HIGH-PH ANION-EXCHANGE CHROMATOGRAPHY

A USEFUL FRACTIONATION METHOD IN THE CHARACTERIZATION OF BACTERIAL EXOPOLYSACCHARIDES, ARABINOXYLANS, AND SUBSTITUTED CELLULOSES

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Although high-pH anion-exchange chromatography (HPAEC) has been known for a long time, it only recently became of practical value with the introduction of efficient anion-exchange resins in pellicular form [1]. The separation is largely determined by the differences in acidity of the hydroxyl groups and their accessibility to the functional groups on the anion-exchange resin. In carbohydrates, the anomeric hydroxyl group is the most acidic one, whereas the differences in pK_a-value between the other hydroxyl groups are much smaller. Substitution of a hydroxyl group obviously blocks the interaction of this potential oxyanion with the anion-exchange resin but, depending on its size and position, it can also reduce the accessibility of other hydroxyl groups to the resin. When the substituent itself is a carbohydrate, additional hydroxyl groups become available for interaction with the resin. In general elutions are carried out with NaOH and an increasing concentration of NaOAc. The resins (CarboPac PA-1 or CarboPac PA-100) enable separation of reducing mono- and oligosaccharides in such a short time-period that previously encountered problems related to the alkaline eluent, such as isomerization and degradation, are usually negligible, but care remains necessary. In any case, in preparative chromatography, collected fractions have to be neutralized directly.

The detection method most frequently applied in combination with HPAEC is pulsed amperometric detection (PAD). The PAD detector generates an electrochemical response to carbohydrates by oxidizing them on a gold electrode using triple pulse amperometry. A repeating cycle of three pulses with different potentials is used to oxidize the carbohydrate on the gold electrode generating the electrochemical response, to remove oxidation products from the gold surface, and to make the electrode surface again ready for the next oxidation, respectively. The most important advantage of this detection method is that it offers the possibility of highly sensitive detection of a wide range of carbohydrates. In case of quantitative analysis procedures the non-uniform molar response for different carbohydrates makes the use of molar adjustment factors necessary. Because only less than 1% of the carbohydrate is involved in the electrochemical reaction, the detection method does not interfere with preparative chromatography.

So far HPAEC-PAD has already been used with great success in the detailed structural analysis of glycoprotein glycans, and subsequently in the development of fast profiling procedures for these oligosaccharides [2-4]. Here a number of applications are presented related to the characterization of polysaccharides of different biological origin.

Exopolysaccharides from Lactic acid bacteria

Fermented food and beverages form a significant part of the food processing industry. In the majority of these fermentations lactic acid bacteria are involved. The prime user of these bacteria is the dairy industry. Well-known examples of fermented milk products are yogurt, sour cream, kefir and buttermilk. Nowadays, the produced exopolysaccharides are becoming of general interest. Hydrocolloids play an important role in the food industry, and it is stated that the *in situ* formed, socially well-accepted, viscous biopolymers may become good alternatives for the present generation of thickeners and stabilizers. Knowing the structures, structure-rheology studies can be carried out, and attention can be devoted to possible modifications to influence the properties of the native polysaccharides.

In Fig. 1 the primary structures of the exopolysaccharides produced by *Lactobacillus delbrückii* subsp. *bulgaricus* rr [5] and by *Lactococcus lactis* subsp. *cremoris* H414 [6] are shown. Both structures were established by monosaccharide analysis, linkage analysis, and one/two dimensional NMR spectroscopy of the native polysaccharide, in combination with characterization of oligosaccharide fragments, obtained by Smith degradation and partial acid hydrolysis, using methylation analysis, fast-atom-bombardment mass spectrometry, electron impact mass spectrometry, and one/two dimensional ^{1}H-NMR spectroscopy. Polysaccharides, consisting of neutral hexopyranose units, are not the most ideal molecules for partial acid hydrolysis studies. In general, highly complicated mixtures of oligosaccharide fragments are generated. It may be clear that these mixtures give rise to many difficulties in separation procedures, whereas sensitive detection is also problematic.

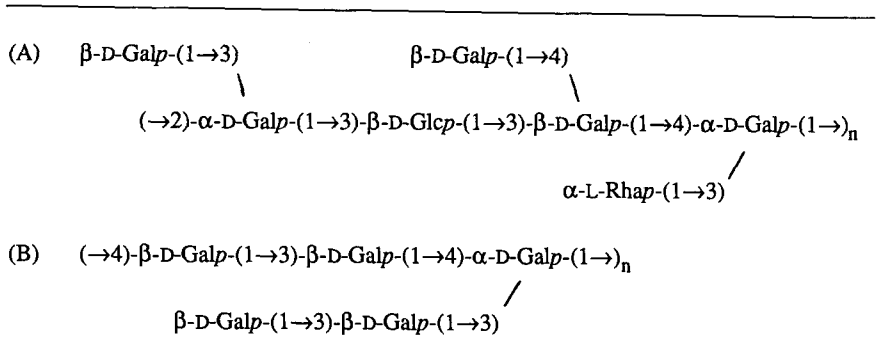

Fig. 1. Primary structures of the exopolysaccharides of *Lactobacillus delbrückii* subsp. *bulgaricus* rr (A) and *Lactococcus lactis* subsp. *cremoris* H414 (B).

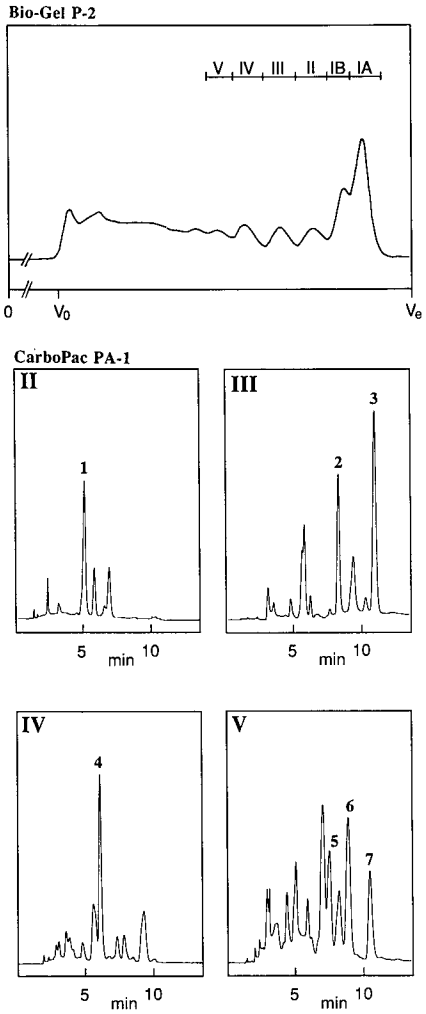

Fig. 2. Bio-Gel P-2 (H_2O) and CarboPac PA-1 fractionation patterns of the mixture of oligosaccharides generated by partial acid hydrolysis of the exopolysaccharide of *Lactobacillus delbrückii* subsp. *bulgaricus* rr. In case of HPAEC, several elution programs were used: program 1 (II), 93:7 eluent A (0.1M NaOH)-eluent B (0.1M NaOH containing M NaOAc) for 0.3 min, then going to 60:40 eluent A-eluent B in 60 min; program 2 (III), 94:6 eluent A-eluent B for 0.3 min, then going to 65:35 eluent A-eluent B in 60 min; program 3 (IV), 97:6 eluent A-eluent B for 0.3 min, then going to 70:30 eluent A-eluent B in 60 min; program 4 (V), 98:2 eluent A-eluent B for 0.3 min, then going to 90:10 eluent A-eluent B in 60 min. The gelfiltration was monitored by refractive index (IA=Gal, IB=Rha) and the HPAEC by PAD (E_1 0.05 V, t_1 300 ms; E_2 0.65 V, t_2 60 ms; E_3 -0.95 V, t_3 180 ms; response time 1 s).
(1) Galβ1-3Gal
(2) Galβ1-4Galβ1-4Gal
(3) Glcβ1-3Galβ1-4Gal
(4) Galβ1-4(Glcβ1-3)Galβ1-4Gal
(5) Galβ1-4(Glcβ1-3)Galβ1-4Galα1-2Gal
(6) Glcβ1-3Galβ1-4Galα1-2(Galβ1-3)Gal
(7) Galβ1-3Galα1-3Glcβ1-3Galβ1-4Gal

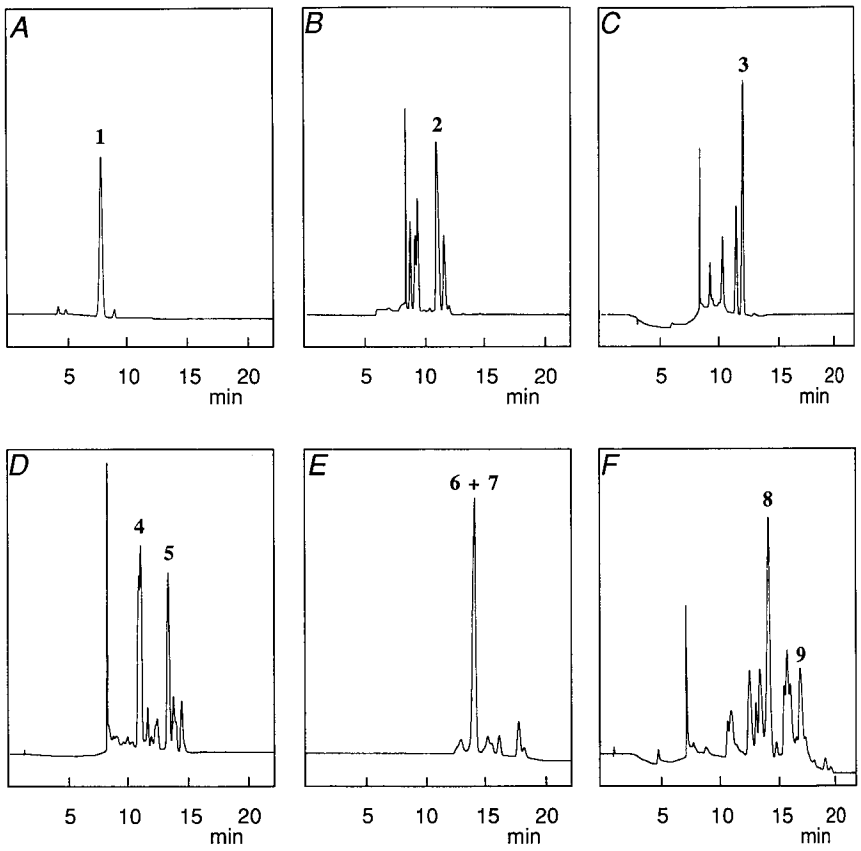

Fig. 3. CarboPac PA-1 (99:1 0.1M NaOH-0.1M NaOH containing M NaOAc for 0.3 min, then going to 50:50 0.1M NaOH-0.1M NaOH containing M NaOAc in 60 min) fractionation patterns of Bio-Gel P-4 fractions A-F of the mixture of oligosaccharides generated by partial acid hydrolysis of the exopolysaccharide of *Lactococcus lactis* subsp. *cremoris* H414. For PAD monitoring, see Fig. 2.
(1) Galβ1-3Gal
(2) Galβ1-3Galβ1-3Gal
(3) Galβ1-3Galβ1-3Galα-4Gal
(4) Galβ1-3Galβ1-3(Galβ1-4)Galα1-4Gal
(5) Galβ1-3Galβ1-3Galα1-4Galβ1-3Gal
(6) Galβ1-3Galβ1-3(Galβ1-3Galβ1-4)-Galα1-4Gal
(7) Galβ1-3Galβ1-3(Galβ1-4)Galα1-4Galβ1-3Gal
(8) Galβ1-3Galβ1-3(Galβ1-3Galβ1-4)-Galα1-4Galβ1-3Gal
(9) Galβ1-3Galβ1-3Galα1-4Galβ1-3Galβ1-4Galα1-4Gal

The application of the HPAEC-PAD technique (CarboPac PA-1) proved to be indispensable in the subfractionation of the Bio-Gel P-4 or P-2 fractions obtained from the complex mixture of oligosaccharide fragments generated by partial acid hydrolysis of both exopolysaccharides. A number of typical HPAEC-PAD fractionation patterns related with the exopolysaccharides of the *L. delbrückii* and *L. lactis* species are shown in Figs. 2 and 3, respectively.

Arabinoxylans

Cereals contain, among other biopolymers, small amounts of arabinoxylans. These polysaccharides are composed of a (1-4)-linked β-D-xylopyranose backbone substituted by α-L-arabinofuranose residues (Fig. 4), and small amounts of non-carbohydrate constituents can occur. Wheat arabinoxylans play an important role in dough development and have a significant effect on loaf properties. These features are especially prominent in flours containing low amounts of protein. The influences of the arabinoxylans are attributed to their high water-binding capacity, which can be tempered by (partial) degradation with endo-1,4-β-D-xylanases.

In view of the interest in the α-L-arabinofuranose distribution along the xylan core and the specificity of different endo-1,4-β-D-xylanases in relation to this distribution, analytical methods have been developed for the characterization of enzymatically generated arabinoxylan oligosaccharides from purified arabinoxylans. Based on

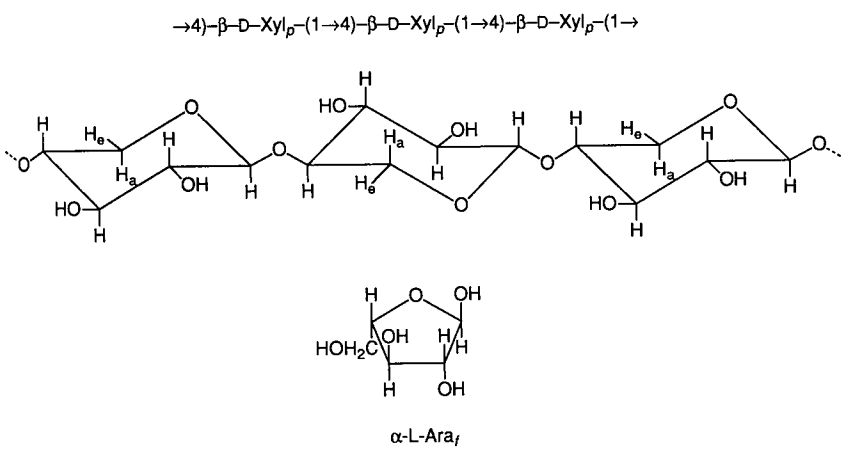

Fig. 4. Structural elements of arabinoxylans.

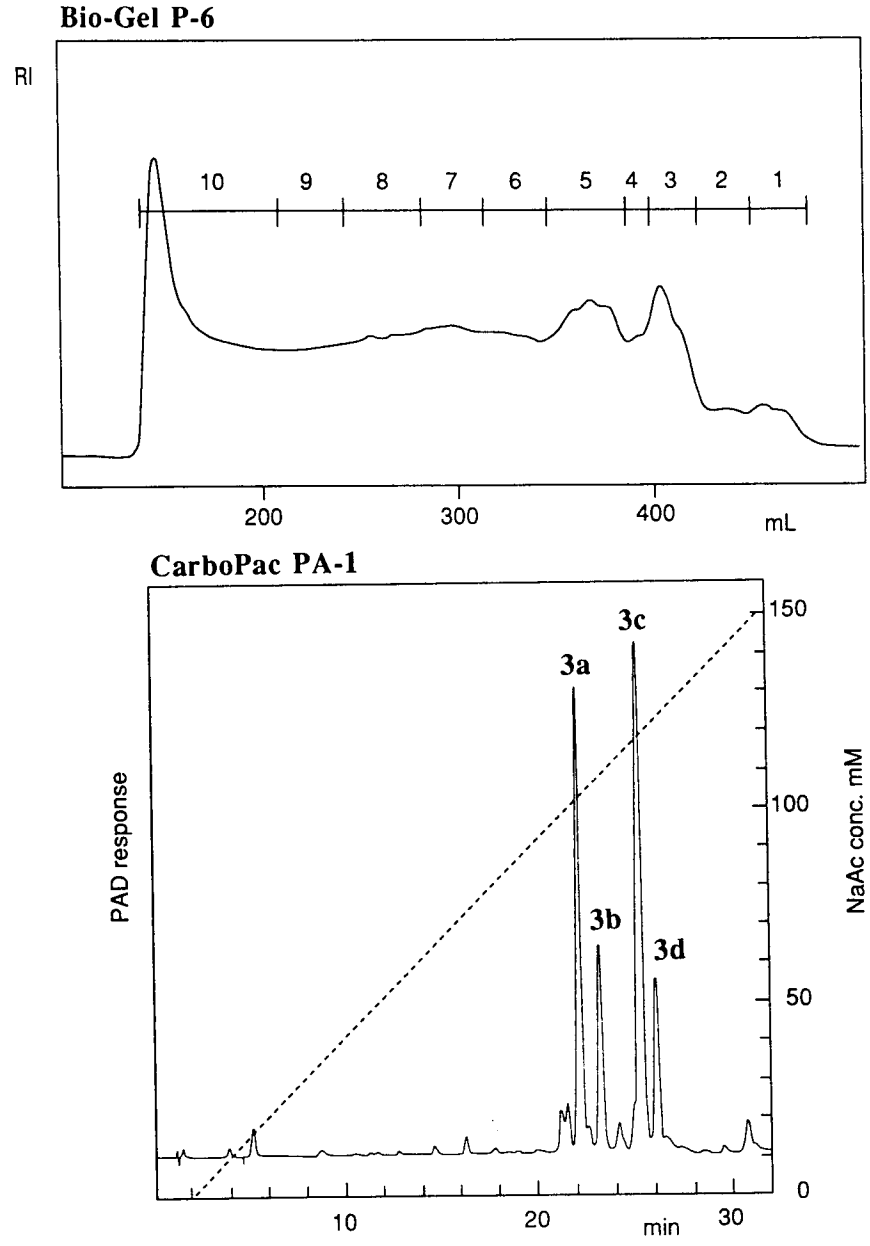

Fig. 5. Bio-Gel P-6 (H_2O) fractionation pattern of oligosaccharides obtained by incubation of arabinoxylan with endo-1,4-β-D-xylanase, and CarboPac PA-1 (0.1M NaOH for 0.3 min, followed by a linear gradient to 4:1 0.1M NaOH-0.1M NaOH containing M NaOAc in 40 min) fractionation pattern of Bio-Gel P-6 fraction 3. The gelfiltration was monitored by refractive index and the HPAEC by PAD (E_1 0.05 V, t_1 300 ms; E_2 0.65 V, t_2 120 ms; E_3 -0.95 V, t_3 60 ms; response time 1 s).

(3a) Xylβ1-4(Araα1-3)Xylβ1-4Xylβ1-4Xyl
(3b) Xylβ1-4(Araα1-3)Xylβ1-4Xylβ1-4Xylβ1-4Xyl and Xylβ1-4Xylβ1-4(Araα1-3)Xylβ1-4Xylβ1-4Xyl
(3c) Xylβ1-4(Araα1-2)(Araα1-3)Xylβ1-4Xylβ1-4Xyl
(3d) Xylβ1-4(Araα1-2)(Araα1-3)Xylβ1-4Xylβ1-4Xylβ1-4Xyl and Xylβ1-4Xylβ1-4(Araα1-2)(Araα1-3)Xylβ1-4Xylβ1-4Xyl

the detailed one/two dimensional ^{1}H-NMR analysis of a large series of arabinoxylan oligosaccharides increasing in complexity, a NMR structural-reporter-group concept could be developed [7,8], which made it possible to identify new members of the arabinoxylan oligosaccharide family and to deduce rules for the substrate specificity of different xylanases [9,10]. For the development of this NMR method, the availability of highly purified arabinoxylan oligosaccharides was a prerequisite. Using conventional chromatographic/detection procedures, it proved to be very difficult to isolate highly purified oligosaccharides from the enzymatic digests. The application of Bio-Gel P-6 gel-permeation chromatography, followed by HPAEC-PAD yielded high-quality samples suitable for structural analysis. A typical example of this serial chromatography is shown in Fig. 5, clearly demonstrating the possibilities and impossibilities of the separation of strongly related oligosaccharides on CarboPac PA-1.

Substituted celluloses

Cellulose ethers are polysaccharide derivatives capable of forming viscous solutions which have found widespread use in various industrial applications ranging from food and printing to oil recovery. Carboxymethylcellulose (CMC) is, in terms of production quantity, the most important water-soluble cellulose ether. Sulfoethylcellulose (SEC) is considered to be a possible replacement for CMC in some applications. CMC is prepared by conversion of cellulose with sodium chloroacetate, whereas SEC is obtained by reacting cellulose with ethylene sulfonic acid. In principle, eight glucose monomers can occur as constituents, namely, unsubstituted, monosubstituted (2-, 3-, and 6-), disubstituted (2,3-, 2,6-, and 3,6-), and trisubstituted (2,3,6-) D-glucose. The relative amounts of the constituting

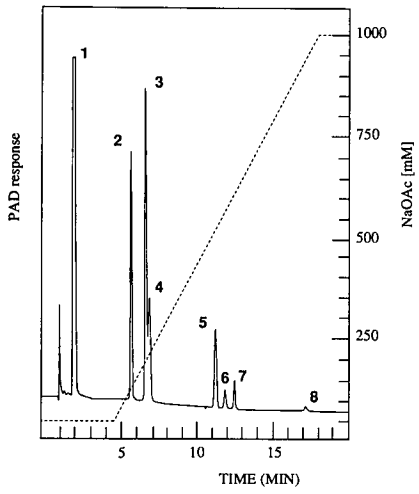

Fig. 6. CarboPac PA-1 chromatogram of a CMC hydrolysate using a gradient of NaOAc (dotted line) in 0.1M NaOH at a flow rate of 4 ml/min. (1) Glc; (2) 6-CM-Glc; (3) 2-CM-Glc; (4) 3-CM-Glc; (5) 2,6-CM-Glc; (6) 3,6-CM-Glc; (7) 2,3-CM-Glc; (8) 2,3,6-CM-Glc.

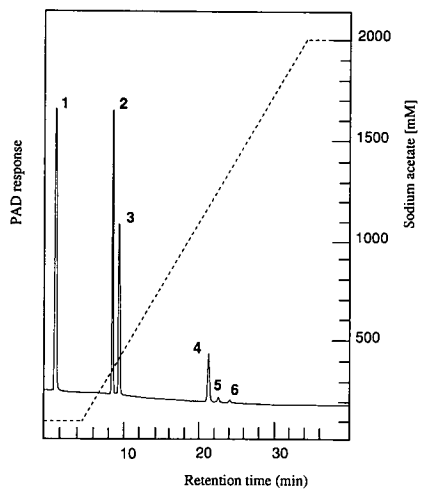

Fig. 7. CarboPac PA-1 chromatogram of a SEC hydrolysate using a gradient of NaOAc (dotted line) in 0.1M NaOH at a flow rate of 4 ml/min. (1) Glc; (2) 6-SE-Glc; (3) 2-SE-Glc; (4) 2,6-SE-Glc; (5) 3,6-SE-Glc; (6) 2,3-SE-Glc.

monomers are of interest in process-control and in understanding the product properties. Therefore, reliable and fast methods to determine the substituent distribution are essential.

In case of CMC, mixtures of monomers, obtained by hydrolysis of CMC, have so far been quantitatively analyzed by gas-liquid chromatography (GLC), ^{13}C-NMR spectroscopy and ^{1}H-NMR spectroscopy. In Fig. 6 a typical example of the application of HPAEC-PAD for composition analysis of CMC is given. As is evident from this chromatogram, a good separation between the different glucose monomers is obtained. The compounds elute in groups according to the number of substituents, indicating that the interaction with the anion-exchange resin is dominated by the carboxymethyl groups. The HPAEC-PAD method has important advantages over the previously reported methods. There is no need for derivatization, whereas each monomer is represented by a single peak in the HPAEC profile [11].

In Fig. 7 a typical example of a HPAEC trace of a hydrolysate of SEC is depicted, showing the same excellent separation as discussed for CMC hydrolysates [12]. It must be noted that due to the type of substituent, SEC hydrolysates can not be analyzed by GLC procedures.

Acknowledgements

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