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Preparative and analytical separation of oligosaccharides from κ-carrageenan

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SUMMARY

The separation of the enzymic degradation products of κ-carrageenan, as studied by size-exclusion chromatography on Bio-Gel P-6 and reversed-phase high-performance liquid chromatography on μBondapak C_{18}, resulted in a homologous series of oligomers in addition to non-homologues substances. On the former column steric exclusion competed with ion exclusion originating from solute–solute and solute–gel interactions. Competition was reduced with increasing ionic strength of the eluent. Separation on μBondapak C_{18} was influenced by the type of salt, indicating that the reversed-phase separation was not solely based on solute–column interactions.

INTRODUCTION

During the last few years, galactan oligosaccharides have gained considerable interest as they are used as model compounds for ^{13}C NMR and Raman spectral band assignment of native polysaccharides[1–3]. Since the tetramer and longer chain oligomers undergo a conformational transition as a function of temperature[4] and in the presence of certain counter ions[5], they can be used to provide evidence for structural changes[6] occurring in the polymer solution. In contrast to the polymer, the oligomers have a well characterized structure. Initially in the hydrolysate of κ-carrageenan a homologous series of four oligomers was detected using thin-layer chromatography (TLC) on cellulose[7]. These sulphated oligosaccharides were isolated by dry-column cellulose chromatography. Size-exclusion chromatography (SEC) on Sephadex G-25 resulted in the separation of a disaccharide and a tetrasaccharide[8]. However, on analysis with Bio-Gel P-6 the presence of up to ten oligomers in the hydrolysate was reported and the separation was studied as a function of the ionic strength of the eluent[9]. In this paper, the preparative separation parameters involved in the separation of the oligomers from the hydrolysate and the use of reversed-phase high-performance liquid chromatography (HPLC) as a fast and suitable adequate technique for the analysis of this type of sample are considered.

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EXPERIMENTAL

Materials

Standard oligomer samples for HPLC were purchased from Grampian Enzymes (Aberdeen, U.K.). The hydrolysate was a kind gift from C. Rochas (Grenoble, France). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

SEC

SEC was performed with two Pharmacia (Uppsala, Sweden) columns. One (850 × 26 mm I.D.) was filled with a Bio-Gel P-6 (Bio-Rad Labs., Richmond, CA, U.S.A.) packing, particle size 200–400 mesh and the other (700 × 15 mm I.D.) with Sephadex G-50 Super Fine (Pharmacia) gel. Detection was performed with an R410 differential refractive index (RI) detector from Waters Assoc. (Milford, MA, U.S.A.). The fraction collector and pump were also from Pharmacia. The flow-rate of the Bio-Gel P-6 column was maintained at 0.45 ml min^{-1} and 5-ml samples were injected. Samples of 0.5 ml were injected onto the Sephadex G-50 column and the flow-rate was 0.15 ml min^{-1}.

HPLC

The HPLC system from Waters Assoc. was equipped with an R410 detector. All data were treated with a 740 data module. Two Model 510 HPLC pumps were connected in series and a flow-rate of 0.5 ml min^{-1} was usually used. All operations were made with the isocratic operating mode of an automatic gradient controller. Injections were carried out with a U6K injector.

All hydrolysate samples were dissolved in 1 ml of the eluent, centrifuged and filtered (Millipore, 0.45 μm) before injection (10 μl) onto a μBondapak C_{18} column coupled with a C_{18} guard column (Waters Assoc.). The system was run at ambient temperature. The eluent was prepared with ultrapure water (Milli-Q), filtered (0.22 μm) and degassed with a Millipore vacuum filtration apparatus.

TLC

The samples were applied to Kieselgel 60 Fertigplatten (200 × 100 mm) (Merck) with a calibrated glass capillary and were placed in a closed glass tank. Butanol–acetic acid–water (2:1:1) was used as the solvent system and detection was performed with naphthoresorcinol–sulphuric acid reagent[7].

RESULTS AND DISCUSSION

Preparative separation

κ-Carrageenan is an alternating copolymer consisting of mainly → 4)-3,6-anhydro-α-D-galactopyranosyl-(1 → 3)-β-D-galactopyranosyl 4-sulphate-(1 →. The repeating unit is called neocarrabiose 4-sulphate (Fig. 1) and the oligomers will be denoted by their degree of polymerization (DP), characterized by *n*, the number of repeating units. Previously, it was shown that the enzyme κ-carrageenase hydrolyses κ-carrageenan in a random-*endo* manner[10]. It has been reported that a homologous series of κ-carrageenan oligomers can be separated from the hydrolysate on a Bio-Gel P-6 column using sodium nitrate solution as eluent[9].

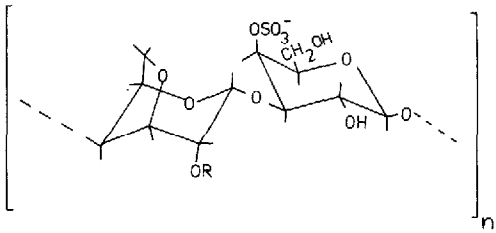

Fig. 1. Idealized repeating structure of κ-carrageenan (R = H).

In Fig. 2 the logarithm of the distribution coefficients (K) for the separation of different oligomers on Bio-Gel P-6 with $5 \cdot 10^{-2}$ and $1 \cdot 10^{-1}$ M sodium nitrate solution at 20 and 35°C are plotted as a function of n. K is calculated using the expression

$$K = \frac{V - V_0}{V_T - V_0} \tag{1}$$

where V, V_0 and V_T are the elution volume of a particular oligomer, the high-molecular-weight enzyme-resistant fraction and the salt, respectively. Because in SEC the

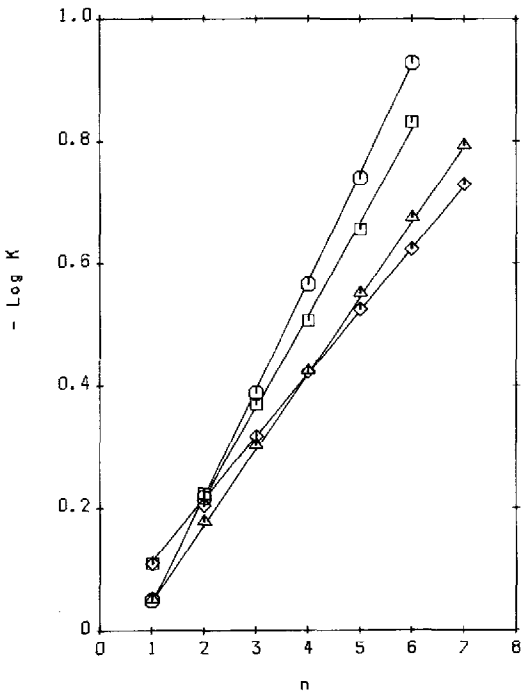

Fig. 2. Negative logarithm of the distribution coefficient (K) of κ-carrageenan oligomers on Bio-Gel P-6 as a function of the degree of polymerization in 10^{-1} M salt solution at (△) 20°C and (◇) 35°C and in $5 \cdot 10^{-2}$ M salt solution at (○) 20°C and (□) 35°C.

separation depends on the hydrodynamic volume of the solutes[11], the straight lines observed in Fig. 2 indicated the presence of homologous substances. In contrast to the literature[9], the curves in Fig. 2 did not overlap at either 20 or 35°C. According to the SEC theory of polyelectrolytes[12,13], an eluent salt concentration of $5 \cdot 10^{-2}$ M is sufficient to screen the electrostatic repulsions between the solutes.

Also for charged oligomers a higher salt content in the eluent does not result in large changes in K. This can be illustrated for the separation of the κ-carrageenan oligomers from the hydrolysate on a Sephadex G-50 column (Fig. 3). At both temperatures the influence of the salt content in the eluent was small on Sephadex compared with Bio-Gel. Therefore, on the latter a more complex separation mechanism seems to occur. The large influence of temperature on K further suggests that the separation on Bio-Gel P-6 is not based merely on the hydrodynamic volume of the solutes. Theoretically, K should be independent of temperature. As the temperature and the eluent salt concentration had different effects on the two SEC packings used, it was concluded that solute–gel interactions were involved in the separation mechanism on Bio-Gel P-6. At 20°C the oligomers eluted faster on Bio-Gel with $5 \cdot 10^{-2}$ M than with 10^{-1} M sodium nitrate solution (Fig. 2), which indicated ion exlusion[14] due to electrostatic repulsions between the charged oligosaccharides and the amide groups of the gel. Addition of more salt suppressed this effect. As a result, the solutes are retained much longer in the gel pores. The salt in the eluent is therefore necessary in order to avoid competition between the steric exclusion mechanism and

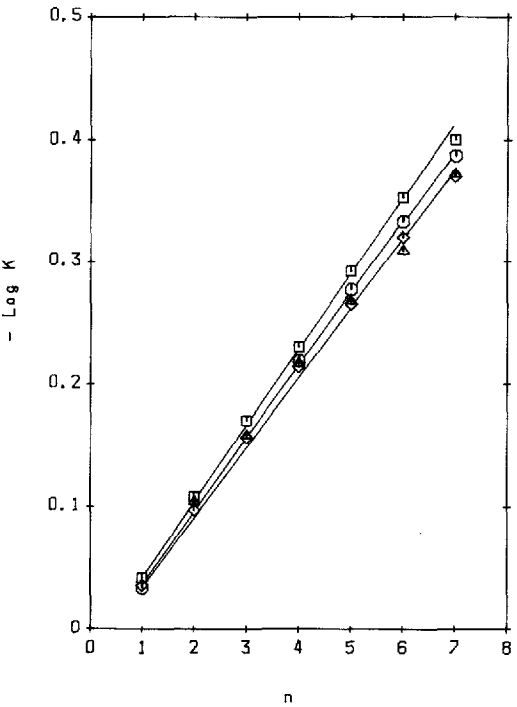

Fig. 3. Negative logarithm of the distribution coefficient (K) of κ-carrageenan oligomers on Sephadex G-50 Super Fine. Symbols as in Fig. 2.

ion exclusion caused by electrostatic repulsions between the solutes and between the solutes and the gel. To overcome both interactions, higher salt concentrations were needed than in the absence of solute–column effects[12,13].

The specific interactions of cabohydrates with polyacrylamide gels are well known and have been reviewed[15]. In agreement with Figs. 2 and 3, the influence of the ionic strength of the eluent on the K values of oligosaccharides separated on Bio-Gel is known to be larger than that on Sephadex[16]. The effect of temperature on the elution volumes of carbohydrates analysed on Bio-Gel has also been shown[17]. It depended on the carbohydrate structure of the solutes[18].

Although K is of considerable importance in the study of the SEC mechanism on a particular column, in preparative SEC mainly the resolution (R) is of interest. The high sample concentrations used in SEC affect the resolution and can give rise to band broadening or peak tailing[19]. On Bio-Gel P-6 increasing concentrations up to 15 mg ml^{-1} resulted only in increased peak heights without a distinct loss of resolution for all oligomers. High sample concentrations for preparative SEC also affect the hydrodynamic size of the solutes and thus K, which may change owing to overloading. The value of K for DP 1 remained unchanged within 1% for concentrations up to 15 mg ml^{-1}. However, with increasing molecular weight the concentration dependence of K increases and the reproducibility of the determined elution volume decreases[19]. Repeated measurements revealed, *e.g.*, that the uncertainty of the determined K for DP 7 is 7% for sample concentrations of 15 mg ml^{-1}, compared with only 1% for DP 1.

Fig. 4 shows the influence of the salt concentration in the eluent and the temperature on the resolution (R) for Bio-Gel P-6. The values of R are plotted against the average molecular weight of two neighbouring compounds (M) in the chromatogram and are calculated according to

$$R = \frac{2(V_2 - V_1)}{W_2 + W_1} \tag{2}$$

where V and W are the elution volume and the peak width at the base, respectively. Better resolution is obtained with 10^{-1} M than $5 \cdot 10^{-2}$ M sodium nitrate solution, especially for the high-molecular-weight oligomers. With 10^{-1} M salt solution oligomers even up to DP 9 were detected. However, with $5 \cdot 10^{-2}$ M salt solution DP 8 and 9 oligomers were never resolved. Therefore, the preparative separation of these oligomers on this column has to be performed with at least 10^{-1} M salt solutions. In a previous study[5] we used ammonium hydrogencarbonate as an electrolyte as it can be partially removed from the oligomer samples during freeze-drying, with ammonia, carbon dioxide and water as decomposition products. The oligomers up to DP 4 are easily separated with both 10^{-1} and $5 \cdot 10^{-2}$ M salt solutions (Fig. 4). Elution with the latter eluent offered the advantage that less salt has to be removed during the very time-consuming desalting step, particularly with these small molecules.

The large influence of temperature on R (Fig. 4) in both eluents is probably a consequence of the non-ideal SEC behaviour. Increasing temperature usually enhances the sample solubility and reduces the solvent viscosity. Both improve the resolution. However, this was not so here. The resolution at 20°C is always higher than at 35°C, for which we have no explanation. Only for the large oligomers in $5 \cdot 10^{-2}$ and 10^{-1} M salt solution did R reach the same values at both temperatures.

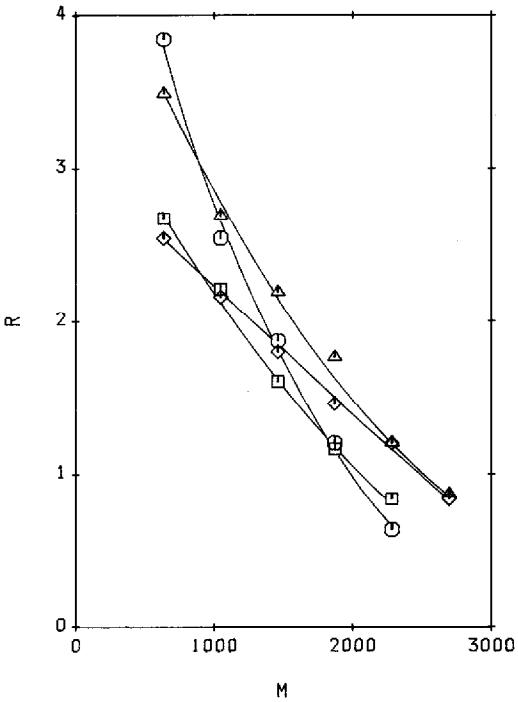

Fig. 4. Resolution (R) of κ-carrageenan oligomers on Bio-Gel P-6 as a function of the average molecular weight of two neighbouring compounds on the chromatogram. Symbols as in Fig. 2.

It is well known that temperature can be used to prevent adsorption of solutes. Small molecules are easily adsorbed on the column, because through permeation they are exposed to a much higher surface area in the packing[20]. In preparative SEC, loss of product due to adsorption should be prevented. However, on Bio-Gel P-6 at 20°C with 10^{-1} M salt solution a peak was recorded after the elution of the salt ($K = 1.14$). Adsorption depended on the ionic strength and disappeared at 35°C.

In order to establish which oligomers were lost at 20°C, the relative peak areas with 10^{-1} M salt solution were compared at both temperatures. Table I shows that no significant difference existed. Therefore, adsorption was probably due to a low-molecular-weight contaminant which at 35°C was eluted with the salt. By ageing and in the presence of traces of acid the oligomers may degrade and the hydrolysis of the 3,6-anhydrogalactose residue results in the formation of galactose and 5-(hydroxymethyl)-2-furaldehyde[9]. The latter compound was responsible for a sometimes slight yellow colour[21] of concentrated oligomer solutions and was adsorbed on Bio-Gel P-2 packing[9]. With $5 \cdot 10^{-2}$ M salt solution at 20°C, owing to the excellent separation of oligomers of DP 1 and 2 (Fig. 4), two other contaminating compounds became apparent at $K = 0.661$ and 0.791. Moreover, detailed examination of the chromatogram revealed the existence of another contaminant between the DP 3 and 4 peaks ($K = 0.330$). These compounds, not previously reported, occur in only minor amounts ($< 0.5\%$ of the total oligomer fraction) and were not isolated. These non-homologous

TABLE I

INFLUENCE OF THE BIO-GEL P-6 COLUMN TEMPERATURE ON THE RELATIVE FRACTION OF ELUTED κ-CARRAGEENAN OLIGOMERS

Eluent salt concentration = 10^{-1} *M*. *n* = Number of repeating units. ± Values are standard deviations calculated from three replicates.

n	*20°C*	*35°C*
1	15.0 ± 0.3	15.7 ± 0.5
2	29.5 ± 0.5	29.8 ± 0.4
3	22.3 ± 0.2	22.3 ± 0.2
4	15.3 ± 0.2	14.4 ± 0.3
5	8.7 ± 0.6	8.3 ± 0.2
6	4.4 ± 0.1	4.2 ± 0.2
>6	2.4 ± 0.1	2.6 ± 0.1
ERF[a]	2.4 ± 0.1	2.6 ± 0.1

[a] ERF = Enzyme-resistant fraction.

substances probably originate from "kinking residues" in the substrate[22] which are insensitive to enzymic hydrolysis.

Analytical separation

Although the analysis of the mixture could be easily performed on the preparative Bio-Gel P-6 column, analytical studies of processes such as κ-carrageenan digestion would be very time consuming as optimum results required 16–18 h. Therefore, reversed-phase HPLC has been suggested for these types of sugars[23]. All separations were effected within 30 min and oligomers up to DP 13 were observed. As in reversed-phase chromatography the separation depends on the hydrophobic interactions between the sample solute and the column, salt was again added to the eluent to screen the electrostatic repulsions between the oligomers. Figs. 5 and 6 demonstrate that the separation improved with increasing sodium nitrate concentra-

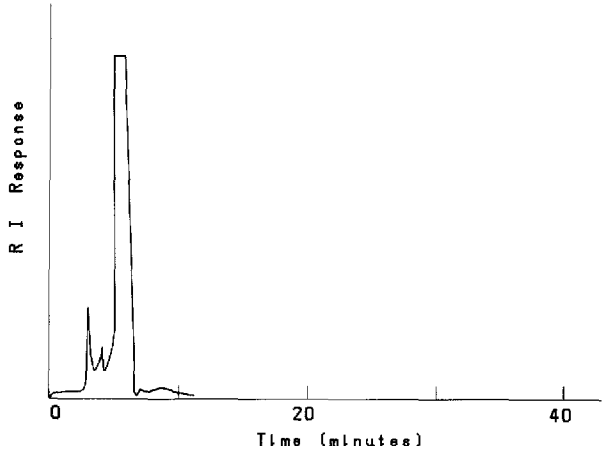

Fig. 5. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers in water.

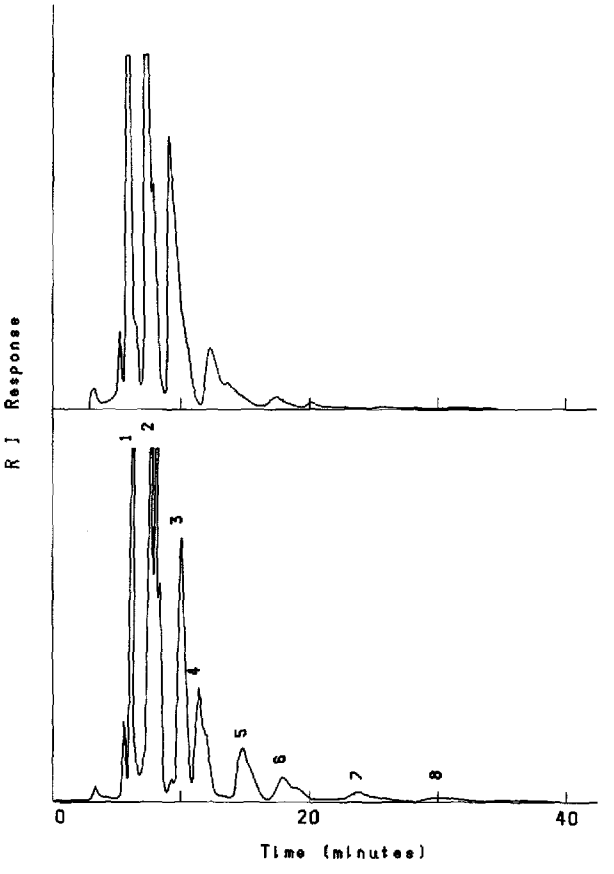

Fig. 6. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers with (top) $5 \cdot 10^{-2}$ *M* and (bottom) $5 \cdot 10^{-1}$ *M* sodium nitrate solution.

tion, which for an optimum separation was distinctly higher than the 10^{-1} *M* used in SEC.

Moreover, the HPLC and SEC traces showed different patterns. In the former instance more peaks were observed and some were broadened owing to the appearance of small shoulders. The peaks in Fig. 6 were assigned using DP 1 and DP 2 standard samples. According to the theory of the retention behaviour of oligomeric series in reversed-phase chromatography[24], the capacity factors of the standards in $5 \cdot 10^{-1}$ *M* sodium nitrate solution were plotted against *n* following the equation

$$\log k = (\log \alpha)n + \log \beta \tag{3}$$

where $\log \alpha$ is the selectivity and β the capacity factor for the hypothetical oligomer with $n = 0$. The capacity factor is defined as

$$k = \frac{V}{V_M} - 1 \tag{4}$$

where V is the retention volume of a particular oligomer and V_M the column dead volume. Based on eqn. 3, the k values of the larger oligomers (n = 3–8) were calculated and compared with the experimental data. The oligomers separated on the HPLC column were also collected and analysed by TLC using the standards as references. Chromatographic movements were observed up to n = 5 and the migration fitted a linear relationship:

$$\log\left(\frac{1}{R_F} - 1\right) = f(n) \tag{5}$$

which indicates the presence of a homologous series.

In contrast with the previously reported HPLC result[23], oligomers of DP 1 and DP 2 were well separated. In addition, we observed a splitting of the DP 2 peak which was due to anomerization. Separation of carbohydrate anomers on HPLC columns is well known[25]. However, in our work it is not yet clear why the anomerization was mostly pronounced for DP 2. When injecting the DP 1 and DP 2 standards only the latter chromatogram showed two peaks, nearly baseline separated with 1 M salt solution. For both the hydrolysate and the pure DP 2 sample the relative peak areas for the first and second peaks were 63.9 ± 0.5% and 36.1 ± 0.5%, in agreement with the β/α anomerization ratio obtained by ^{13}C NMR spectroscopy[4].

Fig. 5 shows that two peaks were eluted before the oligomer of DP 1. The first was due to the presence of salt and corresponded with V_M. The second had a relative peak area of 2.5%, which agreed with the value for the enzyme-resistant fraction eluting at the void volume on Bio-Gel P-6 (Table I). This value was distinctly lower than that of 10.8% reported recently[26]. As it was shown that the enzyme-resistant fraction consisted mainly of irregular galactan polysaccharides, contaminating the carrageenan sample[26], the amount of this fraction depends on the purity of the carrageenan extracted from seaweed.

In all HPLC traces it was observed that the peak width increased with increase in n. Especially for $n > 5$ the peaks became extremely broad, which has also been reported for the separation of other oligomers on this column[27]. This can be explained by the assumption that the larger oligomers gave rise to a decreased diffusion rate through the column packing, which resulted in peak broadening. Also the conformation could have had an effect. We showed that an increased chain length results in a larger conformational freedom. In water the conformation of the oligomer of DP 4 is different from that of DP 1 and the presence of high salt concentrations can induce further conformational changes[5]. The appearance of shoulders on the broad peaks again indicated the presence of the anomers.

The influence of the ionic content of the eluent on the elution of the oligomers on a C_{18} packing was studied with a theory proposed for chromatography with binary mobile phases composed of water and an organic solvent. However, the expressions, such as eqn. 3, remain valid in the extreme case where the concentration of the organic solvent in the mobile phase is zero. In Table II the selectivity of the κ-carrageenan oligomers (log α) and the intercept (log β) are given for two sodium chloride and sodium nitrate eluent concentrations. When log α is positive, the theory predicts that a relatively non-polar repeat structural unit determines the retention. Therefore, it

TABLE II

EXPERIMENTAL VALUES FOR THE SLOPE (LOG α) AND THE INTERCEPT (LOG β) ACCORDING TO THE LINEAR EXPRESSION BETWEEN THE LOGARITHM OF THE CAPACITY FACTOR AND THE DEGREE OF POLYMERIZATION FOR κ-CARRAGEENAN OLIGOMERS WITH VARIOUS SODIUM NITRATE AND SODIUM CHLORIDE ELUENT MOLARITIES

Column: μBondapak C_{18}.

Salt	*Concentration (M)*	*Log α*	*Log β*
$NaNO_3$	$5 \cdot 10^{-2}$	0.138	−0.159
	$5 \cdot 10^{-1}$	0.139	−0.170
NaCl	$5 \cdot 10^{-2}$	0.136	−0.167
	$5 \cdot 10^{-1}$	0.138	−0.112

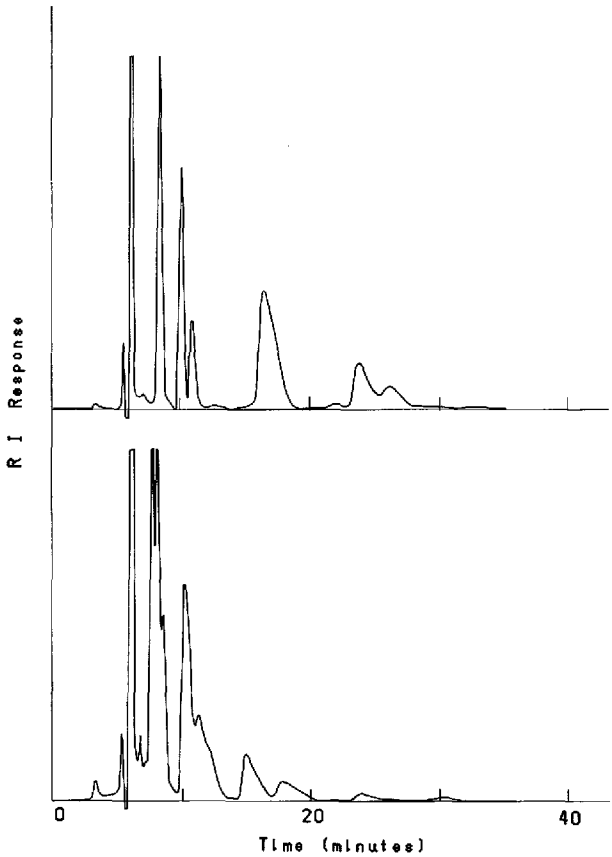

Fig. 7. HPLC on μBondapak C_{18} of the κ-carrageenan oligomers in (top) $5 \cdot 10^{-1}$ *M* and (bottom) $5 \cdot 10^{-2}$ *M* sodium chloride solution.

seems reasonable to assume that the separation is based on the hydrophobic interactions between the C_{18} stationary phase and the repeating anhydrogalacto-pyranosyl unit (Fig. 1).

It is also seen from Table I that the selectivity with sodium chloride and sodium nitrate eluents was constant for both concentrations. This seems normal as the salt was only necessary to screen the electrostatic repulsions. With sodium nitrate, the value of the intercept also remained ralatively constant, which indicated that no large differences in retention volumes were observed with $5 \cdot 10^{-2}$ and $5 \cdot 10^{-1}$ *M* solutions. With sodium chloride, however, we found that a concentration of $5 \cdot 10^{-2}$ *M* was as effective for the separation as $5 \cdot 10^{-1}$ *M* sodium nitrate (Table II and Fig. 7). Indeed, all peaks were well separated, even the anomers of DP 2, but all were detected at larger retention volumes. However, for higher sodium chloride concentrations, only oligomers up to DP 5 were observed and the others probably adsorbed on the (guard) column. With $5 \cdot 10^{-1}$ *M* sodium sulphate, adsorption was even more pronounced (chromatogram not shown) as only oligomers of DP 1 and DP 2 were detected as broad but well separated peaks.

In analogy with the HPLC analysis of other charged compounds[19], the stationary phase of the column plays an essential role in the separation and in some instances gives rise to adsorption. As the chromatographic behaviour of this series oligomers varied with the type of salt in the eluent, we conclude that the separation with eluent salt concentrations above $5 \cdot 10^{-2}$ *M* is not exclusively dependent on the hydrofobic interactions between the anhydropyranosyl units and the C_{18} groups of the stationary phase. The exact influence of the salt on the solute–stationary phase interaction is not yet clear, however.

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