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Note

Preparative separation of oligosaccharides from κ-carrageenan, sodium hyaluronate, and dextran by Superdex™ 30 prep. grade

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Oligosaccharides have recently drawn considerable interest due to important medical effects and indications of their very specific interaction in certain carbohydrate systems with larger molecules of similar structure [1]. Oligosaccharides, as for example galactan oligosaccharides, are also of interest for their use as model compounds for ^{13}C NMR and Raman spectral-band assignment of native polysaccharides [2–5].

Sulfated polysaccharides, like the carrageenans, play a key role in many biological systems and some of their oligomers are gaining increased medical interest due to their action as anti-infectants [6]. The interest in dextran oligomers resides mainly in their use as calibration substances for gel-filtration chromatography (GFC) columns. Thus, it has become increasingly interesting to be able to produce and isolate oligomeric components from polysaccharide samples.

Some separation work to provide the necessary oligosaccharides has already been published [7–9], but, in all cases, the procedures have been fairly time consuming and the amount of oligosaccharide obtained is small. The aim of this note is to describe an efficient and convenient preparative gel filtration column which allows separation of large amounts of oligosaccharides in a relatively short time. Results will be given concerning κ-carrageenan, hyaluronan, and dextran.

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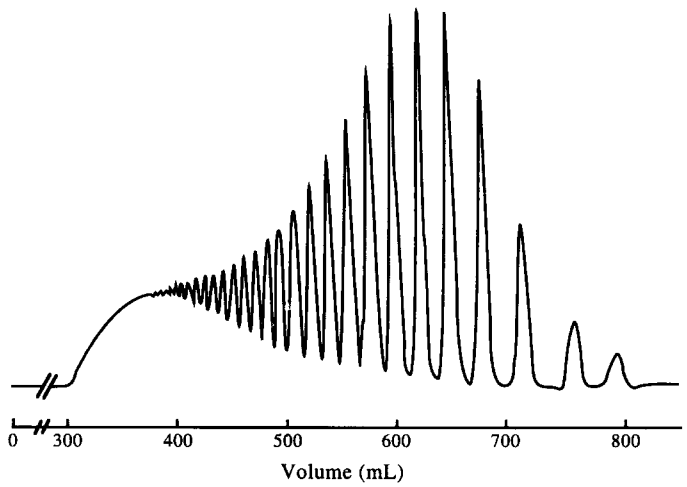

Fig. 1. Elution profile of dextran oligosaccharides.

The preparatory problem is twofold. On the one hand, an originally high molecular weight sample must be degraded under well-controlled conditions to provide an appropriate amount of oligomers. On the other hand, the mixture obtained must be precisely analysed with emphasis on short chain fragments and isolated with high resolution on a preparative scale for specific use in other experiments. Some typical results are presented in Figs 1, 3, and 5. Very good calibration plots are obtained, as seen from Figs 2 and 4.

According to the GFC theory for polyelectrolytes [10,11], an eluent salt concentration of 0.05 M is sufficient to screen the electrostatic repulsions between the solutes. Malfait et al. [8] have investigated the effect of the salt concentration on the separation of charged oligosaccharides using a Sephadex G-50 column. Their results showed that, for a mobile phase concentration higher than 0.05 M, the values of K_{av} were almost constant. In this note, 0.1 M NaCl was chosen as the mobile phase for all experiments.

Fig. 1 shows a chromatogram for dextran oligosaccharides. The last peak corresponds to D-glucose. The preceding peaks contain oligomers with a successively increasing degree of polymerisation (increasing number of glucose monomers). In Fig. 2, all data pairs (K_{av}, log M_w) have been plotted to give a calibration curve; $K_{av} = (V_e - V_o)/(V_c - V_o)$ where V_e, V_o (300 mL) and V_c (1000 mL) are the elution volume of a particular oligomer, the void volume and the geometric bed volume, respectively.

The chromatogram in Fig. 3 shows that the column can separate hyaluronan oligosaccharides containing up to ten disaccharides (HA_{20}). In order to identify each peak, oligosaccharides of defined size were used. These hyaluronan oligosaccharides were prepared according to Smidsrød et al. [12]. If a sample containing a known oligosaccharide is injected alone in the column, there is a shift in the peak position with respect to the mixture which has been explained by Hascall et al. [9]. Therefore, in order to calibrate the column for hyaluronan oligosaccharides, samples injected in the column

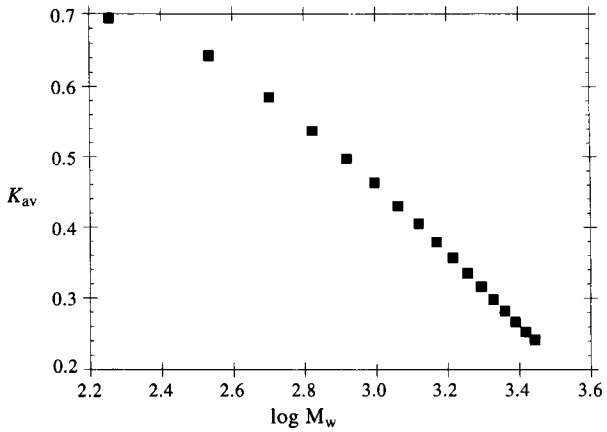

Fig. 2. K_{av} as a function of log M_w for dextran oligosaccharides.

contained known oligosaccharides. The enzymatic degradation of hyaluronan produces a homologous series of oligomers (repeating disaccharide units) with HA_4 as the shortest oligomer [13]. Fig. 3 reveals that there is even a small amount of HA_2. These disaccharides probably derive from chain ends, which is inferred from their smaller amount in comparison with HA_4. Furthermore, in addition to the main peaks in the chromatogram, there are some other peaks which are smaller and well separated from the main peaks in the low molecular weight region. The separation from the main peak becomes less effective in the high molecular region. By using the carbazole method, it was shown that these peaks contained saccharide molecules, very likely oligosaccharides, belonging to chondroitin sulfate which normally is found to some extent in the hyaluronan samples.

K_{av} as a function of log M_w for hyaluronan oligosaccharides is shown in Fig. 4. A comparison of this diagram with the previous one in Fig. 2 shows that, for the same

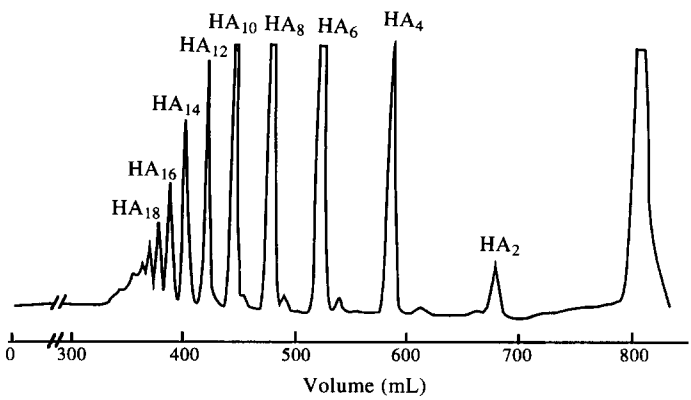

Fig. 3. Elution profile of hyaluronate oligosaccharides.

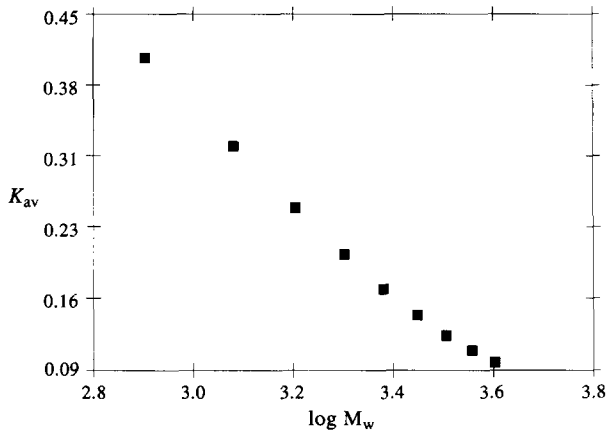

Fig. 4. K_{av} as a function of log M_w for hyaluronate oligosaccharides.

molecular weight, K_{av} is smaller for hyaluronan oligosaccharides than for dextran oligosaccharides. This difference in calibration curves is due to the fact that the charged HA oligosaccharides have a larger hydrodynamic volume as compared to dextran oligosaccharides of equal molecular mass.

By using the calibration curve for the hyaluronan oligosaccharides (Fig. 4), the peaks in the chromatogram belonging to κ-carrageenan oligosaccharides (see Fig. 5) were identified. The κ-carrageenan have a charge density of one charge per disaccharide, which means that each such unit is built up by two galactose parts, one having a sulfate group which is charged and thus has a different molecular weight as compared to the other one. The chromatogram in Fig. 5 shows that, at the low molecular size end, all peaks come at regular intervals, even the last one. No new peaks were detected if samples with increasing degrees of degradation were injected into the column, but the area of the peaks increased for the lower molecular weights. This indicates degradation

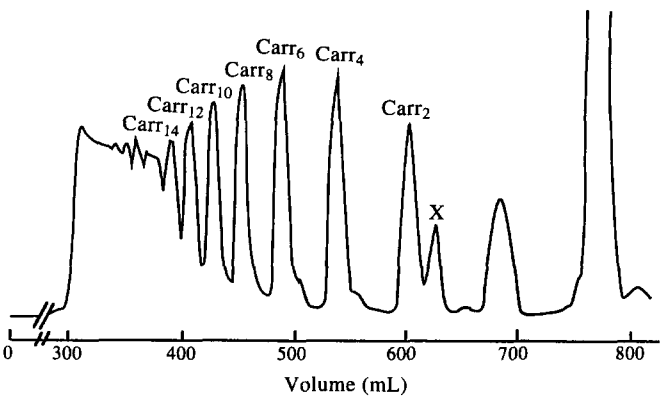

Fig. 5. Elution profile of κ-carrageenan oligosaccharides.

down to dimers. Had, for instance, trimers appeared, they would come in doublets since they would have contained either one or two charged groups, etc. The peak denoted X was not identified and did not give any response to the anthrone reagent. This peak may nevertheless belong to some degradation product. The last peak contains both galactose and salt which was shown by the anthrone method and RI-detection. It was found that the amount of saccharide in this peak was almost the same as in the next to the last peak which corresponds to galactose sulfate. Because of the large hydrodynamic volume of the sulfate groups as compared to the carboxylic groups, κ-carrageenan oligosaccharides up to seven disaccharides ($CARR_{14}$) could be separated, as compared with up to ten disaccharides (HA_{20}) for hyaluronan oligosaccharides.

Fig. 5 reveals that there is some high molecular weight carrageenan substance left after degradation, which appears in the void volume making the separation of higher oligosaccharides difficult. In order to remove the high molecular weight carrageenan, one may extend the degradation process. But at the same time, this would lead to a higher degree of degradation of smaller oligosaccharides. An alternative approach to remove high molecular weight carrageenan, which would further improve the separation of the oligosaccharides, is to use a precolumn containing Sephadex G-50.

It can thus be concluded that the GFC method described is capable of both analytical and preparative separation of dextran, hyaluronan, and κ-carrageenan oligomers with very high resolution. At least the preparative separation can be performed up to twenty monoaccharides with almost baseline separation. Such a separation can easily provide oligomer samples in quantities of 30 mg per oligomer in a single run performed in about 16 h.

1. Experimental

Materials.—κ-Carrageenan (C-1263, Lot no. 19F-0637) and anthrone were purchased from Sigma. Sodium hyaluronate (Healon) and dextran (molecular weights 3.5 kD and 1 kD) samples were gifts from Pharmacia AB, Uppsala, Sweden. Superdex™ 30 prep. grade was supplied by Pharmacia Bioech AB. Superdex™ 30 prep. grade is made up from a composite matrix of cross-linked dextran and agarose with a mean particle size of 34 μm. It has a high chemical stability over a wide pH range, except in the presence of strong oxidising agents. Bovine testicular hyaluronidase (360 IU/mg) was obtained from Sigma. All other chemicals were of analytical reagent grade (E. Merck, Darmstadt, Germany).

Sample preparation.—κ-Carrageenan (8 mg/mL) was dissolved at 50°C in 0.1 M HCl while stirring. The degradation of the polysaccharide was terminated by neutralisation of the solution by NaOH after 7 h. The solution was filtered through a 0.8 μm Millipore filter before use. The concentration of carrageenan-oligosaccharide was measured by a modified anthrone method [14].

Hyaluronate oligosaccharides were prepared by digestion of Healon with bovine testicular hyaluronidase (about 20 IU/mg hyaluronan) in 0.25 M sodium acetate at pH 5.2 for 24 at 37°C. The reaction was terminated by boiling and the sample was centrifuged and filtered through a 0.8 μm Millipore filter. The oligosaccharide content

was measured by the carbazole method [15]. In both cases, the degradation process was investigated carefully with respect to temperature and time to find optimal degradation conditions. The dextran oligosaccharide test sample contained a mixture of equal amounts of dextran 3.5 kD and dextran 1 kD with a concentration of 100 mg/mL.

Gel filtration chromatography (GFC).—Two GFC columns (XK/26, Pharmacia Biotech AB) (1000 × 26 mm ID), where packed according to standard procedure. A Multiref 901 interferometric refractive index (RI) unit from Optilab was used as detector. In the GFC experiments, the mobile phase containing 0.1 M NaCl together with a small amount of NaN_3 was pumped by a P-3500 Pharmacia pump, with a flow rate of 0.60 mL/min. The volume of samples injected were approximately 16 mL (~ 120 mg). In order to inject a higher amount of oligosaccharide, samples needed to be more concentrated. A fraction collector (Frac-200, Pharmacia Biotech AB) was used to collect the fractions. The prefraction was 270 mL and the fraction size was set to 7.2 mL. The experiments were performed at ambient temperature (~ 20°C) which is recommended. The chromatographic performance of the columns was tested by injecting 1.00 mL of an acetone solution (20 mg/mL) into the gel bed. The acetone sample was eluted at 30 cm/h linear velocity (2.5 mL/min) with a UV detector set at 280 nm. The number of theoretical plates per meter was found to be 25000/m for both columns used in this experiment and the peak symmetry was very close to unity.

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