Carbohydrate Polymers **8** (1988) 119–130

Preparative Fractionation of Natural Polysaccharides by Size Exclusion Chromatography

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(Received 6 March 1987; revised version received 15 July 1987; accepted 31 July 1987)

SUMMARY

Preparative fractionation of various polysaccharides by Size Exclusion Chromatography is described. A home-made apparatus, equipped with a set of two columns (each 100 mm i.d. × 600 mm) packed with Toyosoda HW Superfine gels, has been designed for running gram quantities of samples at temperatures from ambient up to 60–80°C in water. Depending on the molecular weight of the substance and on the quality (narrowness) of fraction which is required, a daily throughput of 1–10 grams is reached with a typical flow rate of 500 ml h^{-1}.

Some examples are given for industrial polysaccharides used as food additives to emphasize the opportunities that this technique gives. A study of the change in the composition of alginate and pectins with molecular weight demonstrates how it can give further insight into the structure of water soluble polymers whereas an investigation of the effect of molecular weight on the gel strength of carrageenans illustrates its use in characterizing the molecular weight fraction of the material which is important in an application.

INTRODUCTION

The total market for polysaccharides is growing at the rate of 8–10% a year (Maury & Roque, 1986). Food additives are the major outlet accounting for roughly half of the tonnage. For this purpose, the out-

Carbohydrate Polymers 0144-8617/88/$03.50 —

standing rheological properties and gel forming ability of polysaccharides make them irreplaceable.

Naturally occurring industrial polysaccharides (e.g. carrageenans, alginates, pectins, galactomannans) all exhibit interesting structural features. They are composed of 2 to 5–10 different monosaccharides with variations in composition and structure. One major drawback is that they are subject to seasonal changes. Moreover, various sources (seaweeds, plants, fruits) may provide similar but not identical polysaccharides. They are drastically modified by the industrial extraction process, particularly their molecular weight distribution. Undesirable by-products are sometimes co-extracted, such as coloring matter or neutral polysaccharide ballast. Consequently, quality control tests have to be undertaken to fulfil customer requirements or to market products of constant quality, using standardization procedures.

After an effort was made in our company to develop analytical high performance size exclusion chromatography (SEC) of polysaccharides using Toyosoda PW packings (Lecacheux *et al.*, 1985; Lecacheux *et al.*, 1986), it was decided to start a preparative system for producing gram quantity fractions. By complementing other classic fractionation procedures (e.g. ion exchange), this preparative system was likely to provide unrivalled technology to show molecular weight–property relationships as well as changes in composition with molecular weight.

The need for gram fractions led us quickly to 100 mm i.d. columns by extrapolation from the analytical system. As TSK PW columns are not really commercially available in such large diameters (Sasaki *et al.*, 1985) and the packing material would be too expensive, we had to select another material with the temperature requirement (up to at least 60°C) which would be universally applicable.

A literature survey of the preparative SEC of water soluble polymers revealed several reviews giving general information including available packings and throughputs (Janson, 1984; Regnier, 1984; Lesec, 1985; Knox & Pyper, 1986). New promising gels are described: Hitachi (Kumanotani *et al.*, 1979), Dynospheres (Paulsen *et al.*, 1986), Merckogel PGM 2000 (Hiermann, 1986), Superose 6B (Andersson & Hagel, 1984); however, Sephadex (Jacobsson *et al.*, 1986), Sepharose (Kirk *et al.*, 1969), Biogel (Djordjevic *et al.*, 1986; Konno *et al.*, 1986), Toyosoda PW (Himmel & Squire, 1981; Mumby *et al.*, 1985) and Toyosoda HW (Germershausen & Karkas, 1981; Germershausen *et al.*, 1983; Noelken & Bettin, 1983; Callec *et al.*, 1984) are the most commonly used. Column diameters are around 20 mm with a few exceptions where 100 mm columns have been used (Dulout *et al.*, 1980; Sasaki *et al.*, 1983). Reports are mainly concerned with protein purification or

oligosaccharide fractionation, with one report at 60°C (John *et al.*, 1982) but only a few of them deal with polysaccharides (Anger & Berth, 1985). Owing to the relative similarity between PW and HW packings, we selected the latter to take up the challenge of developing a large scale preparative chromatograph for high molecular weight polysaccharides.

EXPERIMENTAL

Preparative chromatograph

An old Waters GPC 200 apparatus, which had been out of use for many years, was totally renewed for this purpose. A Waters 590 EEF solvent delivery system (0–80 ml min^{-1}) was used in connection with a home-made pulse dampener. A home-designed injector was operated by three electrovalves. The sample loop (200 ml) could be fed with any volume by means of a 50 ml syringe. Column effluent was monitored by a Waters R 403 refractometer and fractions were collected by using a LKB 2211 system equipped with the Superrac kit.

A set of two stainless steel columns (each 100 mm i.d. × 600 mm) were installed in the oven compartment. They were packed by the classic slurry technique, one with Toyosoda HW 55S gel, the other with HW 75S. Figure 1 gives an idea of the large fractionation range that was thus obtained, based on a single injection of a mixture of polyethylene-oxide standards. The injector and column compartments were thermostated electronically.

Routine conditions

Water was taken from a Milli-ro + Milli-q water purification system from Millipore. The eluent was typically 0·1 M $NaNO_3$, except for some runs in pure water. 0·2 g $liter^{-1}$ NaN_3 was added as a bactericide. The temperature was generally 60°C, except for pectins which were run at room temperature. Flow rate was between 500 and 750 ml h^{-1} ($\simeq$ 10 cm h^{-1} linear velocity) and the pressure drop was 0·5–1 bar. Higher flow rates were unfortunately prohibited because there was a sudden increase in pressure. Only one or two injections per day were possible. 100–200 ml of solution at 2–20 g $liter^{-1}$ (0·2–4 g) were injected, depending on the molecular weight range of the sample; 0·1–1 liter fractions were collected. Fractions were concentrated and the solute was recovered by isopropanol precipitation, washing and constant weight drying. The molecular weight distribution was determined by the coupling of SEC

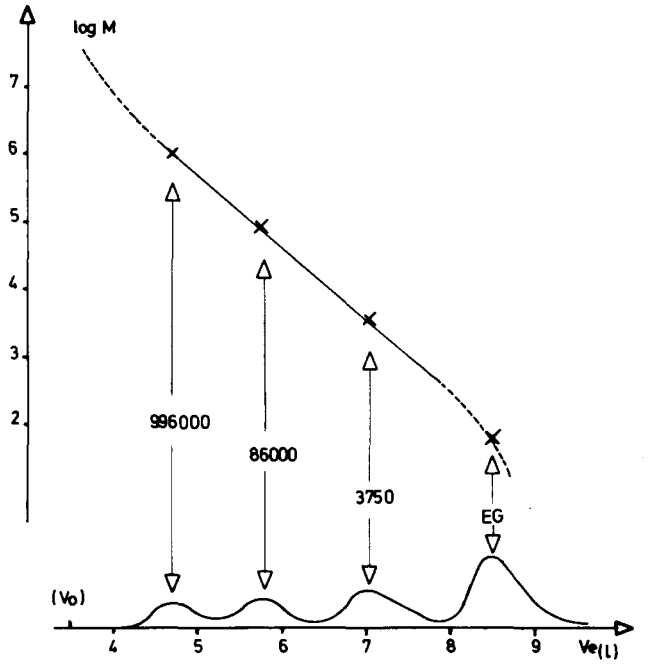

Fig. 1. Calibration of the system with a mixture of polyethylene oxide standards (eluent: water–room temperature; flow rate: 600 ml h^{-1}; sample: PEO standards; injected quantity: 1 g (total)).

and light scattering (Lecacheux *et al.*, 1985); the fractions were then submitted to appropriate analysis.

Analytical methods

In the following section, three examples are given in order to illustrate the opportunities that are created by this preparative system. Further developments, mainly for pectin, are continuing and will be published at a later date.

Samples were mainly commercial products from SATIA which had been extracted by classical industrial processes. Mannuronic/guluronic acid ratio in alginates were established by i.r. spectroscopy. Absorbances at 892 and 904 cm^{-1} were measured and M/G was deduced from a calibration curve.

Neutral sugars in pectin were determined by ion exchange chromatography in borate buffers using the Biotronik LC 5000 instrument. 50 mg of sample were submitted to hydrolysis (8 h at 100°C in H_2SO_4 2 N).

The physical properties of carrageenan gels were studied as a function of molecular weight using the INSTRON 1122 apparatus. Gels (1% carrageenan in 0.1 M KCl) were prepared in cylinders of 13 mm diameter and 20 mm height. The compression rate was 20 mm min^{-1}.

RESULTS

Alginate composition

Among the natural polysaccharides, alginates of plant origin exhibit a very simple structure as they are linear copolymers of mannuronic acid (M) and guluronic acid (G). Their composition is most simply described by the M/G ratio, although sophisticated methods like NMR (Grasdalen *et al.*, 1981; Grasdalen, 1983) or circular dichroism (Craigie *et al.*, 1984) can give information about the non-regular blockwise pattern of M and G along the chain. In bacterial alginates, acetyl groups associated with mannuronic residues have been evidenced. Much is known about the gel properties of alginates, which depend to a large extent on the proportion of G blocks.

A sample from *Laminaria digitata* with a M/G ratio of 1·1 was selected for its very high molecular weight. It was fractionated to check for M/G constancy along the distribution curve. 180 ml of a 4 g $liter^{-1}$ solution were injected. Owing to the very low differences that were observed between fractions, a second injection was performed with some changes in the collector program. Results (weight average molecular weight, M_w, polydispersity index, I, and M/G ratio) are displayed in Table 1. The curve in Fig. 2 is clearly proof that there is a small change in composition, lower molecular weight chains being slightly enriched in guluronic monomer (50%) whereas the highest molecular weight species have a G content of 45% independent of molecular weight. Other samples from various origins will be analysed similarly, to see if there is a general change in M/G ratio with molecular weight.

Insight into pectin structure

Pectin is of great concern to many workers because it can be classified as a polyelectrolyte, a complex polysaccharide, an important food fiber, a major plant cell wall component, and a ubiquitous nutritional factor and gelling agent in foods (Fishman & Jen, 1986).

Pectic substances are predominantly composed of galacturonic acid (or its methyl ester), with the occurrence of neutral sugars — rhamnose,

TABLE 1
Fractionation of Alginate from *Laminaria Digitata*

		M_w	I	M/G
Whole sample		500 000	6	1·10
First injection	No. 1	615 000	2	1·19
	No. 2	435 000	2·5	1·18
	No. 3	395 000	3	1·12
	No. 4	225 000	4	1·09
Second injection	No. 1	1 800 000	1·5	1·20
	No. 2	1 000 000	2	1·21
	No. 3	500 000	3	1·20
	No. 4	315 000	3	1·11
	No. 5	240 000	3	1·08
	No. 6	160 000	3	1·00

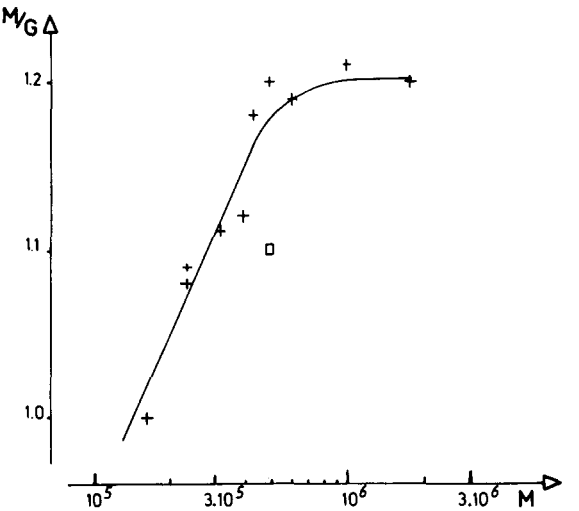

Fig. 2. Change in alginate composition with the molecular weight. (+, fractions; □, whole sample).

arabinose, galactose, glucose, xylose and mannose. The location of each of them — rhamnose in the backbone, the others as side chains or as a free neutral polysaccharide ballast — has been a matter of debate for many years. It is likely that preparative SEC, along with other fractionation methods, will give further insight into pectin structure and composition.

In our laboratory, several pectins from various origins (apple pomace, lime) and with different gel properties have already been fractionated. Here, the results obtained on a sample from lime are given as a representative example. Fractions from four injections (each 0·5 g) were combined in order to obtain enough material for various analyses. Figure 3 gives the refractometric signal (RI) of a preparative injection, the slicing indicates the fractions collected. Analytical absolute distributions of four

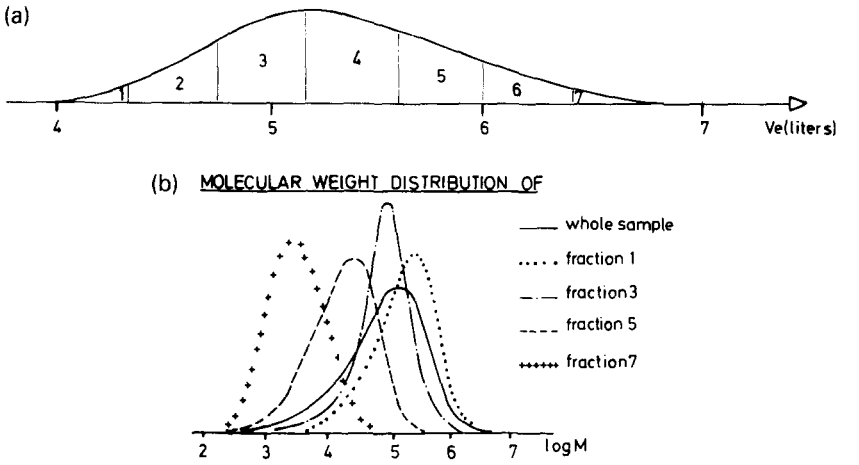

Fig. 3. (a) Elution pattern and fractionation of lime pectin (eluent: $NaNO_3$ 0·1 M — room temperature; flow rate: 500 ml h^{-1}; injected quantity: 0·5 g). (b) absolute molecular weight distribution for a selection of fractions.

TABLE 2
Fractionation of Pectin from Lime (Neutral Sugars in Weight %)

	Weight fraction (%)	$M_w \times 10^3$	*I*	*Rhamnose*	*Galactose*	*Arabinose*	*Glucose*	*Xylose*	*Mannose*	*Total sugars*
Whole sample	—	170	5·5	1·5	3·2	1·8	0·3	0·1	0·1	7·0
1	<0·5	315	3	1·8	3·8	1·7	0·2	0·2	0·2	7·9
2	15	220	2·5	1·7	2·9	1·6	0·1	0·1	0·1	6·5
3	34	130	2	1·6	2·2	1·4	0·1	0·1	0·1	5·5
4	27	80	3	1·4	2·1	1·35	0·1	0·1	0·05	5·1
5	15	47	3	1·35	3·5	1·7	0·2	0·1	0·1	7·0
6	5	20	3·5	1·5	7·2	2·2	0·7	0·1	0·2	12
7	4	8	2·5	1·4	19	2·6	1·4	0·4	1	26

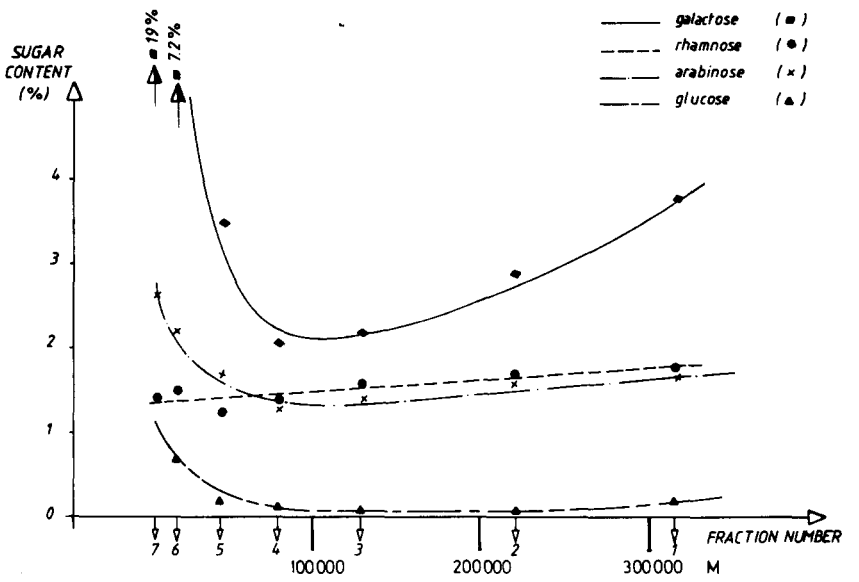

Fig. 4. Neutral sugars in lime pectin along the molecular weight distribution.

fractions are also compared to the whole sample. All the results are collected in Table 2 and neutral sugar contents are plotted against molecular weight in Fig. 4.

Rhamnose exhibits the most regular curve, with a slight increase with molecular weight. Rhamnose/arabinose molar ratio is constant near unity, except for low molecular weight chains where the arabinose percentage clearly increases. Galactose shows a minimum content of 2% for intermediate chains. There is a definite increase in galactose for higher molecular weight pectins but a dramatic increase for low molecular weight samples with values as high as 20% (out of scale in Fig. 4). Glucose, xylose and mannose were found at lower concentration. They are concentrated in the low molecular weight tail, which is probably enriched in free polysaccharide ballast.

Carrageenan fractionation and gel properties

Carrageenans form a group of cell-wall polysaccharides that are extracted from certain red seaweeds. Among this family, both κ- and ι-carrageenans can adopt a double helical structure in solution, allowing the formation of thermoreversible gels.

In order to better understand gel properties, eight fractions of κ-carrageenan from *Euchema cottonii* were prepared. As several grams

were needed, a compromise was taken between quantity and quality (narrowness) of fractions. Fractions 1 and 2 were obtained from a native, very high molecular weight material, whereas (3, 4), (5, 6) and (7, 8) were respectively obtained from three successive products, corresponding to a step by step degradation of the native carrageenan using a chemical process.

As an example, Fig. 5 (RI trace) shows how fractions 3 and 4 were taken from the first step degradation product. In this case, 12 injections of 1·25 g each were combined to get about 5 g of each fraction. 'Fraction' 9 was in fact the last step of the degradation process. Its polydispersity was considered low enough to avoid fractionation. This work, including fractionation and analysis of fractions, was very time-consuming as it required full use of the apparatus for two months. Weight average molecular weights and polydispersities are collected in Table 3.

As a preliminary test, gel properties were measured in 0·1 M KCl using all samples except number 9 which did not form any gel, even at 2% concentration. Young's modulus, E (in Newtons cm^{-2}), and rupture strength, F (in Newtons), are plotted in Fig. 6 against molecular weight. In spite of some scatter in the experimental points (the handling of gels

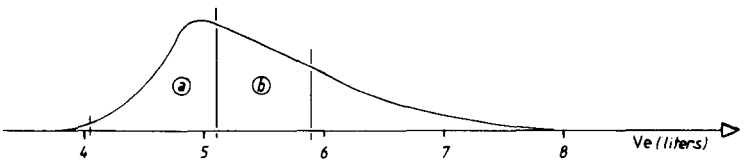

Fig. 5. Preparative RI trace of the carrageenan leading to fractions 3(a) and 4(b) (eluent: $NaNO_3$ 0·1 M — 60°C; flow rate: 600 ml h^{-1}; injected quantity: 1·25 g).

TABLE 3
Gel Properties of Carrageenan Fractions

Fraction	$M_w \times 10^3$	*I*	*E* ($N\,cm^{-2}$)	*F* (*N*)
1	960	4	2·9	13·5
2	560	5	3·2	9·1
3	350	3	3·6	8·2
4	260	4	3·3	4·6
5	100	2	2·6	3·0
6	72	2·5	1·5	1·3
7	60	2	1·3	0.86
8	33	2·5	0·35	0·15
9	6·3	2·5	No gel	No gel

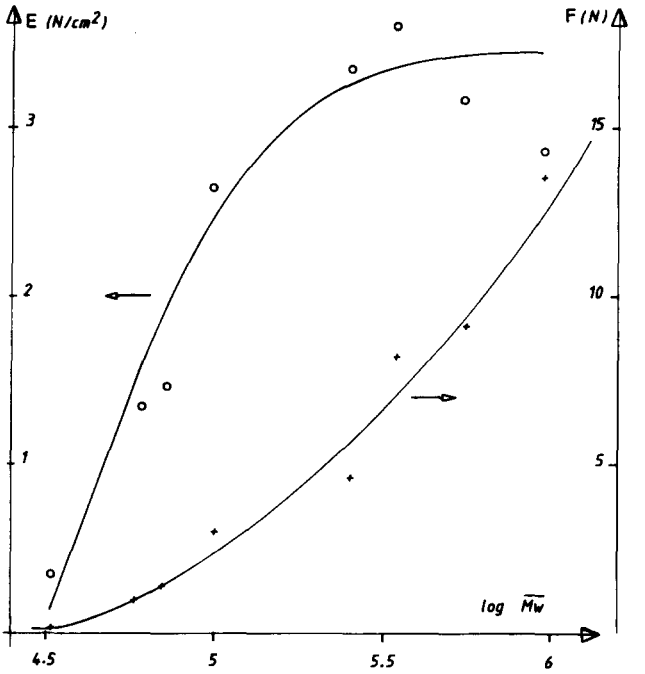

Fig. 6. Young's modulus, E, and rupture strength, F, plotted against molecular weight for carrageenan gels.

was not very easy), two trends are clearly evidenced. Rupture strength increases regularly with molecular weight, whereas Young's modulus increases sharply but then levels off for molecular weights beyond about 300 000.

CONCLUSION

A preparative SEC chromatograph with a large throughput has been described and some simple applications have been given to demonstrate its potential uses. It is probable that such a system, in connection with other analytical methods, will allow a deeper knowledge of natural polysaccharides as well as other water soluble polymers, by indicating the actual part of the molecular weight distribution which is important for an application property or by showing structural features along the molecular weight distribution. Standards of very low polydispersity ($I < 1{\cdot}2$) for calibration or any other purpose may also be obtained. Although pectin is the main interest of the authors, fractionation of small quantities

(preferably less than a gram) of various polysaccharides can be carried out at other companies' request.

ACKNOWLEDGEMENTS

Thanks are due to P. Garrigou, G. Lacombe and R. Prechner for their technical assistance during the development of the chromatograph. The authors are indebted to Y. Mustiere and C. Vive-Lesperance for part of the experimental work.

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