times of each peak obtained were converted to glucose units (GU) in RP mode for the pyridylated isomaltotrioligosaccharides (G1-3) and to relative retention time (RRT) in AE mode against pyridylated-isomaltotriose, and then the address data [GU, RRT] were plotted on a graph. This two-dimensional mapping method was found useful for a rapid qualitative evaluation of the chemical structure of trisaccharides formed in yogurt.

Key words: galactosyllactose; HPLC; two-dimensional mapping; pyridylation

The two-dimensional mapping analysis by HPLC is a known technique for the structural evaluation of N-glycosylated sugar chains bound to glycoproteins. In this method, pyridylation is widely used as a derivation technique for sugar chains having a reducing-end residue because of its high sensitivity with fluorescence. Although the method has recently been adopted for analyzing O-glycosylated sugar chains, an improved method has not previously been reported for the low-molecular-weight free oligosaccharides that exist in milk and milk products.

Galactosyllactoses (GLs) are also known as milk sugars existing in human milk which are thought to be biosynthesized from lactose (galactopyranosylβ1→4glucopyranose, Galβ1→4Glc) by galactosyltransferase in mammary epithelial cells. In fermented milk products such as yogurt, GLs are known to be produced by transgalactosylation of β-galactosidase(EC 3.2.1.23) by lactic acid bacteria. Although GLs are also known to have growth-promoting activity against *Bifidobacterium* as bifidus factors and the major oligosaccharide except lactose and galactose, especially in yogurt, no research concerning their rapid qualitative evaluation has been reported. In this report, we describe the development of a new two-dimensional mapping method to evaluate GLs in commercially available yogurt.

2-Aminopyridine (2-AP) was purchased from Wako Pure Chemical Industries (Osaka, Japan), and sodium cyanoborohydride was obtained from Aldrich Chemical Company (Milwaukee, WIS, U.S.A.). β-Galactopyranosyl(1→3)-β-galactopyranosyl(1→4)-glucopyranose (Galpβ1→3-Galpβ1→4Glc, 3'-GL), Galpβ1→4Glpβ1→4Glc (4'-GL), Galpβ1→4Galpβ1→4Glc (4'-GL), Galpβ1→6Galpβ1→4Glc (6'-GL) and Galpβ1→4(Galpβ1→6Glc) (6-GL) were kindly presented by Dr. Shinji Hata (Faculty of Dentistry, Tohoku University, Japan). Isomaltose (Glcpa1→6Glc), isomaltotriose (Glcpa1→6Glcpa1→6Glc) and isomaltotetraose (Glcpa1→6Glcpa1→6Glcpa1→6Glc) were purchased from Seikagaku Co. (Tokyo, Japan). The other chemicals used were of analytical grade.

The oligosaccharides were pyridylated by the method of Hase. The pyridylated (PA)-oligosaccharides were separated from the reaction mixture by HPLC in gel permeation (GP) mode with a Superoxide Peptide HR 10/30 column (10×300 mm, 13 μm particle diameter; Pharmacia Biotech, Uppsal, Sweden) at room temperature. Elution was performed isocratically with a 0.01 M ammonium hydrogen carbonate buffer (pH 7.9) at a flow rate of 0.25 ml/min. The PA-oligosaccharides were separated by HPLC in two different modes A and B. Mode A: HPLC in reverse-phase (RP) mode was done with a Wakosil-II 5C18RS column (4.6×250 mm, 5 μm, Wako) at 45°C. Elution was conducted isocratically with a 0.2 M sodium citrate buffer (pH 4.5) at a flow rate of 0.5 ml/min. Mode B: HPLC in anion-exchange (AE) mode was done with a Palpak Type A column (4.6×150 mm, 8 μm; Takara, Shiga, Japan) at 65°C. Elution was conducted isocratically with a 0.7 M potassium-borate buffer (pH 9.0)/acetonitrile (90:10, v/v) at a flow rate of 0.3 ml/min. The PA-oligosaccharides were monitored by a F-1080 fluorescence detector (Hitachi, Tokyo, Japan) at 320 nm excitation and 400 nm emission.

The milk carbohydrate fraction was extracted from commercial yogurt (plain type) with 4 volumes of chloroform/methyl alcohol (2:1, v/v) by our previous method. After isolating the trisaccharides by HPLC in GP mode with the Superdex Peptide HR 10/30 column at room temperature, eluting with Milli-Q water at a flow rate of 0.25 ml/min, the sugars were pyridylaminated and then analyzed in the same way against standard GLs.
The retention time of each PA-trisaccharide by RP-HPLC was converted to glucose units (GU) which are defined by comparing with the retention time of PA-glucose oligomers (G=1, glucose; G=2, isomaltose; G=3, isomaltotriose; and G=4, isomaltotetraose). The retention time of each PA-trisaccharide by AE-HPLC was also converted to relative retention time (RRT) against PA-isomaltotriose (G=3) which was eluted just before the four standard GLs. Finally, both results obtained from the two different modes of HPLC were plotted as the inherent address [GU, RRT] for each sugar on a two-dimensional map.

The two-dimensional mapping analysis of PA-sugars by HPLC has usually been applied for more than pentasaccharides, because of difficulty with mutual and complete separation between a great excess of 2-AP and a trace amount of the low-molecular-weight PA-oligomer (<G=4) by gel filtration. Furthermore, it is time-consuming to wash an open column to completely remove 2-AP in many samples. The contamination of 2-AP usually interferes with a precise HPLC analysis in any analytical mode. In this experiment, the complete removal of 2-AP was rapidly achieved by GP-HPLC within 3 hrs in a Superdex Peptide HR10/30 column whose theoretical plate is more than 30,000 (N/m) and by using an ammonium hydrogen carbonate buffer as the mobile phase to reduce the charge of 2-AP.

After removing 2-AP by GP-HPLC, four PA-GLs were analyzed by HPLC. RP-HPLC, using a 10 mm phosphate buffer (pH 3.8) as a condition for the conventional method of sugar chain analysis, resulted in all peaks being overlapped and eluting as a single peak. Good baseline separation in RP mode was achieved within 25 min by adopting 0.2 m citrate (pH 4.5) as the eluting buffer, which was originally used by Takemoto et al. for a PA-alditol analysis, and by selecting a column with suitable end-capping proportions (Fig. 1-a).

The Wakosil-II 5C18RS column used in this experiment could separate 2-AP as a sharp peak eluted first, unlike the other C-18 columns tested with a different ratio of end-capping (data not shown). As the peak of 2-AP was separately eluted first from the GL peaks, accurate quantitative and qualitative data could be obtained with this column. HPLC in the normal mode under the conditions used for the conventional method of sugar analysis could not separate the four PA-GLs. This problem was resolved by introducing HPLC conditions with an anion-exchange column that is used for the sugar compositional analysis of PA-monosaccharide in a glycoconjugate. Fig. 1-b shows a typical chromatogram in AE mode with complete separation of four PA-GLs achieved within 80 min. Finally, the two-dimensional map with completely separated addresses for the four PA-GLs was successfully plotted as shown in Fig. 2.

Figure 3 shows the typical elution chromatograms for the PA-trisaccharides prepared from commercial yogurt by HPLC in the RP and AE modes. RP-HPLC gave five peaks (1-5) including two large peaks (2 and 5) in the chromatogram (Fig. 3-a). The retention times of peaks 1, 2, 3, 4 and 5 were converted to GU values as 1.16, 1.32, 1.69, 1.88 and 2.06 (Glc=1.00, isomaltose=2.00, isomaltotriose=3.00), respectively. AE-HPLC gave five peaks (1-5) including two large peaks (3 and 4) in the

![Fig. 1. High-performance Liquid Chromatograms of 4 Kinds of PA-galactosyllectose (GL, Standard Trisaccharide) by Using Two Different Modes.](image)

![Fig. 2. Two-dimensional Map of 4 Kinds of PA-galactosyllectose (GL, Standard Trisaccharide) Prepared from the Results of HPLC Analyses in the RP and AE modes.](image)
Fig. 3. High-performance Liquid Chromatograms of the PA-trisaccharide Fraction Prepared from Commercially Available Yogurt in Two Different Modes.

a: Reverse-phase (RP) mode; arrows indicate the eluting position of PA-glucose (G = 1, rt = 17.3 min, GU = 1.00), PA-isomaltose (G = 2, rt = 22.3 min, GU = 2.00) and PA-isomaltooltriose (G = 3, rt = 36.7 min, GU = 3.00). b: Anion-exchange (AE) mode; the arrow indicates the eluting position of PA-isomaltooltriose (G = 3, rt = 21.4 min, RRT = 1.00). 2-AP, 2-aminopyridine.

chromatogram (Fig. 3-b). The retention times of peaks 1, 2, 3, 4 and 5 were converted to RRT values compared to PA-isomaltooltriose (rt = 21.44 min., RRT = 1.00) as 1.69, 1.93, 2.24, 2.80 and 3.06, respectively. Finally, the address for each peak was compared to the 2-dimensional map (Fig. 2) and their chemical structures were evaluated. In this yogurt, the most dominant trisaccharide (peak 5 in Fig. 3-a, peak 3 in Fig. 3-b, address [GUx, RRTy = 2.06, 2.24]) and the second most dominant one (peak 2 in Fig. 3-a, peak 4 in Fig. 3-b, address [GUx, RRTy = 1.32, 2.80]) were identified as 3'-GL and 6'-GL, respectively. The ratios of 3'-GL and 6'-GL present were estimated as 65.7% and 27.5%, respectively, in the whole trisaccharide by calculating from the data on the AE-HPLC chromatogram (Fig. 3-b). The minor components of 6-GL (peak 1 in Fig. 3-a, peak 5 in Fig. 3-b) and 4'-GL (peak 3 in Fig. 3-a, peak 1 in Fig. 3-b) were also detected with an unknown trisaccharide (peak 4 in Fig. 3-a, peak 2 in Fig. 3-b).

Kimura et al. have recently reported the structural determination of galacto-oligosaccharides in Oligomate-50 (a product of Yakult Pharmaceutical Co.), which are made from lactose by the action of β-galactosidase from Aspergillus oryzae and Streptococcus thermophilus, by RP-HPLC after pyridylation and two-dimensional NMR spectroscopy. Our method with 2-dimensional HPLC mapping proved useful for a rapid evaluation of trisaccharides which had been isolated from commercial yogurt (Fig. 3) and Oligomate 50 (data not shown) after gel filtration and pyridylation.

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