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Preliminary report on fractionation of fucans by ion-exchange displacement centrifugal partition chromatography

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Abstract

A new method combining ion-exchange displacement chromatography with centrifugal partition chromatography (CPC) was used for the fractionation of partially depolymerized fucans (polysulphated polysaccharides). The ion-exchanger was Amberlite LA2, a high-molecular-mass liquid secondary amine miscible with most common organic solvents and immiscible with aqueous solutions. Ion-exchange displacement centrifugal partition chromatography was performed with LA2 in methyl isobutyl ketone (MiBK) as the stationary phase, water as the mobile phase, Cl^- as the carrier and OH^- as the displacer. A complex mixture of partially depolymerized fucans was resolved into adjacent families characterized by their peak molecular mass and polydispersity. The Dubois test (sugar) and the azur A test (SO_3^-) confirmed the displacement mode of the process, and size-exclusion chromatographic controls confirmed its efficiency. © 1998 Elsevier Science B.V.

Keywords: Fucan; Polysaccharides; Countercurrent chromatography; Displacement chromatography

1. Introduction

Fucans, a family of sulphated polysaccharides extracted from brown seaweeds, have been attributed various biological activities (e.g. antithrombotic, anticoagulant, antiinflammatory, antitumoral, and antiviral) [1,2]. They can be classified into three major groups: (1) the fucoidan or homofucan group, mainly composed of sulphated fucose units; (2) the ascophyllan group with a polyuronide backbone branched with short chains of neutral or sulphated units (xylose, galactose and fucose) and (3) the glycuronofucogalactans with a linear chain of galactose units branched with fucose and uronic acids [3–5]. Several structures have been proposed for fucoidans [6]. High-molecular-mass fucans and partially depolymerized ones have been isolated in our laboratory and subjected to various assays [7]. However, their purification and characterization have proved difficult, which led us to use ion-exchange chromatography combined with centrifugal partition chromatography in an attempt to improve their purification.

Countercurrent chromatography (CCC) or centrifugal partition chromatography (CPC) generally refers to support-free liquid–liquid chromatography with two immiscible solvents or solutions. An instrument keeps one liquid stationary while the other liquid is pumped through it, and the chromatographic process occurs between the two liquid phases. The historical inventor of CCC is Dr. Yochiro Ito, whose theoretical and instrumental contribution has resulted

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in approximately 200 papers. Several books have been published [8–12], in which many examples of separations in various fields can be found.

Displacement CCC was first performed through acid–base equilibrium and referred to as ''pH zone refining CCC'' [13–16]. As in conventional displacement chromatography, the feed components emerge from the CCC column as adjacent ''square wave zones'' of homogeneous material.

1.1. Principle of ion-exchange displacement centrifugal partition chromatography of partially depolymerized fucans

In contrast to ionizable molecules usually purified by pH zone refining, partially depolymerized fucans (F_i^-, whatever the number of negative charges) are not ionizable but ionic. For this reason, we began studies on true ion-exchange by using a liquid weak anion-exchanger called Amberlite LA2 (Rohm & Haas), a high molecular mass, oil soluble secondary amine with excellent solubility in most common organic solvents and extremely low solubility in aqueous solutions. These solubility characteristics, together with the ability of secondary amine to react with acids to form the corresponding amine salt, allow LA2 to be used as a weak anion-exchanger. Typically, LA2 is dispersed in an organic solvent, such as methyl isobutyl ketone (MiBK), and protonated by an aqueous HCl solution:

$$\overline{LA2} + HCl \xleftarrow{} \overline{LA2H^+, Cl^-} \quad (1)$$

(Overlined molecules or ions are those in the organic phase).

Ion-exchange between Cl^- and a fucan, F_i^-, will occur as follows:

$$F_i^- + \overline{LA2H^+, Cl^-} \Leftrightarrow Cl^- + \overline{LA2H^+, F_i^-} \quad (2)$$

with a corresponding selectivity coefficient:

$$K_{Cl}^{F_i} = \frac{(\overline{LA2H^+, F_i^-})(Cl^-)}{(F_i^-)(\overline{LA2H^+, Cl^-})}$$

[1]Ion-exchange between two fucans, F_i^- and F_{i+1}^-, can be described by the following equation:

$$F_{i+1}^- + \overline{LA2H^+, F_i^-} \Leftrightarrow \overline{LA2H^+, F_{i+1}^-} + F_i^- \quad (3)$$

with a selectivity coefficient:

$$K_{F_i}^{F_{i+1}} = \frac{(\overline{LA2H^+, F_{i+1}^-})(F_i^-)}{(F_{i+1}^-)(\overline{LA2H^+, F_i^-})}$$

Let us choose OH^- as a displacer ion in the mobile phase; OH^- will displace all anions from the stationary phase through deprotonation of the weak anion-exchanger:

- in the absence of fucans:

$$OH^- + \overline{LA2H^+, Cl^-} \xleftarrow{} \overline{LA2} + Cl^- + H_2O \quad (4)$$

  which is basically the inverse of Eq. (1).

- in the presence of a fucan:

$$OH^- + \overline{LA2H^+, F_i^-} \xleftarrow{} \overline{LA2} + F_i^- + H_2O \quad (5)$$

In a typical development run, the CPC column is initially filled with the stationary phase containing $\overline{LA2H^+, Cl^-}$. The fucan mixture, F_1^- to F_n^-, is then introduced at the beginning of the CPC column. The CPC column is rotated (see Section 2), and development starts with an aqueous mobile phase containing OH^-. Provided that the selectivity coefficients, $K_{F_i}^{F_{i+1}}$, are different from 1 and greater than 1, the displacer will force the desorption of fucans, which develop into adjacent square wave zones of homogeneous material. If the CPC column is long enough, the system will reach an isotachic state moving at the velocity of the displacer, with the least strongly absorbed fucan appearing first in the displacement train (Fig. 1). At the front of the train, Cl^- anions are displaced by the less retained fucan, F_1^-, and eluted as NaCl, if Na^+ has been chosen as the co-ion in the mobile phase.

At the end of the train, the most retarded fucan, F_n^-, is forced into the mobile phase through deprotonation of the weak anion-exchanger. In the whole train, the conservation of ionic charge means that the molarity (in equivalent l^{-1}) is that of the displacer in the mobile phase, (OH^-).

In the i^{th} zone, $\overline{LA2H^+, F_i^-}$, in the organic stationary phase, is in equilibrium with H^+ and F_i^- in the aqueous mobile phase, H^+ being present through the acid-base equilibrium:

[1]In this paper, ion concentrations are expressed as equivalent l^{-1} or meq ml^{-1}. All equilibria are written in the same way, i.e. one equivalent Cl^- exchanges with one equivalent F_i^-.

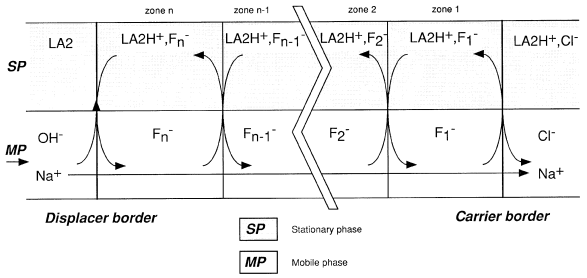

Fig. 1. Schematic representation of an isotachic state obtained in ion-exchange displacement chromatography. If the column is long enough, each zone contains only one species and is demarcated by two well-defined boundaries. Cl^- is the carrier ion (the less retained ion) and OH^- is the displacer ion, which displaces the most retained ion through deprotonation of the weak anion-exchanger.

$$\overline{LA2H^+, F_i^-} \Leftrightarrow \overline{LA2} + H^+ + F_i^- \quad (6)$$

The corresponding equilibrium constant is the following:

$$K'_{a,F_i} = \frac{(\overline{LA2})(H^+)(F_i^-)}{(\overline{LA2H^+, F_i^-})} \quad (7)$$

where K'_{a,F_i} is the conditional acidity constant of $LA2H^+$ in the presence of F_i^-.

By neglecting the autodissociation of water, we can approximate $(\overline{LA2}) = (H^+)$, hence:

$$K'_{a,F_i} = \frac{(H^+)^2(F_i^-)}{(\overline{LA2H^+, F_i^-})} \quad (8)$$

The distribution ratio, $D = [(\overline{LA2H^+, F_i^-})/(F_i^-)]$, being constant in the whole isotachic train, and determined by the ratio $(LA2H^+)$ to (OH^-), the pH of the mobile phase in the i^{th} zone is:

$$pH_i = \frac{1}{2}(pK'_{a,F_i} - \log D) \quad (9)$$

The pH of the eluted mobile phase should thus be an indication of the stability of $\overline{LA2H^+, F_i^-}$ or, in other words, of the conditional acidity constant of LA2 in the presence of F_i^-. If the CPC column is long enough to allow these steady-state equilibria to occur, we should observe an elution profile, as shown in Fig. 2.

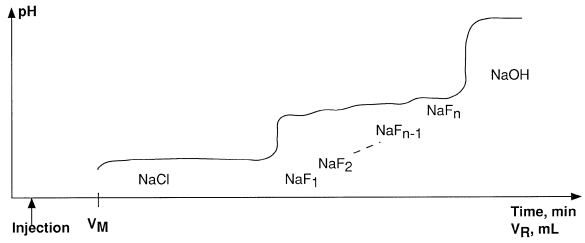

Fig. 2. The elution profile in ion-exchange displacement chromatography once complete development has been achieved. Each zone is characterized by a pH which provides information on the stability of the pair $LA2H^+, F_i^-$ in the organic stationary phase.

2. Experimental

2.1. Chemicals

Sodium hydroxide (32%) was purchased from Riedel–de Haën (Seelze, Germany); hydrochloric acid (37.5%) from Panreac (Barcelona, Spain), and methyl isobutyl ketone (puro) from Carlo Erba (Rodano, Italy). Azur A (ref A 6270) was obtained from Sigma (St. Louis, MO, USA), and phenol (ref 20 597 234) from Prolabo (Paris, France). Water was deionized using a Milli-RO® 4 water purification system (Millipore).

A sample of Amberlite LA2 was kindly provided by the manufacturer (Rohm & Haas, Philadelphia, PA, USA).

2.2. Fucans

Fucans were extracted from the brown seaweed *Ascophyllum nodosum*, as previously described [17]. The fucan extract was depolymerized by a radical process using the method of Nardella et al. [7]. A low-molecular-mass fraction was isolated, for which the peak-molecular mass (determined by HPSEC) was around 9 000 and the chemical composition 31.2% fucose, 4.9% uronic acid, 9.6% sulphur and $<0.1\%$ nitrogen.

2.3. CPC

2.3.1. Apparatus

A series 1000 HPCPC (Sanki Engineering, Nagaokakyo, Kyoto, Japan) was used [18]. This bench top CPC ($30\times45\times45$ cm, ≈60 kg) has a column consisting of a stacked circular partition disk rotor

containing 2136 channels with a total internal volume of 200 to 240 ml, depending on the unit (our unit has 204 ml). The column was connected to the injector and the detector through two high pressure rotary seals. The partition disks were engraved with $1.5\times0.28\times0.21$ cm channels connected in series by $1.5\times0.1\times0.1$ cm ducts. A 4-port valve included in the series 1000 allowed the HPCPC to be operated in either descending or ascending mode. The HPCPC was connected to a Rheodyne model 7110 injector (10 ml sample loop), and two model 510 pumps with an automatic gradient controller (Waters, Milford, MA, USA).

2.3.2. Liquid phases

2.3.2.1. *Organic stationary phase*. A 10% (v/v) solution of LA2 in MiBK was first saturated with water. Since the average molarity of pure LA2 is 2.25 meq ml^{-1}, the resulting organic phase was 0.225 meq ml^{-1}. A volume *V* of this organic phase was then equilibrated with an equivalent volume of water containing a sufficient amount of HCl to protonate LA2 fully.

2.3.2.2. *Aqueous mobile phase*. A 0.025 *M* (0.05 *M* for the blank run) solution of NaOH was saturated with a 10% solution of unprotonated LA2 in MiBK. As LA2 and MiBK are practically insoluble in water, a small amount of organic phase was sufficient to saturate the aqueous phase fully.

2.3.3. Monitoring the effluent

A microflow pH sensor (Broadley–James Corporation, Santa Ana, CA, USA) was used for continuous monitoring of effluent pH. This 89 mm×∅ 18 mm body was connected directly in-line to 1.59 mm (1/16″) O.D. tubing. The pH sensor contained a combination pH electrode, and the internal volume for the effluent was only 50 μl. The pH sensor wetted-materials were glass, ceramic, polypropylene and fluororubber. The pH sensor supported 0 to 25 ml min^{-1} flow-rates and was connected to a P600 pH meter (Consort, Turnhout, Belgium), giving a 0 to 2 V analogue output for recording. A Kipp and Zonen recorder was used.

2.3.4. Analytical controls

2.3.4.1. *Carbohydrate analysis*. Total carbohydrate concentrations were determined in each fraction by the phenol–sulphuric acid method of Dubois et al. [19]. The starting material was used for calibration.

Sulphate contents were estimated by Azur A assay [20]. A 50 μl sample was added to 1 ml of Azur A solution (10 μg ml^{-1}). The initially blue solution instantaneously turned violet (in the presence of sulphated sugars). Absorbance was read at 535 nm.

2.3.4.2. *Sulfur content*. Elemental analysis of sulfur was performed for several selected fractions (tubes 26, 33, 37, 41, and 50) by the ''Service Central de Microanalyse du CNRS'' (Gif sur Yvette, France).

2.3.4.3. *Chloride detection*. The presence or absence of Cl^- was checked in each fraction, using $AgNO_3$ after acidification with acetic acid, if necessary, as for the last fractions.

2.3.4.4. *Molecular mass determination*. An aliquot of each fraction was analyzed by high-performance size-exclusion chromatography (HPSEC) in 0.2 *M* NaCl using a 30×0.32 cm I.D. Superdex column (Pharmacia PC 3.2/30, Uppsala, Sweden) at a flow-rate of 0.1 ml min^{-1}. Column calibration was performed with standard pullulans. Pullulans are neutral glucans, whereas fucans are highly negatively charged oligosaccharides. Consequently, such a calibration, which does not allow an exact measure of fucan molecular mass, was used only for relative comparisons. Area measurements, calculations of molecular mass, and polydispersity were determined using Turbochrom software (Perkin–Elmer, Norwalk, CT, USA).

2.3.4.5. *Electrophoretic analysis*. This assay was performed on a cellulose acetate membrane (4.7× 1.5 cm, Sartorius, Goettingen, Germany) previously soaked in 0.1 *M* NaOH (pH 6.6). Fucan solutions (2 μl, 10 mg ml^{-1}) were deposited on the cathode side. Runs were performed with the same buffer. After the electrophoretic run (1 H, 200 V), the membrane was immersed in a staining solution [0.05 *M* $MgCl_2$; 0.025 *M* NaOAc; 0.2% (w/v) alcian blue; 50% (v/v) EtOH–H_2O] for 30 min and then washed three times

with the same buffer without alcian blue. Finally, the membrane was soaked for 2 min in MeOH and then for 15 s in 25% (v/v) AcOH in MeOH and dried between two glass plates. Fucans appeared as very broad blue bands.

3. Results and discussion

Figs. 3 and 4 show two original pH recordings of a displacement run: the first was a blank run (displacement of Cl^- by OH^-) and the second corresponded to the injection of 800 mg of a mixture of fucans. For these two runs, the rotational speed of the CPC unit was 1 200 rpm, and the stationary phase fraction (S_F) was around 75% at the beginning of the experiment. A constant bleeding of the stationary phase was observed during the displacement process, corresponding to 4 to 5% of the stationary phase in the eluted mobile phase. This had no effect on pH detection, and this 4% stationary phase volume could easily be removed from collected fractions after decanting. No trace of liquid ion exchanger was found in the aqueous solutions.

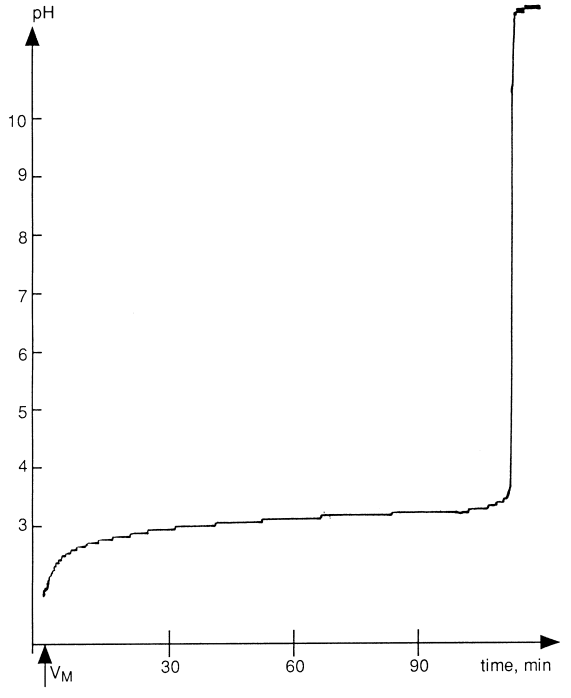

Fig. 3. Ion-exchange displacement centrifugal partition chromatography: blank run. Stationary phase: 10% (v/v) $LA2H^+, Cl^-$ in MiBK, saturated with water. Mobile phase: 0.05 *M* aqueous NaOH, saturated with 10% (v/v) LA2 in MiBK. Column: CPC instrument, $V_c = 204$ ml; rotational speed: 1 200 rpm. Flow-rate: $F = 5$ ml min^{-1} – Descending mode. Mobile phase volume in the CPC column: 55 ml at the beginning of the experiment, 77 ml at the end (due to constant bleeding of the stationary phase).

The record of the first run resembled the neutralization curve of a weak acid ($LA2H^+$) by a strong base (OH^-). Eluent pH was acidic and equal to the expected pH of an aqueous solution in equilibrium with $LA2H^+$ in MiBK. The shape of the second record, from 160 to 205 min, was characteristic of a typical displacement chromatographic run [13], corresponding to a continuous displacement of adjacent or partly overlapped fucan families, as confirmed by electrophoretic analysis. A first test to check for Cl^- in the effluent showed that Cl^- disappeared completely in 170 min (tube 23), corresponding to the appearance of the first fucan, as predicted by the displacement model. The sugar test (Dubois) and $-SO_3^-$ test (Azur A) were then performed from time 0 to time 160, and the presence of neutral sugars (Dubois-positive and Azur A-negative fractions) was detected at the beginning of elution. This means that neutral sugars were present in the fucan sample; they were eluted in the classical elution mode. Since they are non-ionic, and have no affinity for the organic stationary phase, they were eluted in the « void » volume of the CPC column.

Various analyses were then performed on collected fractions between 160 and 210 min.

3.1. Sugar test (Dubois) and $-SO_3^-$ test (Azur A)

The Dubois assay was constantly positive from tube 21 to tube 60. Fig. 5 shows the $-SO_3^-$ test superimposed on the pH curve of the effluent. The Azur A test gave a fair estimation of the molarity of $-SO_3^-$ and, as predicted by the displacement model, the molarity (in equivalent l^{-1}) was roughly constant in the main part of the chromatogram. This means that all the $-SO_3^-$ sites, or a constant ratio (fucans are highly branched polysaccharides in which some $-SO_3^-$ could be completely hidden) played an active role in the ion-exchange process.

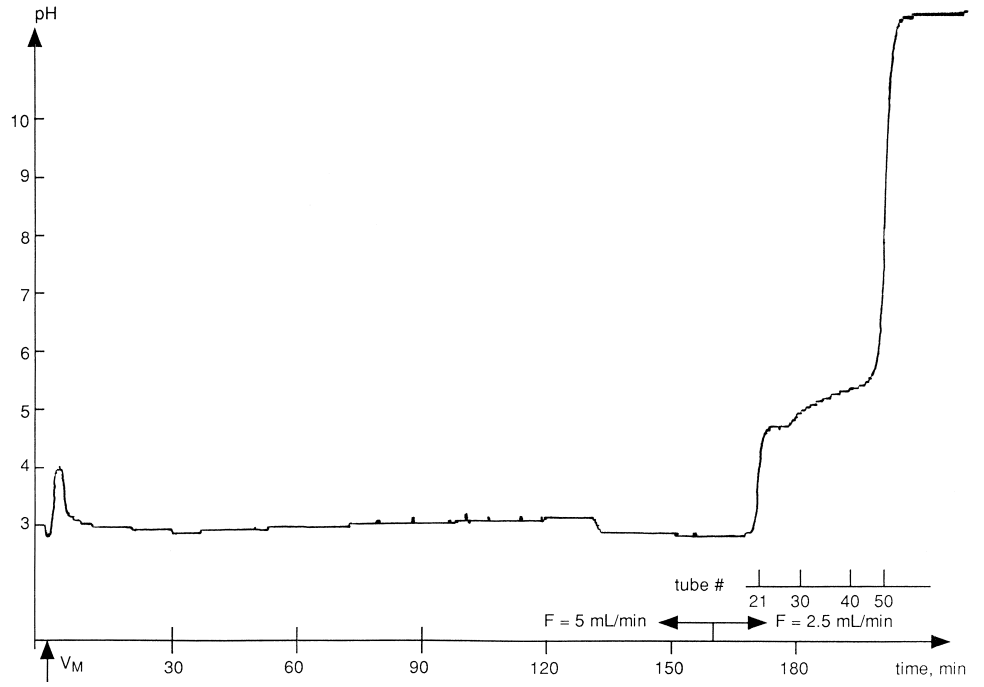

Fig. 4. Ion-exchange displacement centrifugal partition chromatography of a fucan mixture. Stationary phase: 10% (v/v) $\overline{LA2H^+, Cl^-}$ in MiBK, saturated with water. Mobile phase: 0.025 *M* aqueous NaOH, saturated with 10% (v/v) *LA*2 in MiBK. Column: CPC instrument, $V_c = 204$ ml; rotational speed: 1200 rpm. Flow-rate: $F = 2$ ml min^{-1} from time 0 to time 5 min, 5 ml min^{-1} from time 5 to time 160 min, then 2.5 ml min^{-1} – Descending mode. Mobile phase volume in the CPC column: 40 ml at the beginning of the experiment, 70 ml at the end (due to constant bleeding of the stationary phase). Injected sample: 800 mg of a fucan mixture (see Section 2) in 8 ml of the protonated stationary phase and 2 ml of water.

3.2. HPSEC analysis

SEC analysis was performed for every fraction collected. Fig. 6a shows five typical SEC patterns of CPC fractions, and Fig. 6b the SEC pattern of the unfractionated fucan sample. Fig. 7 shows the relative peak-molecular mass, M_p, superimposed on the pH curve of the effluent. It can be seen that fucan elution starts with low-molecular-mass polysaccharides (tubes 22, 24), followed immediately by a transition zone (e.g. 27) in which both the low-molecular-mass and the highest molecular mass polysaccharides are found. Three characteristic families were then eluted (tubes 32–54) close to each other, considering their peak molecular mass, but different in both their SEC shapes (all remarkably symmetrical) and the corresponding pH of their elution zones (pH 5.13, 5.44, 7.2).

Fig. 8 shows the peak areas superimposed on the pH curve. It is admitted that, in a broad range of molecular mass for the same family of polymers, the variation of the refractive index is roughly proportional to the concentration (mg ml^{-1}) of polymers. Most of the fucans injected came out between tube 32 (pH 5.0) and tube 54 (pH 7.2). Tubes 22–31 containing the low and highest molecular mass polysaccharides, represented only 17% (w/w) of the polysulphated matter.

Table 1 shows the results corresponding to families I to III. Relative peak-molecular masses decreased from Family I to Family III. As Family III was more retained, there should have been more sulphates per fucose unit in this family than in Family I. In fact, the sulphur content increased from tube 33 (Family I, 10.2%, w/w, STDV 0.06) to tube 50 (Family III, 11.26%, w/w, STDV 0.08). Other observed values of 5.95 (0.05), 10.4 (0.06) and 11.36 (0.1) for tubes 26, 37 and 41, respectively, increased as expected. There was no significant difference between tube 41 (11.36%) and tube 50

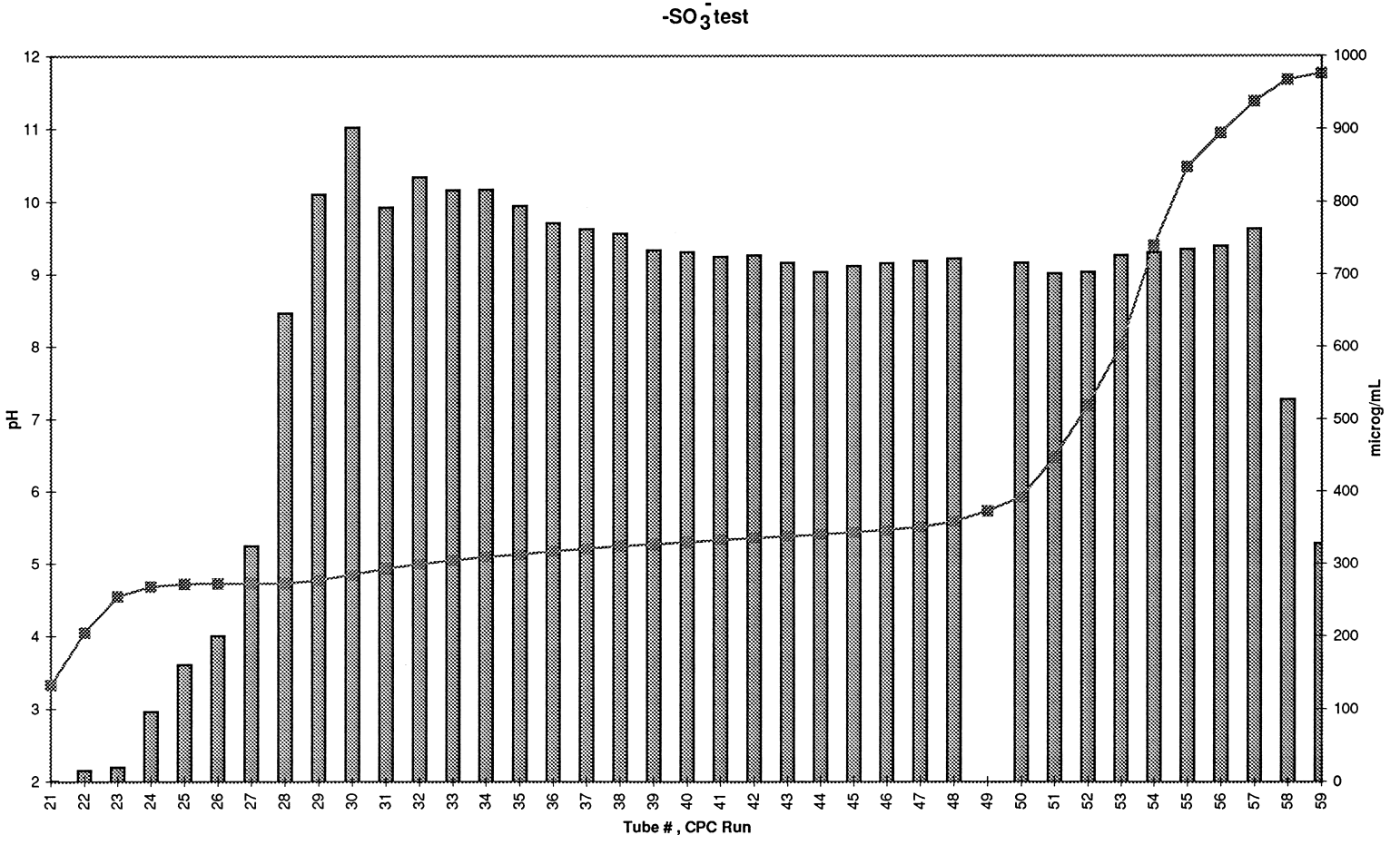

Fig. 5. $-SO_3^-$ test (Azur A), on CPC fractions 21 to 60. From tube 28 to tube 60, the molarity of $-SO_3^-$ is roughly constant, as predicted by the displacement model. Fucans present in tube 21 to 27 may contain some other ionic functions such as COO^- since the presence of Cl^- is not detected after tube 22.

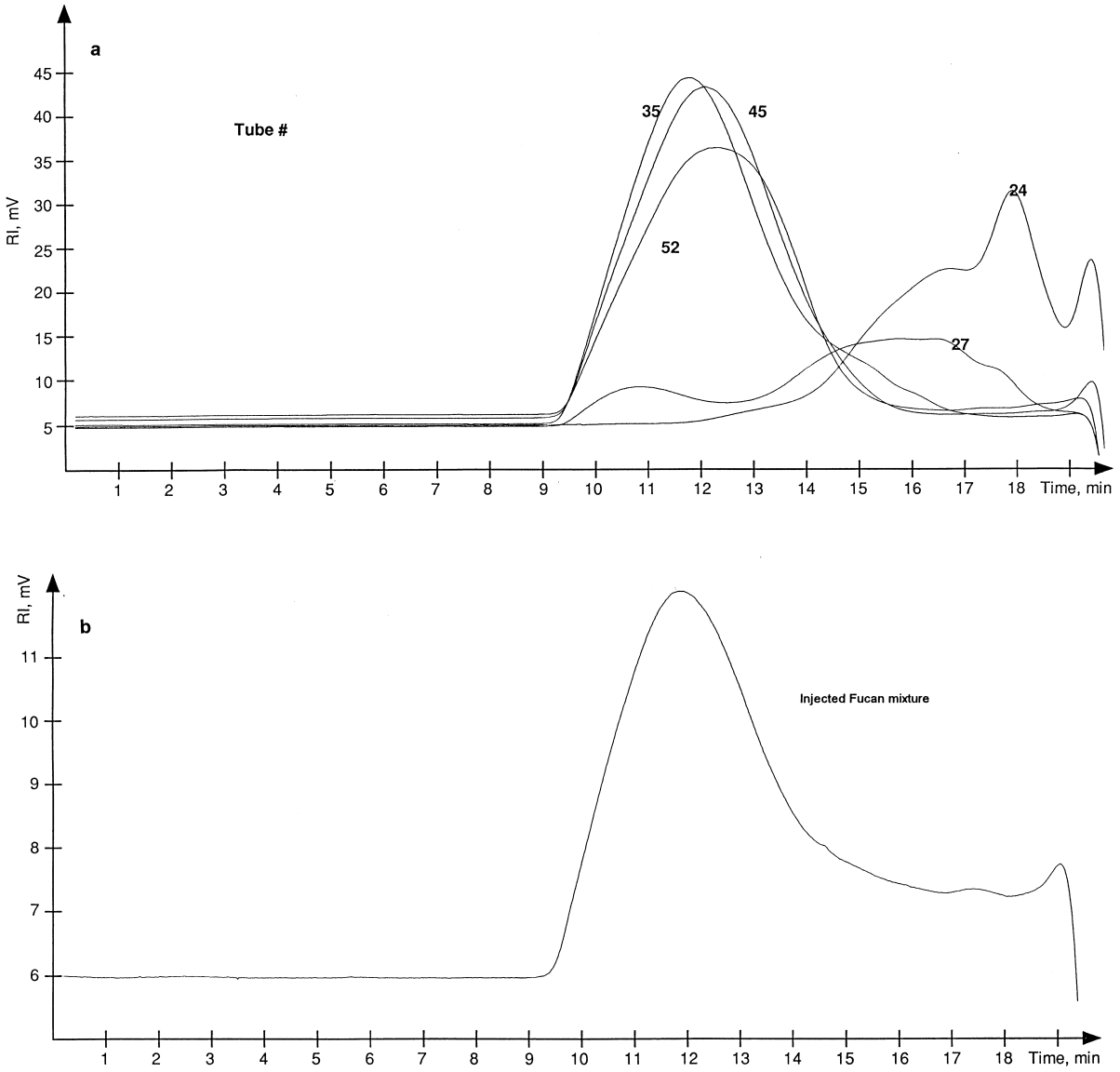

Fig. 6. (a) SEC chromatograms of five fractions representative of five typical zones of the CPC run. Tube # refers to the X scale of Fig. 5. (b) SEC chromatogram of the original sample. See Section 2 for SEC conditions.

(11.26%), although the latter had a much higher retention volume. Further structural studies should clarify this point since sulphate accessibility is also an important parameter.

Owing to the low difference in molecular mass, it was impossible to separate these three families by preparative SEC. Anion-exchange chromatography on solid resins gave no satisfactory separation, probably because a rigid matrix cannot adopt the right shape to interact differentially with very similar molecules and thus to discriminate between them. Obviously, such a limitation does not exist with a liquid ion-exchanger.

Prior to this study, ion-exchange countercurrent chromatography of glycosaminoglycans in the elution mode was used by Hurst et al. [21], who

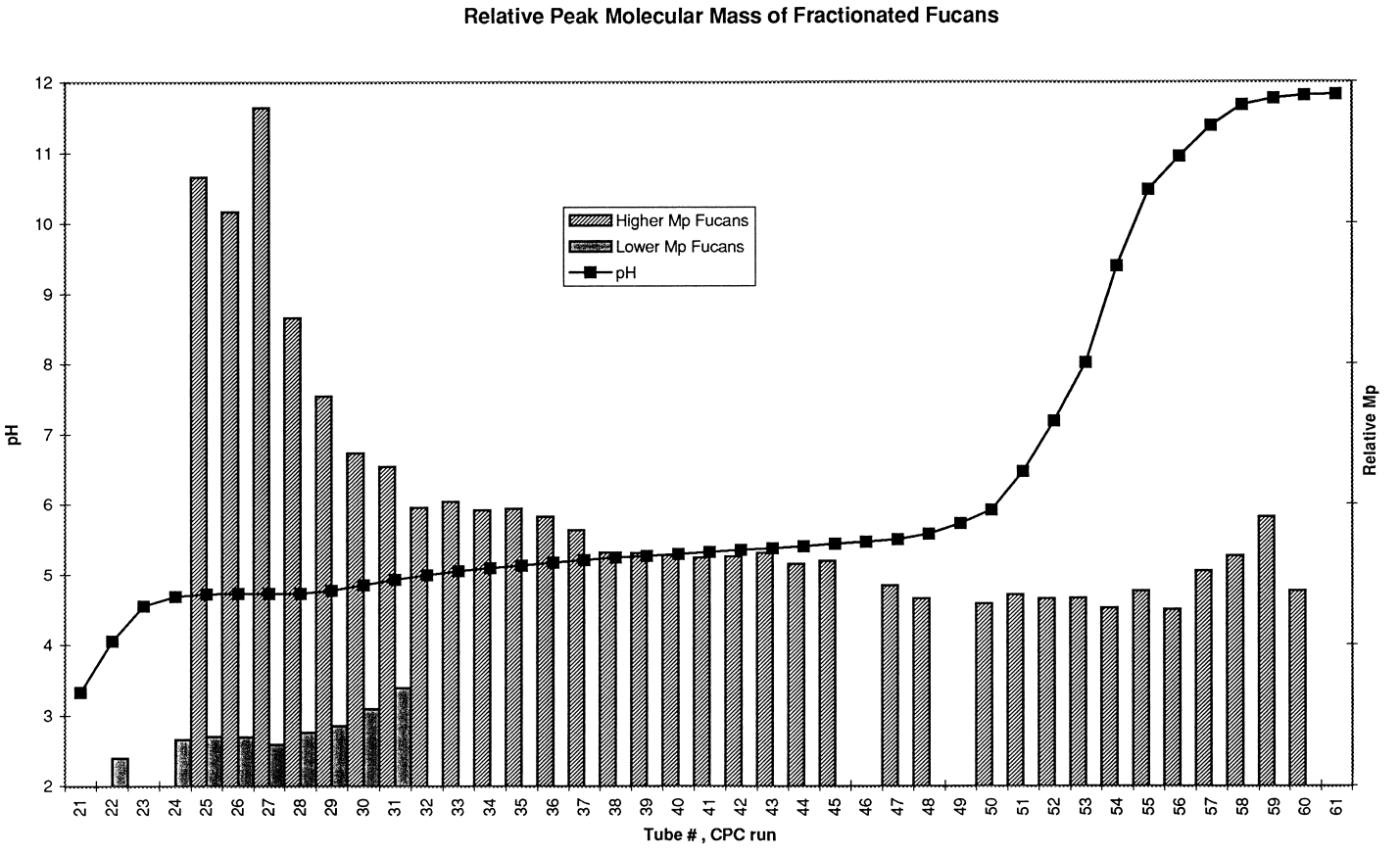

Fig. 7. Changes in peak molecular mass, M_p, during the CPC run. From tube 32 to 60, M_p varies little, but the corresponding SEC chromatographic shapes are different (Fig. 6a).

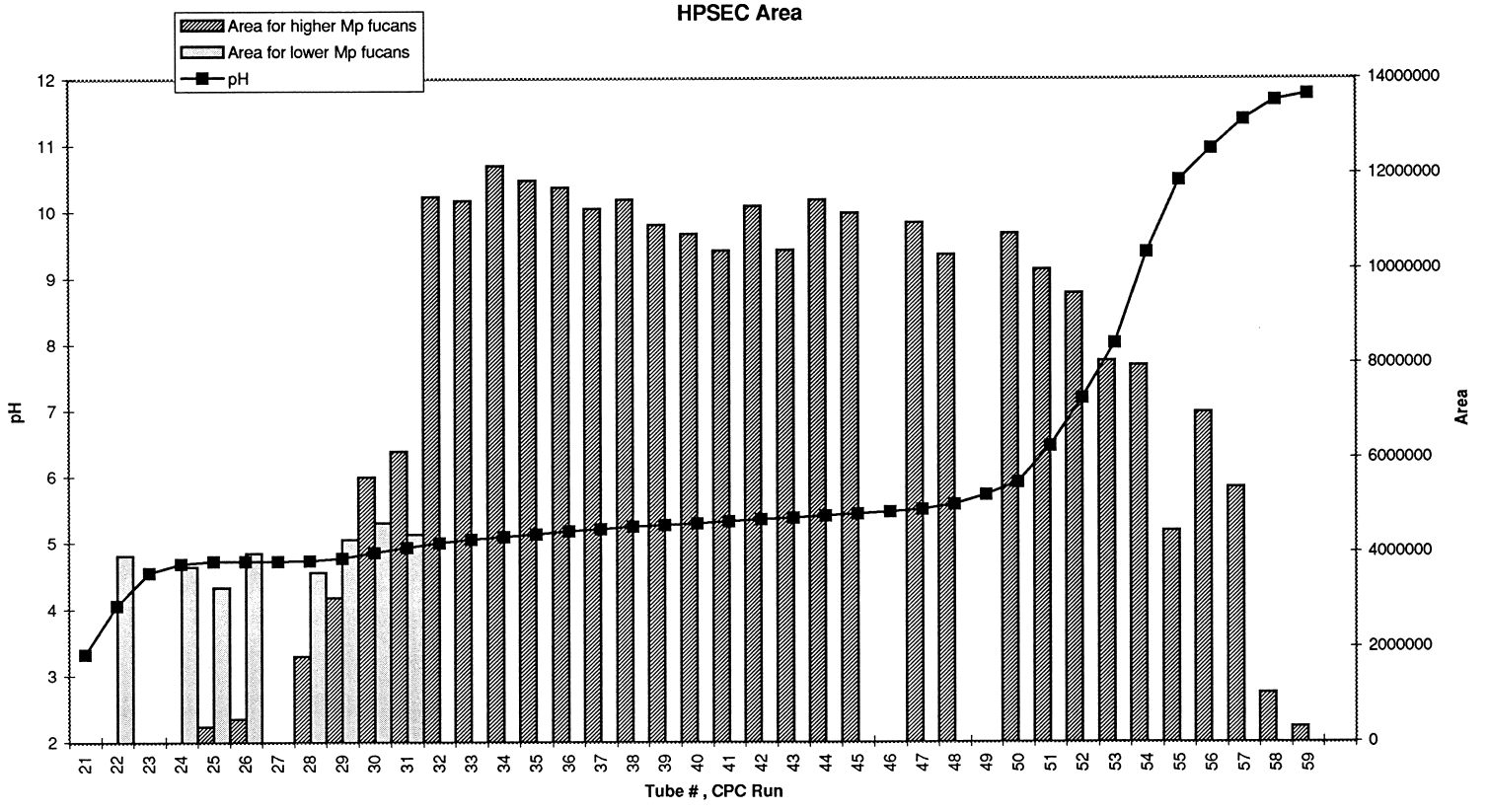

Fig. 8. Changes in SEC peak areas during the CPC run. Most of the sulphated fucans come out between tube 32 and 54, the pH record indicating several fucan families that follow each other in quick succession.

Table 1
Some characteristics of the three fucan families purified by CPC

Family I	Family II	Family III
Average M_p[a]	Average M_p[a]	Average M_p[a]
9500	7900	7100
stdv	stdv	stdv
630	580	980
Average polydispersity	Average polydispersity	Average polydispersity
2.80	2.29	2.51
stdv	stdv	stdv
0.32	0.07	0.37
% polysulphated matter	% polysulphated matter	% polysulphated matter
27.8	33.3	22.0

[a] As noted in Section 2, average M_p must be used only in a relative manner.

employed the hexadecylpyridinium ion and a sodium chloride gradient in a biphasic 1-butanol/water system and observed the beginning of fractionation.

Use of the displacement mode allowed us to discriminate clearly between the various components of the complex mixture of fucans. In fact, this preliminary work provided significant data. There were some non-sulphated polysaccharides in the fucan sample, and ≈83% of the polysulphated matter was rather homogeneous regarding the peak molecular mass, even though it can be regarded as containing three equally present families which need to be studied further. The parameters influencing purification are now under investigation in our laboratory.

4. Conclusion

Ion-exchange displacement centrifugal partition chromatography allowed us to fractionate a sample of polysulphated polysaccharides, which are very polar molecules, on the basis of the molecular mass and the sulphate content of each molecule. This technique is easy to set up, and can be used for other anionic species, provided that they partition into the organic phase containing LA2 under mildly acidic conditions and are back-extracted to the aqueous phase under mildly basic conditions.

It is well-known that column regeneration is the major drawback of conventional displacement chromatography with a solid stationary phase. This drawback does not exist in displacement CPC, since it is quite easy to change the used stationary phase to a new one by pumping it into the CPC column. The used stationary phase can then be regenerated outside the column by washing steps and acidification.

Last but not least, the cost of the liquid stationary phase (MiBK with 10% (v/v) LA2) is extremely low when compared to that of conventional solid exchangers used in HPLC.

Thus anion-exchange fractionation performed with a CPC system seems to be a very simple tool for fractionation of complex mixtures of anionic species from complex media. The homogeneity of fucan fractions needs to be confirmed by further characterization by NMR spectroscopy.

Acknowledgements

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