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Note

A rapid method for the separation and analysis of carrageenan oligosaccharides released by *iota*- and *kappa*-carrageenase

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Abstract

Based on the improved performances in speed of chromatographic separation on Superdex-type materials (Pharmacia) compared to conventional media such as Sephadex and Bio Gel-type, a rapid size-exclusion chromatography (SEC) method was developed for the separation and analysis of carrageenan oligosaccharides. It was used to evaluate the elution profiles of hydrolysates produced by carrageenases specific for *kappa*- and *iota*-carrageenans. Oligosaccharide peaks ranging from di- to dodeca-saccharides were obtained in about 20 min on an analytical scale, whereas preparative runs were completed in a few hours. The method may also be used to monitor polysaccharide degradation. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Carrageenan; *Iota*-carrageenase; *Kappa*-carrageenase; Sulphated neocarrabiose oligosaccharides; Size-exclusion chromatography; NMR

Carrageenans are water-soluble galactans extracted from some red seaweeds. They have a repeating backbone of 3-linked β-D-galactose (G-units) and 4-linked α-D-galactose (D-units), containing a varying degree of sulphate. In the following text a short hand notation system based on a letter code system has been applied.[1] Carrageenans with the ability to form a water gel contain 4-linked units of 3,6-anhydro galactose (A) in place of the D-units, and may occur with the idealised repeating structures A-G4S (*kappa*-) or A2S-G4S (*iota*-), but molecular hybrids and other sulphate substitutions such as the so called precursors D6S-G4S (*mu*-) and D2,6S-G4S (*nu*-), may be found depending on the biological source.[1]

NMR- and IR-spectroscopic analyses of galactans give information about their main structural characteristics.[2] Depolymerisation and a subsequent chromatographic separation are necessary to detect residues and sequences occurring in low proportions. In an unspecific acidic degradation, and a following derivatisation, constituent sugars and even linkages and sulphate substitution patterns may be determined by GLC or GLC–MS methods.[3] Meth-

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ods based on reductive hydrolysis have been developed to determine complete sugar composition, including units of 3,6-anhydrogalactose.[4,5] Further developments have allowed the substitution pattern of variously sulphated di- and tetrasaccharidic fragments of the carrabiose type to be determined.[6] In order to identify minor structures in the samples, the use of specific carragenolytic enzymes isolated form micro-organisms, i.e., carrageenases, are very useful.[7] Quite recently the genes encoding these enzymes have been cloned, and the recombinant forms of His-tagged carrageenases expressed in *Escherichia coli* and purified in large quantities and stable forms.[8,9] By degrading carrageenans with structure-specific carrageenase, a series of sulphated oligosaccharides with a neocarrabiose repeating backbone are formed (OLIG). The action pattern of the so-called *lambda*-carrageenase is less well defined. However, since carrageenan molecules in general are hybrid molecules, some parts of the molecular chains are resistant to hydrolysis, and large fragments (ERF) may be present in the crude hydrolysates. Furthermore, even oligosaccharides with irregular sequences, such as A–G4S–A2S–G4S–A-G4S (commercially available from *Chondrus crispus*, gametophytes) or A–G–A–G4S (from *Furcellaria lumbricalis*) can be formed depending on the substrate, since for at least some *kappa*-ase preparations the enzymic site is restricted to only a certain short length.[10] The full specificity and the lack of complete degradation, which may be caused by product inhibition or transglycolsylation, need to be studied further.

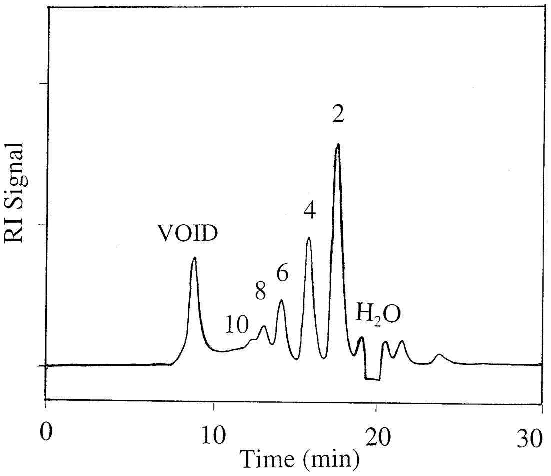

Fig. 1. Analytical scale SEC separation (Superdex Peptide HR 10/30 in 0.1 M $NaNO_3$) of a *kappa*-carrageenase hydrolysate obtained from KA-carrageenan. Void refers to the polymer fraction whereas numbers refer to the degree of polymerisation of sulphated neocarrabiose type oligosaccharides. The negative RI-signal response at ca 20 min is a result of the sample having been applied to the column as a solution in pure water.

When analysing enzymic hydrolysates, most attention has been on the OLIG fraction, especially since a full chromatographic separation based on size-exclusion (SEC) into distinct pure fragments, if present, is simple. The pioneer works were performed by the use of Sephadex G25,[11] as well as with Bio Gel P6[12] or Bio Gel P4.[10] Although these matrices allow sufficient separation, the time consumption is considerable; a typical column configuration consisting of two serial connected columns (900 mm × 26 mm i.d.) and a flow rate at about 25 mL/h typically results in 1–2 days run time. Superdex 30 (Pharmacia) has been tested on acidic hydrolysates of *kappa*-carrageenan, presumably carrabiose oligosaccharides, by applying two serially connected columns (1000 mm × 26 mm i.d.), operated at a flow rate 0.60 mL/min.[13] Time consumption was about 18 h for a full fractionation, and only a slight improvement in speed compared to the Bio Gel and Sephadex based systems[10–12] was obtained. In addition, reverse-phase HPLC (C18-column) has been used on an analytical scale.[14]

In our present work both Superdex 30 and prepacked columns with a similar content (Superdex Peptide-HR) were tested both for analytical and preparative applications. We found a huge improvement in speed compared to the old materials and systems. In this work, some examples are given for neocarrabiose type oligosaccharides, and some results describing the time course and the different hydrolytic abilities of a *kappa*-carrageenase and an *iota*-carrageenase preparation are given.

The separation of $(A–G4S)_n$ $n = 2- > 5$ (4-sulphated neocarrabiose type oligosaccharides of *kappa*-carrageenan enzymic hydrolysate) on a Superdex Peptide HR column was achieved in 20 min (Fig. 1). Comparing their retention times with those of commercial A–G4S-type standards identified the fractions. Identifications were also done by ^{1}H NMR[15]

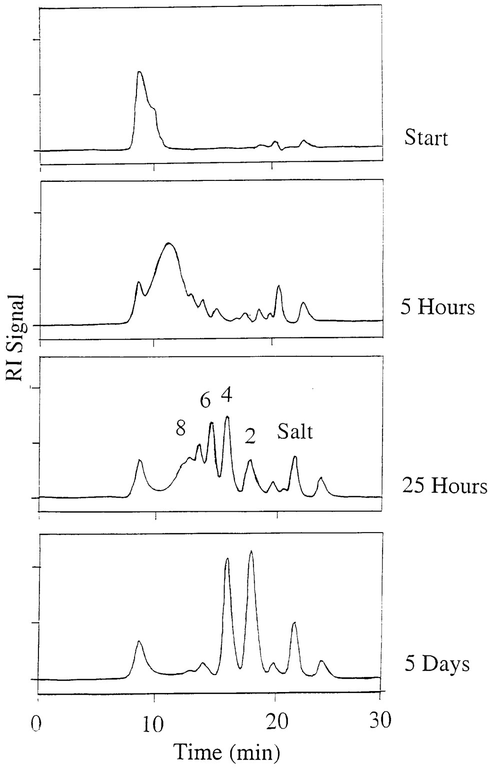

Fig. 2. SEC profiles of the oligosaccharide distribution at different times during enzymic degradation of a commercial KA-carrageenan.

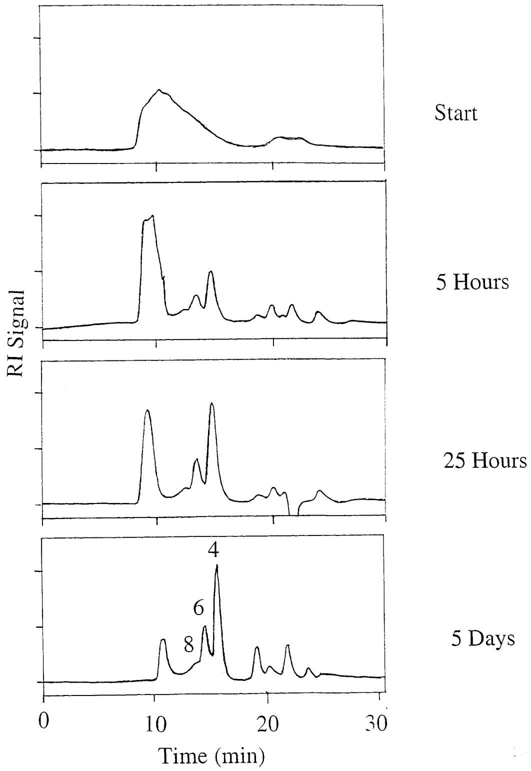

Fig. 3. SEC profiles of the oligosaccharide distribution at different times during enzymic degradation of a commercial ES-carrageenan.

after pooling several identical fractions and freeze-drying. The disaccharide is well separated from salt and buffer components originating from the solution for the enzymic degradation. The system is well suited to directly follow the liberation of oligosaccharides during enzymic hydrolysis (Fig. 2). It can be seen that initially the A–G4S-type carrageenan (*kappa*) is degraded into relative large oligosaccharides by the action of the *kappa*-carrageenase. It is interesting to note that a quite defined enzyme resistant, high-molecular weight fraction is formed at an early stage. At a later stage, short oligosaccharides (Dp 4–8) are dominating whereas the end products are mostly di- and tetrasaccharides. The system might be useful for the study of product inhibition as well as simple kinetics (Fig. 2). For the hydrolysate of A2S–G4S-type (*iota*) carrageenan, the smallest fragment formed upon treatment with *iota*-carrageenase was a tetrasaccharide (see Fig. 3).

In the semi-preparative configuration oligosaccharides at a mg scale were obtained in one single run taking about 1 h. In Fig. 4 a typical enzymic degradation mixture from A–G4S-type carrageenan is shown, including an enzyme resistant fraction. Finally in the preparative run 50–250 mg samples of carrageenan hydrolysates were applied depending on the viscosity of the sample and fractions containing as much as 100 mg were collected (Figs. 5 and 6). As reported previously for the native enzyme from the bacterial strain 1,[16] recombinant *iota*-carrageenase did not produce any disaccharidic (i.e., A2S–G4S) fragments (Fig. 6). The smallest fragment was identified as the tetrasaccharide A2S–G4S–A2S–G4S, by ^{1}H NMR (Fig. 7).

The system is also well suited to separate oligosaccharides originating from alginic acid. In addition, by using the semipreparative column (XK-16/70), baseline separation was obtained for di- and trisaccharides of N-acetyl glucosamine which elute at 95 and 85 mL in pure water, respectively (data not shown). The practical fractionation range for carrageenan oligosaccharides, $(\text{A–G4S})_n$ given in M_r, is about 400 (disaccharide) to 3200 (hexadecasaccharide). This is substantially different from the range given by the producer based on peptides, i.e., 75–12500. This is in accordance with the earlier findings that 'cut off' values for dialysis membranes and chromatographic systems must be critically evaluated when dealing with negatively charged oligosaccharides as compared to peptides.[17]

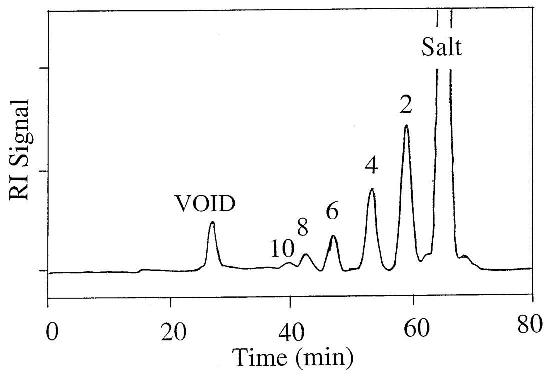

Fig. 4. Fast, semi-preparative SEC (Superdex 30 in 0.1 M NaCl) of a hydrolysate obtained after prolonged enzymic degradation of KA-carrageenan.

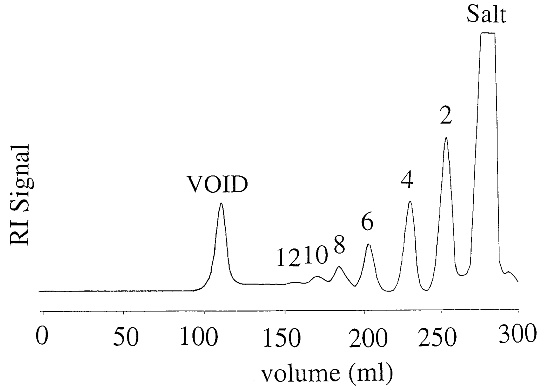

Fig. 5. Fast, preparative scale SEC (Superdex 30 in 0.1 M NaCl) of 48SOL fraction of a KA-hydrolysate. Samples were solubilised in the eluent.

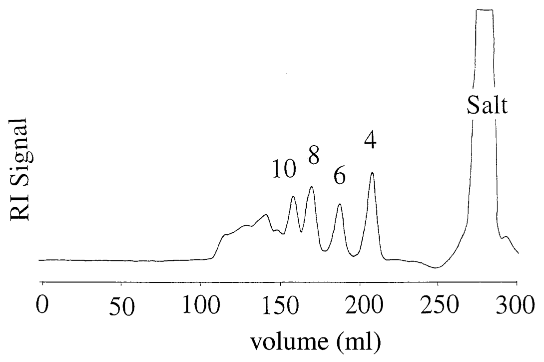

Fig. 6. Fast, preparative scale SEC (Superdex 30 in 0.1 M NaCl) of 48SOL fraction of a ES-hydrolysate. Samples were solubilised in the eluent.

To summarise, the chromatographic materials show good mechanical stability and have been used for at least 100 repeated runs with no difficulties. No efforts were made at this stage to further optimise the systems demonstrated. Recent work has been done to demonstrate the direct analysis of the *kappa*-carrageenan oligosaccharide fractions by electrospray-ionisation mass spectrometry (ESI-MS), without the need of further desalting or derivatisation.[20]

1. Experimental

Carrageenan substrates.—Carrageenans were extracted at neutral pH from *Kappaphycus alvarezii* (KA) which had not been subjected to treatment with hot alkali. Commercial *iota*-carrageenan, from alkali treated *Eucheuma spinosum* (EP), were obtained from Copenhagen Pectin Factory (Genuvisco X-S908).

Carrageenase.—Purified *kappa*-carrageenase was obtained from large-scale fermentation of *kappa*-carrageenan with *Pseudomonas carrageenovora*.[18] *Iota*-carrageenase was obtained from a culture of the recombinant *E. coli* BL21(DE3) harbouring pLysS plasmid and transformed with a pET20b vector containing the coding region of the mature *iota*-carrageenase as described previously.[8] Cells were submitted to osmotic shock in order to release the periplasmic proteins in 0.1 M Tris–HCl buffer (pH 7.2) containing 0.5 M sucrose and 1 mM EDTA.[8]

Enzymic degradations.—Enzymic digestions were performed at 30 °C on 0.5% (w/v) solutions of carrageenans in 100 mM NaCl and 5 mM $NaHCO_3$ in 0.5% (w/v) NaN_3.

Fractionation based on EtOH solubility.—For the samples used for the preparative run, a crude fractionation of the hydrolysates was obtained by adding an equal volume of EtOH into the hydrolysates while stirring. The soluble fraction was termed 48SOL, whereas the insoluble fractions were not further analysed in this work.

Column dimensions, chromatographic support and eluents.—Analytical runs were performed on a commercially available

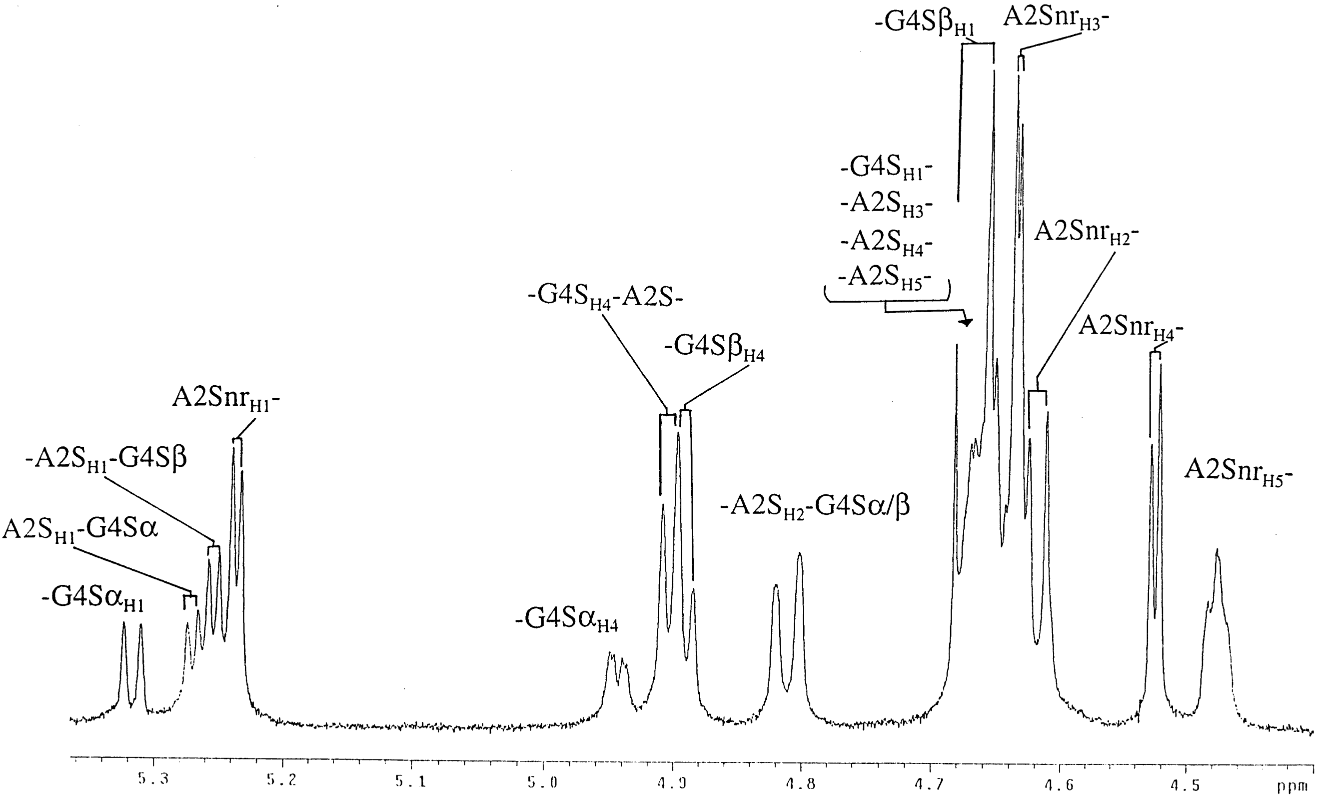

Fig. 7. Region of the 300 MHz ^{1}H NMR spectrum of a tetrasaccharide of the *iota*-type. A2S denotes 4-linked 3,6 anhydrogalactose 2-sulphate and G4S 3-linked D-galactose 4-sulphate, respectively. A hyphen denotes remaining neighbour residues and α and β denote the effect of the anomeric configuration at the reducing sugar residue on a specific proton on this residue or its neighbour.

Pharmacia Superdex Peptide HR 10/30 (Gel height 300 × 10 mm i.d.) by eluting with 0.1 M $NaNO_3$. Samples (250 μL) were injected by a loop and run at 1 mL/min with approximately 10 kg/mm^2 reading of backpressure. Semipreparative runs performed on Pharmacia XK-16/70 (Superdex 30 Prep Grade, Gel height 620 × 16 mm i.d.) and preparative GPC was performed on Pharmacia XK-26/70 (Superdex 30 Prep Grade, Gel height 620 × 26 mm i.d.) eluted with 0.1 M NaCl at a flow rate 1.7 ml/min. Both types were at ambient temperature, approximately 22–26 °C.

Pumps and detectors.—Analytical and semipreparative run were performed on a Shimadzu HPLC with a Shodex RI SE-61 RI-detector with an 8 μL flow cell. The software LC10AT handled flow control and data acquisition. For preparative runs a Pharmacia High load P50 was used in combination with a Shimadzu RID6A RI-detector with a 15-μL flow cell and a Pharmacia LKB Superfrac, operating in drop count mode.

Desalting.—The salt content originating from the eluent was reduced by solubilising freeze dried fractions and applying to a Pharmacia HiPrep 26/10 (Gel height 100 × 26 mm i.d.), eluted with water, 0.1 M NaCl or 0.1 M $(NH_4)_2CO_3$ depending of oligosaccharide length and structure.

Analysis of fragments.—Fragments were analysed (10 mg/mL D_2O) by ^{1}H NMR on a Varian Mercury 300 system, operated at 80 °C and typically 32 repetitions. Spectra were recorded by using a 2-s delay, applying a 45° pulse, an acquisition time of 4 s and 1000 Hz sweep width. Chemical shift for R-A2S-G4S$_{H\text{-}1}$α was set to 5.32 ppm.[15] Assignments were done by comparison with resonances and coupling constants for A–G4S-type oligosaccharides and data published for polymeric A2S–G4S-type carrageenan.[19]

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