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Flow field-flow fractionation and characterization of ionic and neutral polysaccharides of vegetable and microbial origin

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Abstract

The flow field-flow fractionation (FlFFF) analysis of a variety of neutral as well as ionic polysaccharides from plants and micro-organisms shows the generally broad distribution in molecular size of these polymers. This result is also obtained on a commercial sample of pullulan whose size distribution appears much wider than that of any of five standard fractions of the same polymer. Clear evidence of some physico-chemical properties of the polysaccharides is given by the study of the effect of the carrier ionic strength on salep, oxidized salep and konjac, carboxymethylcellulose and hyaluronic acid. While neutral polysaccharides, regardless of their origin, only slightly change size distribution in the presence of a simple electrolyte in solution, charged polymers, either naturally charged or chemically ionized, consistently exhibit very low retention levels in water which dramatically increase even at low salt concentrations. Exclusion mechanisms, including steric effects, are shown to be responsible for the anticipated retention times in water of these species that assume the expected statistical coil behavior only when electric charges are screened by the added electrolyte. Under these conditions, higher retention levels are obtained because the volume adjacent to the accumulation wall becomes more accessible to the sample during relaxation. On the basis of these findings, the elution behavior of a number of polysaccharide samples in-laboratory obtained from the fungus *Aureobasidium pullulans* under different incubation conditions is attributed to the presence of species varying in physico-chemical properties and molecular size.

Keywords: Flow field-flow fractionation; Field-flow fractionation; Polysaccharides; Pullulan; Salep; Konjac; Carboxymethylcellulose; Hyaluronic acid; Exopolysaccharides

1. Introduction

A procedure to extract starch from cereals has been known since the 2nd century BC. Polysaccharides however, have attracted novel scientific interest in the last few decades when the potential of custom-made products with peculiar structural and functional properties has begun to be realized. A variety of these biocompatible and biodegradable, naturally occurring polymers may currently be obtained with given composition and molecular mass through biotechnological processes. Unlike other biopolymers such as proteins whose genetically controlled synthesis yields perfectly monodisperse products, polysaccharides are most often found as polydisperse mixtures of species with varying molecular size and composition. As the most widely

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occurring components in living systems, both of animal and vegetable origin, polysaccharides may be synthesized inside the cells (capsular polysaccharides, CPS), outside (exopolysaccharides, EPS) or in stepwise processes involving capsular production of some precursor and a final stage in the exocellular matrix. Depending on their role of either structural support, protective agents or energy supply they may be major components of cell walls (as in plants and bacteria), of the exocellular matrix, or rather be accumulated inside the cells [1].

1.1. Plant polysaccharides

By far the prevailing structural polysaccharide found in vegetable systems, cellulose forms as much as 98% of the cotton fibre. Nevertheless, it is usually obtained from wood which only contains 40–50% of the polymer. Unexpectedly, this linear β(1-4) linked highly crystalline polymer of glucose is also found in the exocellular matrix of some lower invertebrate species.

One of the few existing homopolysaccharides besides cellulose, widely occurring as reserve material, is mannan the (1-4) linked polymer of β-D-mannose. Similar to cellulose both in stereochemistry and structure, it gives rise to a large number of copolymer carbohydrates when some of the mannose units are replaced, in an apparently random fashion, by (1-4) linked D-glucose residues [2]. Glucomannans found in the hemicellulose as well as in the roots, bulbs and tubers of many plants are generally considered linear random block copolymers even though species with sidechains of α-(1-6) linked D-galactose units are known. A polysaccharide of this type the salep glucomannan, produced by orchid tubers (*Orchis morio, Tubera salep*) as reserve material [3] and used as low-calorie dietary product, is regarded as a neutral linear molecule with ca. 1:3.6 glucose/mannose ratio [4] in which the sporadic lateral chains (ca. seven per molecule) do not seem to be long enough to affect molecular conformation. A more extensively employed glucomannan referred to as konjac mannan [2,5–7], is obtained from the tuber of *Amorphophallus konjac*, a plant of the family Araceae containing ca. 60–80% of the polysaccharide. This water-soluble glucomannan, used in traditional oriental cuisine for over 1000 years as flour and reported to have hypocholesteroleric activity [8], has recently been introduced into Europe and the USA as a thickening additive for gravies and sauces as well as a dietary product. On enzymatic hydrolysis, konjac glucomannan yields a variety of different oligosaccharides suggesting the absence of a simple repeat structure. Although the structure of konjac glucomannan has not yet been unambiguously determined, the mannose/glucose ratio seems to be rather consistently 1.6 with about one acetyl group every 17 monomers [2], conferring solubility to the molecule.

1.2. Microbial polysaccharides

Polysaccharides from micro-organisms such as archaeobacteria, soil bacteria, unicellular algae, and fungi, have been shown to be highly attractive for their peculiar features [9]. Due to the uncommonly high number of starting monosaccharides, these polymers possess remarkable structural and compositional diversity. The composition and generally highly regular structure, which imparts to them better rheological properties, seem to be determined by the micro-organism strain as well as by the growth conditions [9,10]. The production of these macromolecules may hence be customized by carefully selecting the cell strain and controlling the incubation conditions or, as more recently reported, by genetically modifying specific micro-organisms [11]. A particularly attractive feature of the microbial exopolysaccharides is that they may be easily obtained from cell cultures by simply removing the micro-organisms by centrifugation and then purifying the product after precipitation from the culture fluid. Of all non-ionic polysaccharides the fungal pullulan from *Aureobasidium pullulans* is by far the most studied and best characterized [10]. Even though there is no evidence of toxicity to men and animals, pullulan seems to have toxic activity to some plants, either when they are infected by the fungus, or if an extremely diluted solution of the commercial polysaccharide is injected directly into the plant lymphatic system [12]. Pullulan is not the only exopolysaccharide produced by the ubiquitous *A. pullulans*. This fungus with an impressive metabolic and ecological versatility is also known for synthesizing acidic polysaccharides and β-glucans

[13]. In contrast, pullulans appear to be α-glucans which, unlike the β-glucans produced by several fungi, are synthesized only by two fungal strains, namely *A. pullulans* and *Tremella mesenterica*. The ratio for the α(1-4)/(1-6) glucosidic linkages has suggested a polymaltotriose structure for pullulan, which thus should assume a linear conformation and the behavior of a flexible random coil in solution. Although the linear structure for pullulan is currently widely accepted, occasional branching has been reported together with variations in composition. Based on these and other experimental results, it has been speculated that pullulan is not the only exopolysaccharide secreted by *A. pullulans*. Indeed the polysaccharide output by this fungus seems to depend in composition, molecular mass and yield on several fungal growth conditions, including pH, phosphate content in the medium, type and concentration of the carbon source, incubation time and temperature [14]. Pullulan from *A. pullulans* has also been obtained using a number of different substrates [15].

One of the major components of the cell surface in humans as well as in animals are glycosaminoglycans, among which hyaluronan (also hyaluronic acid) is widely studied. Hyaluronan is a highly regular, linear polymer of a β(1-4) linked disaccharide repeat structure containing a unit of D-*N*-acetylglucosamine β(1-3) bonded to D-glucuronic acid [16]. Since the p*K* of the carboxyl group is 3–4, at pH 7 hyaluronan is a polyanion. Unlike other polyanionic glycosaminoglycans such as chondroitin sulfate, keratan sulfate and heparan sulfate that can exist in a large number of chemical structures, only one form of hyaluronan is known. For this reason, it is a highly biocompatible macromolecule widely used in biomedical applications as well as being a starting component of novel biomaterials [17]. Refined investigations of this macromolecule generally considered an expanded random coil polymer in solution [18] suggest the presence of an extensive intramolecular hydrogen-bonded structure that also involves water molecules. Both the intramolecular H-bonds and the water bridges between acetamido and carboxylate groups would strongly contribute to stiffen the chain [19]. A flat, tape-like secondary structure of hyaluronan recently elucidated, exhibits extensive hydrophobic patches of about eight CH units [20]. These structures combined with the presence of charged sites on the carboxyl have led to attribute amphiphilic properties to this polymer with the hydrophobic patches responsible for its aggregation behavior [21].

In this work, we report results of the flow FFF analysis of a number of neutral as well as charged polysaccharides of different origin, composition and conformation. Through the determination of the coil statistical size in water as well as in aqueous electrolyte solutions, we show the capability of flow FFF to manifest the polyelectrolytic as well as the neutral character of these macromolecules and to provide evidence for any aggregation behavior of the amphiphilic species.

2. Theory

The analytical technique named field-flow fractionation (FFF) encompasses a number of separation methods characterized by the transverse compression of samples induced by an external field orthogonal to a laminar non-uniform flow. The extensive literature published since the theoretical fundamentals of FFF were first introduced [22] has shown its extensive applicability. Versatility in analyzing aqueous as well as non-aqueous systems, accuracy in yielding a sample's physico-chemical parameters, and flexibility in retention control are outstanding features of field-flow fractionation, which has been successfully applied to the separation of charged and uncharged species of any conformation and shape in an unparalleled dimensional range [23,24]. Because of the nature of the field, flow FFF is the most extensively employed subtechnique for the analysis of macromolecules of any origin from biological [25,26] to synthetic [27,28]. Although the theory of FFF has been dealt with elsewhere [28,29], we briefly outline the theoretical treatment that allows evaluation of molecular size parameters from retention data.

Because of the high ratio between the thickness *w*, and the other two dimensions, namely the length *L* and the breadth *b* of an FFF channel, the velocity distribution of a stream of liquid flowing along the longitudinal axis is parabolic. The separation of sample components is accomplished, under the effect of the field, by the segregation of molecules differing

for some property into fluid laminae of dissimilar velocities. The convective flux of particles (or molecules) induced by the field, which compresses them on the channel accumulation wall, and the counteracting diffusive flux yield an exponential concentration distribution whose mean thickness ℓ is given by

$$\ell = \frac{kT}{F} \tag{1}$$

where k is the Boltzmann constant, T the absolute temperature and F the field-force. By recognizing that a force acting on a particle is related to its velocity U through the friction coefficient f as $F = fU$ and using this correlation in Eq. (1), the following expression for ℓ is obtained

$$\ell = \frac{kT}{fU} \tag{2}$$

Eq. (2) shows the dependence of ℓ on a particle characteristic ratio kT/f which is Einstein's expression for the diffusion coefficient D. The latter inserted into Eq. (2) gives

$$\ell = \frac{D}{U} \tag{3}$$

By measuring the protrusion of molecules into the channel, where the carrier liquid velocity varies with the distance from the walls, ℓ determines the zone elution velocity hence retention time t_r. A general expression for t_r shown in Eq. (4) relates this parameter to the emergence time of a non-retained species t^0 and to the dimensionless space constant λ defined as ℓ/w

$$\frac{t_r}{t^0} = \frac{1}{6\lambda\,[\coth(1/2\lambda) - 2\lambda]} \tag{4}$$

λ appears to be a function of the particle/molecule hydrodynamic size when D in Eq. (3) is replaced by the Stokes–Einstein equation for the diffusion coefficient $D = kT/3\pi\eta d_h$, where η is the fluid viscosity. If U is obtained from the volumetric crossflow-rate as $U = \dot{V}_c/bL$ and w is scaled to the channel void volume V^0 according to $w = V^0/bL$, the dependence of λ on the analyte hydrodynamic diameter d_h appears

$$\lambda = \frac{kT}{3\pi\eta d_h}\frac{V^0}{\dot{V}_c w^2} \tag{5}$$

The hydrodynamic size of the eluting molecules may thus be computed as

$$d_h = \frac{kT}{3\pi\eta}\frac{V^0}{\dot{V}_c w^2}\frac{1}{\lambda} \tag{6}$$

after the λ value has been obtained from measured retention times.

3. Experimental

3.1. Apparatus

The F1000 flow FFF channel from Postnova Analytics (Salt Lake City, UT, USA) previously described [29] has been used with two different types of ultrafiltration membranes laid on the accumulation wall. Of these membranes, both of regenerated cellulose from Millipore (Bedford, MA, USA) the PLBC had a 3 kDa molecular weight cut-off, whereas for the PLAC, this value was 1 kDa. The two HPLC pumps providing the transport flow and crossflow were, respectively a Shimadzu LC-9A (Kyoto, Japan) and a Perkin-Elmer Series 2 (Norwalk, CT, USA). The correct mass balance of the liquid entering and exiting the channel flow line was obtained by adjusting a variable back-pressure regulator (Alltech Associates, Deerfield, IL, USA) placed at the channel outlet, with the aid of a second, constant back-pressure regulator from Upchurch (Oak Harbor, WA, USA) connected to the crossflow outlet. The on-line tee-union, equipped with a silicon septum, serving as injector, was separated from the channel inlet by a zero dead volume filter of 0.45-μm pore size from Upchurch. Detection was obtained by the differential refractive index detector RID 10-A from Shimadzu. The polysaccharide elution profiles were stored on computer and processed using in-house data reduction software. The stop-flow procedure was always carried out using the theoretically calculated stop-flow time.

3.2. Reagents and samples

All the polysaccharide samples were provided by Professor Vittorio Crescenzi of the University ''La Sapienza'' of Rome (Rome, Italy). The standard pullulans with molecular masses 12 200, 48 000, 100 000, 186 000 and 380 000 Da were from Showa Denko K.K. (Tokyo, Japan), whereas the commercial sample of the same polymer, denominated PF-20, was produced by Hayashibara Co (Tokyo, Japan). The sample of salep glucomannan, labelled B, was in-laboratory obtained by extraction from the tubers of the orchid *T. salep*, following a procedure reported in the literature [30], whereas the salep C from *O. morio* is a commercial product available in natural product stores. An aliquot of the salep C subjected to TEMPO mediated C6 oxidation with sodium hypochlorite and bromide at pH 9.5 [31] yielded the corresponding oxidized polyuronic acid with an estimated degree of oxidation of ca. 80%. The same oxidising procedure applied to a sample of konjac glucomannan extracted from Nutricol flour (FMCO, Brussels, Belgium) yielded a degree of oxidation similar to that obtained for the salep C. The two samples of hyaluronic acid of different molecular mass were produced by Fidia Advanced Biopolymers (Abano Terme, PD, IT) from micro-organisms. No information from the producer was available for the commercial sample of carboxymethylcellulose (CMC) from BDH (Germany).

This work also reports the study by flow FFF of a number of mixtures of exopolysaccharides obtained from the ATCC 42023 strain of *A. pullulans* [32]. The polysaccharides produced according to a reported procedure [14] were separated from the fungal growth medium by precipitation after different fermentation periods. All polysaccharide samples except the $EPS_{GLU\text{-}GA}$ were obtained employing only glucosamine as the nutrient. Samples EPS_2, EPS_3 and EPS_5 were collected after 2, 3 and 5 days of fungal growth, respectively without changing either the nutrient or the pH. The polysaccharide mixtures denominated $EPS_{GA\text{-}GA}$ and $EPS_{GLU\text{-}GA}$ were precipitated from the culture broth after 7 days, but different culture liquids and pH conditions were employed. Specifically, to obtain the $EPS_{GA\text{-}GA}$ sample, the *A. pullulans* was incubated for 2 days at pH 2 using glucosamine as the nutrient. The pH was then increased to 6 without changing the carbon source. The sample $EPS_{GLU\text{-}GA}$ was produced by the same micro-organism under identical conditions used for the $EPS_{GA\text{-}GA}$, but after 2 days the glucosamine substrate was replaced by glucose. Concurrently the pH was raised to 6. A list of all the samples investigated showing their origin and producer is reported in Table 1. All samples ready for analysis as

Table 1
Polysaccharide samples, their origin and supplier

Name	Origin	Producer
Pullulan P10	*A. pullulans*	Showa Denko, Japan
Pullulan P50	*A. pullulans*	Showa Denko, Japan
Pullulan P100	*A. pullulans*	Showa Denko, Japan
Pullulan P200	*A. pullulans*	Showa Denko, Japan
Pullulan P400	*A. pullulans*	Showa Denko, Japan
Pullulan PF-20	*A. pullulans*	Hayashibara Co., Japan
Salep B Glucomannan	*T. salep* tubers	In-laboratory obtained by Professor Crescenzi
Salep C Glucomannan	*O. morio* tubers	Natural product store
Sodium hyaluronate A and B	Micro-organisms	Fidia Advanced Biopolymers, Italy
Carboxymethylcellulose		BDH, Germany
Konjac Glucomannan	*Amorphophallus konjac* tubers	FMC Co, Belgium
Exopolysaccharides EPS	*A. pullulans*	In-laboratory produced by Professor Crescenzi

powder or lyophilized material were dissolved in the same liquid used for the flow FFF experiments at a concentration of about 1 mg/ml. The total injected mass of polymer was typically 3 μg unless otherwise stated. Sample solutions were stored at 4 °C for a few days. After this period of time, they were freshly prepared. Prior to injection, samples were allowed to reach room temperature which was also the working temperature of the flow FFF system. The distilled water serving as both the carrier liquid and sample medium was deionized and filtered through an ion-exchanger and ultrafiltration device from USF (Ransbach-Baumbach, Germany) before use. The sodium sulfate chosen to adjust the ionic strength was from Carlo Erba (Milan, Italy). For the extrapolation of sample parameters, the temperature measured during the experiments was always used.

4. Discussion

FFF theory allows to derive values of the hydrodynamic diameter of an analyte from retention parameters without the need for reference materials. Absolute values of d_h are therefore obtained by this technique since no calibration with known standards is required.

The hydrodynamic size of non-rigid particles does not strictly coincide with any geometrical parameter but it rather reveals the behavior of an object suspended in a liquid when it moves under the effect of an external force. It is known from polymer theory that the hydrodynamic dimension of flexible chain macromolecules such as most polysaccharides (and which is generally much larger than that of the molecules of the surrounding medium) is primarily determined by the polymer conformation but is strongly affected by interactions among polymer segments and between the latter and the solvent molecules. Changes in the medium solvating power, which may be induced by addition of an electrolyte and are particularly pronounced in water, greatly influence the relative extent of these interactions. Introduction of charged sites into a macromolecule has an even stronger effect on its overall hydrodynamic behavior. The tremendous increase in free energy, in this case, may only be compensated by chain elongation and the macromolecule appears to have a more or less rigid conformation depending on the type of monomer and charge density. The evaluation of the hydrodynamic diameter of macromolecules hence gives clear indications of the solution behavior of a polymer.

4.1. Pullulan PF-20

The flow FFF analysis of five narrowly-disperse pullulan standards with molecular weights 12 200, 48 000, 100 000, 186 000 and 380 000 Da shows their narrow distribution as illustrated in Fig. 1 also by their gaussian symmetry. In contrast, a commercial sample of pullulan investigated by flow FFF under identical experimental conditions reveals in the profile displayed in Fig. 1 the wide molecular size distribution of the polysaccharide obtained from the same *A. pullulans*. Both the dimensional range, much larger than that of any pullulan standard, and peak asymmetry indicate the polydispersity in size, which ranges from a few nanometers to about 45 nm. The effect of the medium ionic strength (I) on this neutral polysaccharide was also studied, and is illustrated in Fig. 2 by the size distribution curves obtained in solutions of sodium sulfate of increasing

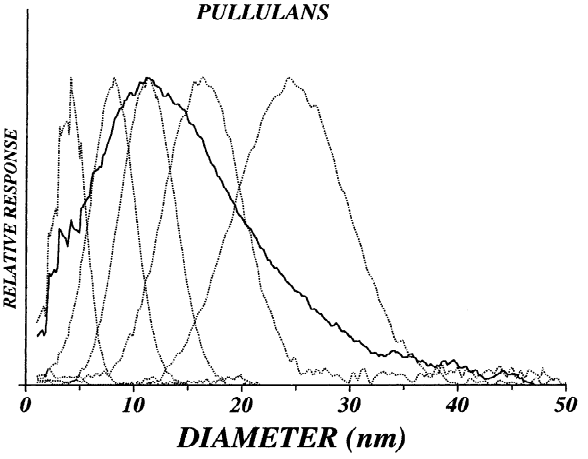

Fig. 1. Size distribution of the 10-μg injection load of the pullulan commercial sample PF-20 (solid curve) overlaid to the distributions of the five pullulan standards (dotted curves). Injection mass for each standard was 3 μg; axial flow-rate and crossflow-rate were 0.2 and 0.6 ml/min, respectively, in the water carrier.

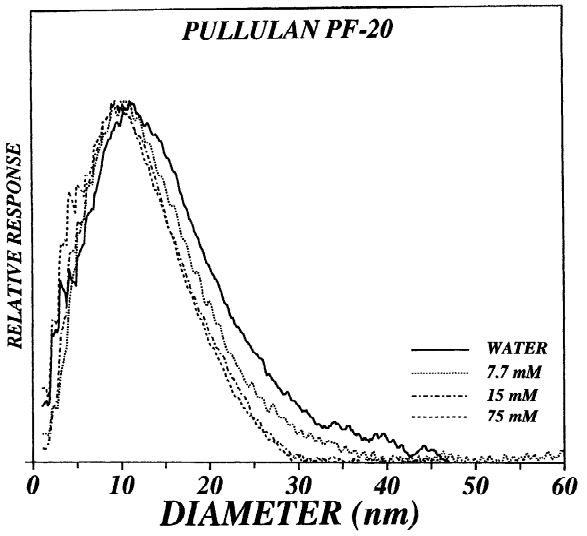

Fig. 2. Overlaid size distributions of the pullulan PF-20 obtained in Na_2SO_4 aqueous solutions of the ionic strength shown in the figure; 10 μg of sample were always injected. Flow conditions as in Fig. 1.

concentration. Inspection of Fig. 2 shows a slight narrowing of the size distribution of the PF-20 when the electrolyte is added to the aqueous medium in the amount needed to obtain 7.7 m*M* ionic strength and, on further increase in the ionic strength to 15 m*M*, a more limited reduction of the size interval is observed. The latter however does not appear to vary remarkably beyond this salt concentration. Addition of an electrolyte to a polymer solvent, particularly when this is water, reduces its solvating power thereby making polymer–solvent interactions less favoured than in the absence of electrolyte. This phenomenon, which induces reduction of the macromolecule's volume to an extent that depends on the polymer properties, is strongly magnified for polyelectrolytes. For these polymers, in fact, the presence of an electrolyte in the solubilizing medium also modulates the effective macromolecular charge. Therefore, because of the neutral character of the pullulan polysaccharide, the rather modest effect of the added salt on the size distribution is not unexpected. As discussed later, charged polysaccharides have a dramatically different behavior in water compared to that in salt solutions.

4.2. Salep

Contrary to pullulan, salep is an eteroglucan. Two samples of this copolymer of glucose and mannose expected to have neutral character [2] were analyzed by flow FFF. Specifically, a commercial product marketed as dietary food, the salep C, and a sample prepared in-laboratory from orchid tubers of *T. salep*, the salep B, were fractionated using water and Na_2SO_4 aqueous solutions of increasing ionic strength. From the distribution curves of the salep C displayed in Fig. 3, it appears that similarly to the pullulan bearing neutral monomers, the presence of an electrolyte in the solubilizing medium has a moderate effect on this sample's molecular size, very slightly affecting only larger molecules. From the investigation of the two samples of this glucomannan carried out using water as eluent and shown in Fig. 4, the salep B appears to exhibit a dimensional distribution similar to that of salep C, which roughly spans from 2 to 30 nm, but with a long tail of very low concentration extending up to 50 nm probably due to a different purification procedure.

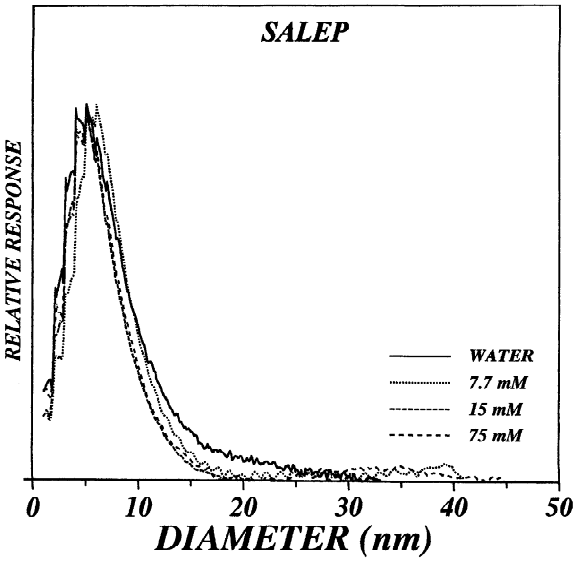

Fig. 3. Overlaid size distributions of 5 μg of the glucomannan salep C extrapolated from FlFFF retention parameters in solutions of the ionic strength reported in the figure. Flow conditions for all the experiments were the same as in Fig. 1.

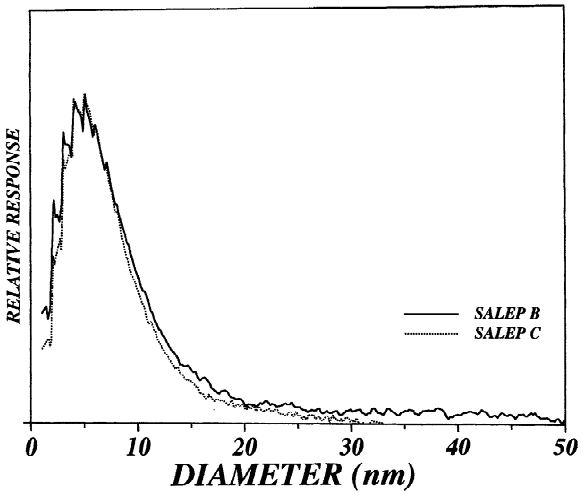

Fig. 4. Superimposition of the size distribution of the glucomannan samples of salep B and C acquired in water. Sample load was 3 μg for each polymer. Same flow conditions as in Fig. 1 for all the experiments.

Oxidation of the primary hydroxyl groups of the sugar units to carboxyl converts the salep C to the corresponding charged polysaccharide. The fractograms obtained using pure water as carrier liquid, displayed in Fig. 5a, show the glucomannan's elution profile before and after sample oxidation. The time-based curves rather than the size distributions, are shown in this illustration because the very low retention undergone by the oxidized sample would not yield meaningful values of the molecule hydrodynamic size. At a first examination, the dramatic effect of oxidation evidenced by the anticipated retention time could be attributed to either the effect of the charged sites, introduced into the macromolecule by the oxidation reaction, or to a possible chain degradation. Reduced retention of charged samples in aqueous flow FFF previously reported [27–29] has been attributed to the increased elevation of the sample eluting zone, which drives molecules into regions of faster streamlines where they are swept at higher velocities down the channel. Augmented molecular effective volume caused by the double layer (whose thickness decreases with the ionic strength), chain expansion by intramolecular repulsion, and particle–particle electrostatic interactions, are considered responsible for the enlargement of the zone in the aqueous flow FFF of polyelectrolytes. These phenomena, generally correlated, are identified as volume exclusion effects. In the case of

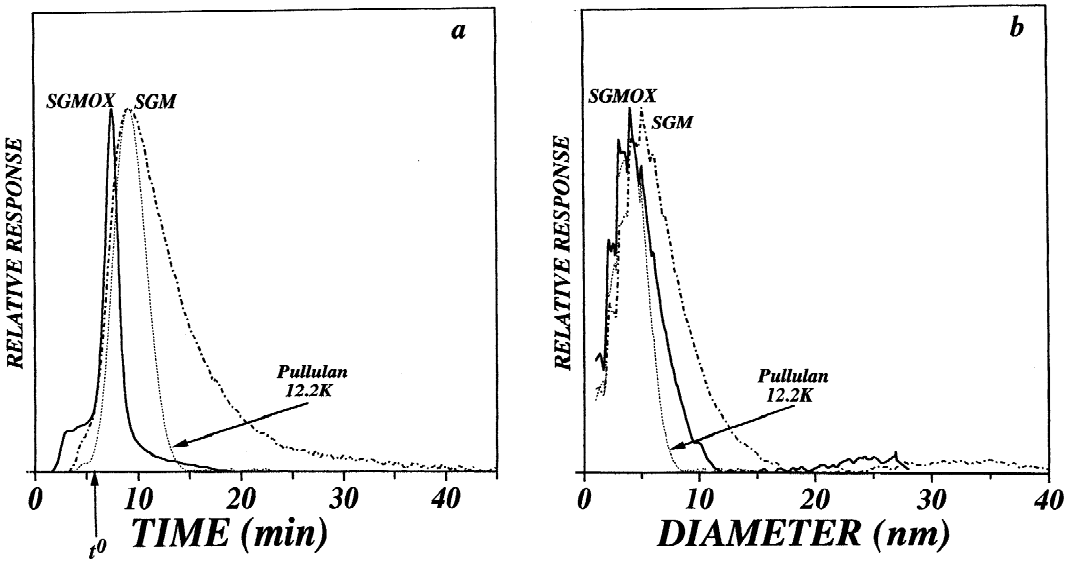

Fig. 5. (a) Time-based flow FFF fractograms of oxidized salep C (SGMOX) and its parent sample (SGM) and of the pullulan standard of 12 200 Da. The water eluent was supplied at the same axial and crossflow-rate used in Fig. 1. (b) Size distributions of the untreated salep C (SGM), oxidized salep C (SGMOX), and of the pullulan standard of nominal molecular mass 12 200 Da. All the profiles were obtained from FlFFF in Na_2SO_4 at ionic strength 75 m*M* with the channel flow-rate of 0.2 and crossflow-rate of 0.6 ml/min.

polysaccharides, exclusion of volume may be dramatically magnified by the large and stiff repeat units, which increase the polymer persistence length and hence the overall molecular volume. Therefore, introduction of electrically charged sites into these macromolecules is expected to induce considerable chain expansion and stiffening by intra-molecular repulsion. These effects however, should be reduced by the screening action of a simple electrolyte to an extent that depends on the polyelectrolyte charge density and structure, and on the salt concentration.

Analysis of the oxidized salep C in aqueous sodium sulfate at 75 m*M* ionic strength shown in Fig. 5b, allows to rule out serious chain degradation. From the size distributions of the oxidized salep C (SGMOX) and of its parent molecule (SGM), it appears that the ionic strength (Fig. 5b) induces a remarkable broadening of the distribution of the polyelectrolytic carbohydrate, which, contrary to that registered in water (Fig. 5a), almost reproduces that of the 12 200 Da pullulan and overlays that of the original sample in the lower dimensional range. A similar influence of the simple electrolyte added to the carrier liquid was not observed for the neutral pullulan PF-20 or for the untreated salep C (Figs. 2 and 3). The enlargement of a polymer molecule, in particular when exposed to electrolyte solutions, is known to generally originate from aggregation phenomena induced by hydrophobic interactions established between neutral molecules or at points where in a long chain macromolecule hydrophobic patches are present. The increase in the measured molecular dimensions registered for the polyelectrolytic salep in salt solutions would therefore be expected for the native neutral polymer rather than for the corresponding polyelectrolyte. Broadening of the polyelectrolyte's size distribution in salt solution may thus be reasonably attributed to the reduction of the exclusion phenomena following decreased intra- and inter-molecular electrostatic interactions. The higher flexibility, derived from the reduced intra-particle repulsions, allows the macromolecules to assume a random coil configuration and hence to be compressed into elution zones of lower thickness. The decrease in the double layer thickness further contributes to this mechanism. Both these effects bring about a higher level of retention and larger measured molecular dimensions. Fig. 5b however, indicates that the oxidized salep has a narrower size distribution range than its parent molecule. Although a 75 m*M* ionic strength might not be enough to completely eliminate charge effects, partial degradation undergone by this sample during oxidation cannot be ruled out either. Further analyses of charged polysaccharides discussed later have shown that the very early elution exhibited by the polyelectrolytic salep acid is typical of all ionic carbohydrates.

4.3. Konjac

Konjac, the other glucomannan analyzed by flow FFF, seems to generally have a much higher molecular mass than the salep [2,5], and most of its solution properties are thought to depend on the large molecular mass. Although konjac is a water-soluble polysaccharide, complete dissolution in this solvent may be achieved only under sonication [6] and gives highly viscous solutions. In addition to molecular size, partial aggregation is also considered responsible for the solution viscosity of konjac [2]. The flow field-flow fractionation has been carried out in water on the polyelectrolytic konjac obtained following the same oxidation reaction used for the salep C. Contrary to the neutral polymer, dissolution in water of the corresponding polyelectrolyte is fast and quantitative. As already found for the oxidized salep, this ionic polysaccharide also undergoes dramatic exclusion effects such that the fractogram could not be converted to a size distribution. In fact, as evident in Fig. 6a, where the flow FFF fractogram of the oxidized konjac (KGMOX) is superimposed on that of the 12 200 Da pullulan, the konjac appears to elute as a narrow peak almost corresponding to the void volume. Substitution of the water carrier with a Na_2SO_4 aqueous solution of $I=75$ m*M* drastically changes the elution profile of the konjac polyion, which, as manifested in Fig. 6b, now appears to distribute in a much broader size range than that of the lower molecular mass pullulan. Assuming that the phenomena excluding most of the polyelectrolyte molecules from the channel accumulation wall when water only is used are minimized by the addition of the simple electrolyte to the medium, the size distribution determined for the konjac at 75 m*M* ionic strength is more indicative of the effective

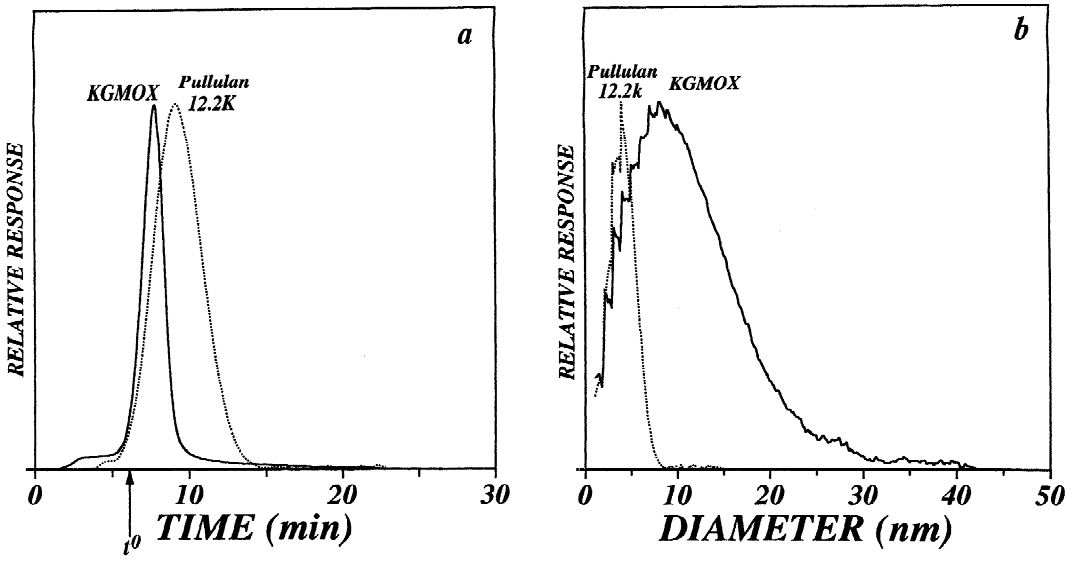

Fig. 6. (a) Aqueous flow FFF fractograms of the oxidized konjac glucomannan (KGMOX) and 12 000 Da pullulan standard. (b) Superimposed size distributions of the oxidized konjac (KGMOX) and of the 12 200 Da pullulan obtained in aqueous Na_2SO_4 at ionic strength 75 m*M*. The injected load for the KGMOX was 10 μg whereas 3 μg were used for the pullulan. Flow conditions as in Fig. 5 for all the experiments.

molecular dimensions. Therefore, a comparative analysis of Figs. 6b and 5b indicates the remarkable difference in the size distribution of the two oxidized glucomannans, the salep and the konjac. The evidence of a much higher molecular weight for the konjac is well in agreement with the literature data. However, it is noticed that because of the molecular degradation often occurring during oxidation, quantitative evaluations obtained on the oxidized samples cannot be extended straightforwardly to their parent molecules.

4.4. Carboxymethylcellulose

Similarly to the ionic carbohydrates previously illustrated, also the polyelectrolyte carboxymethylcellulose exhibits a very low retention level in water as shown in Fig. 7a. The CMC peak however, although very early eluting, bears a long tail suggesting the presence of species of large molecular dimension. At the ionic strength of 7.7 m*M*, this polymer appears to follow the same pattern exhibited by the oxidized konjac and salep and shows a distribution over a wide size range as indicated by the curve displayed in Fig. 7b. It also appears from this figure that the broad and irregular size distribution slightly narrows when the ionic strength is raised to 15 m*M* but it changes considerably when the salt concentration is increased to yield an ionic strength of 75 m*M*. Polyelectrolyte coil contraction under the effect of the added salt could explain the data obtained at both salt concentrations. However, in the 75 m*M* ionic strength solution the variation in the peak profile is so noticeable to suggest an effectively decreased contribution by higher molecular mass species. Since aggregation by CMC at this ionic strength may not be ruled out, species of higher dimensions could be retained by the on-line filter or adsorb onto the ultrafiltration membrane.

4.5. Sodium hyaluronan

The aqueous flow FFF analysis of two samples of different molecular mass of hyaluronan extracted from micro-organisms is illustrated in Fig. 8a. Both fractions, regardless of their molecular mass, elute in a narrow, almost unretained peak, which however, contrary to the pullulan, extends in a long tail at higher retention times. The very low retention reminiscent of the behavior of the ionic polysaccharides

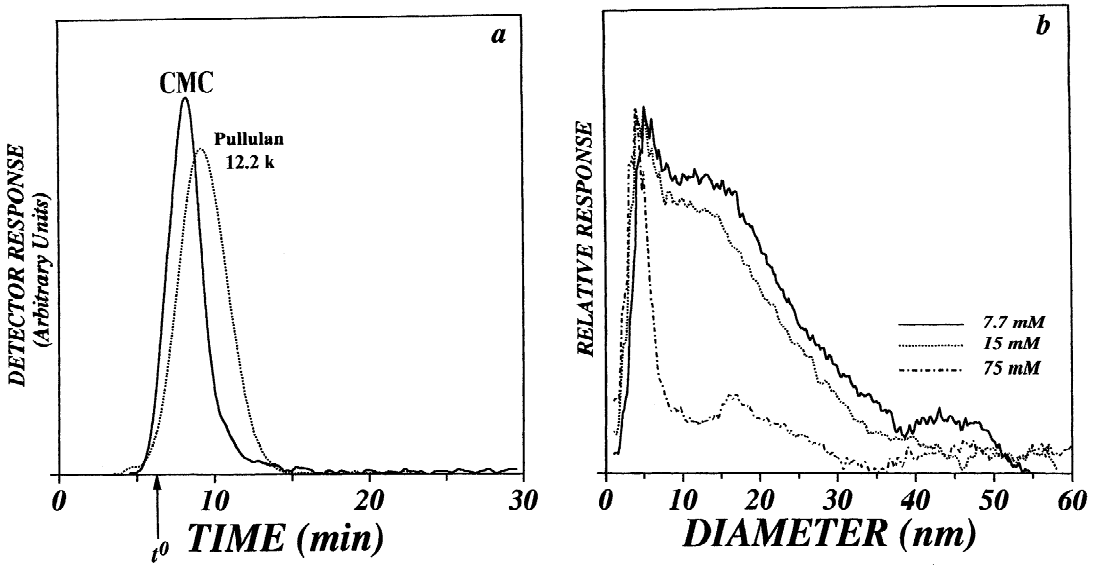

Fig. 7. (a) Overlaid flow FFF fractograms of 3 μg of a commercial sample of carboxymethylcellulose and of 12 200 Da pullulan obtained in water. (b) Size distributions of 15 μg of the carboxymethylcellulose sample, obtained from FlFFF in aqueous sodium sulfate at the ionic strength shown in the figure. Flow conditions as in Fig. 5.

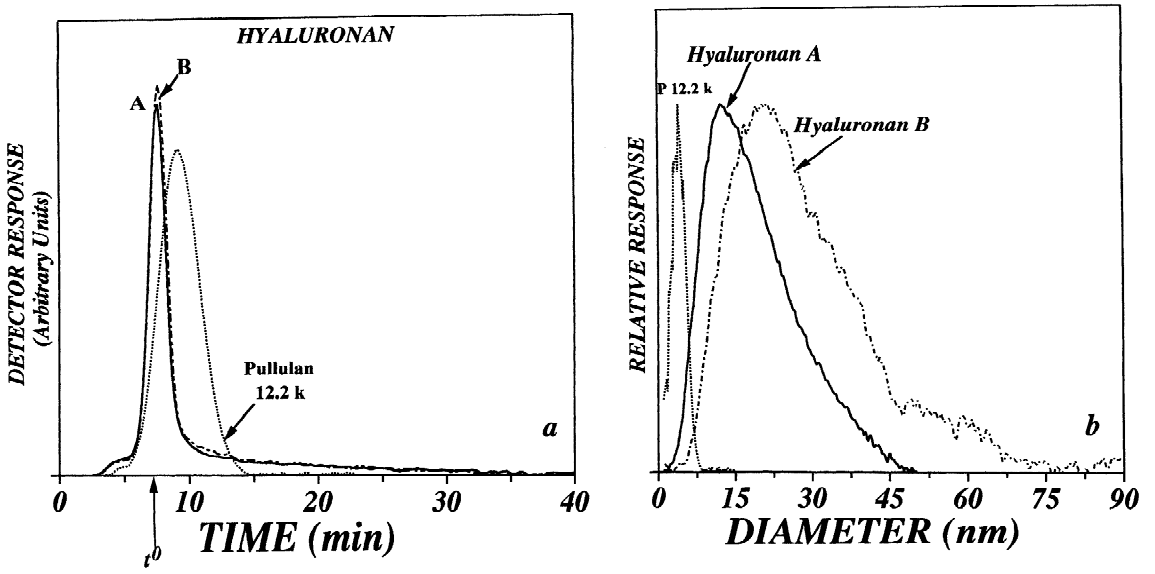

Fig. 8. (a) Time-based fractograms of the samples A and B of hyaluronic acid superimposed on the fractogram of the 12 200 Da pullulan collected using DI water as carrier liquid. Flow-rate and crossflow-rate were, respectively, 0.2 and 0.6 ml/min. Sample load was 3 μg for each polymer. (b) FlFFF derived size distributions of hyaluronan A and B and of the 12 200 Da pullulan standard. The Na_2SO_4 aqueous solution at ionic strength 15 m*M* was supplied at a channel flow-rate of 0.2 ml/min and a crossflow-rate of 0.6 ml/min. The injected load for both the hyaluronan samples was 10 μg.

previously presented was not unexpected for this polymer. It is however worth noting that in the water solvent exclusion phenomena hamper the retention mechanism of this polysaccharide to the point of hiding any difference in molecular mass. It appears from these results that secondary effects in the aqueous flow FFF of charged polysaccharides are predominant and more strongly affect separation than in the case of the synthetic polystyrene sulfonate which, on the contrary, shows a selective, although poorly reproducible elution in water in the molecular mass range 65 000–700 000 Da [27]. Considering the hyaluronan molecular structure and charge distribution, steric exclusion, besides inter-particle charge repulsion, may strongly contribute to its behavior in water. The polymer chain expanded by intra-molecular electrostatic repulsion and hydration appears in fact to behave more as a rigid rod stiffened by the bulky glycan repeat units. The above considerations are fully confirmed by the analysis of the same hyaluronate samples carried out at ionic strength 15 m*M*. As may be seen in Fig. 8b, addition of a simple electrolyte drastically modifies the elution profiles of the two hyaluronan fractions, which now both appear well separated from the pullulan 12 200 Da and demonstrate the overall higher molecular size of fraction B. The effect of higher salt concentrations has been investigated for both samples of this polysaccharide. From Fig. 9a displaying the size distribution of the hyaluronan A, it appears that an increase in the medium ionic strength to 75 m*M* noticeably reduces its molecular size range. However, whereas at the lower ionic strength this sample size distribution spans continuously from ca. 4 to 50 nm, a bimodal profile at the higher ionic strength shows a distinct second peak in the 30–50 nm range. While the reduction in the coil statistical size at the higher ionic strength was expected for this polyelectrolyte, the behavior shown at 75 m*M* ionic strength appears closer to that of amphiphilic polymers [19] whose tendency to aggregation, thus increase in size, is well documented [19,33,34]. A closer inspection of Fig. 9a would suggest that aggregates of this polymer are formed mainly by higher molecular mass species as may be inferred by the decrease in the relative concentration of these particular components in the first peak, which then appear to contribute to the second peak. This assumption seems to be confirmed by the behavior of the higher molecular mass hyaluronan fraction analyzed in salt solutions of increasing concentration. Species of higher molecular mass of

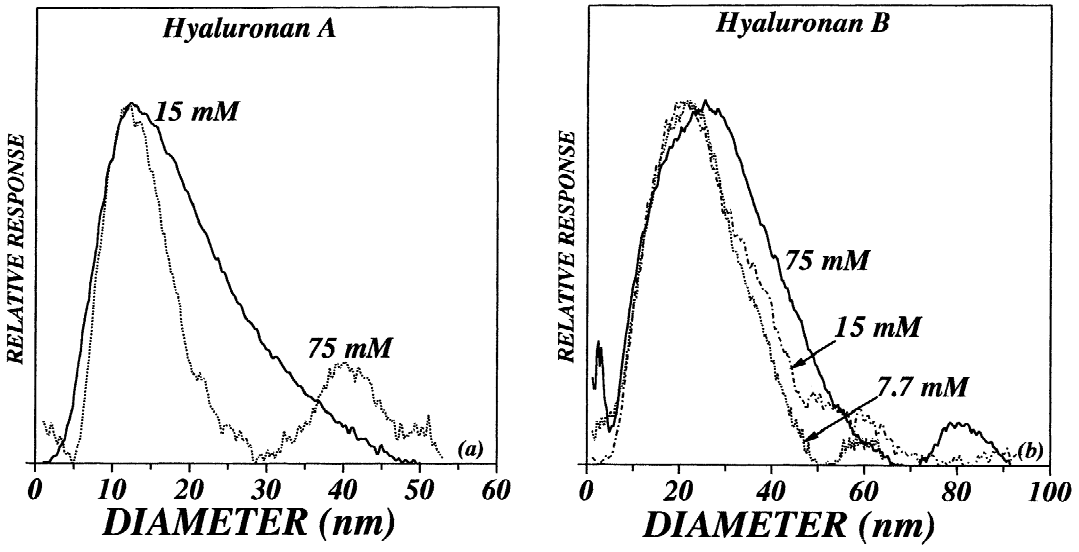

Fig. 9. (a) Overlaid size distributions of the hyaluronan A obtained in solutions of the ionic strength shown in the figure. (b) Size distributions of the hyaluronan B extrapolated by FlFFF in aqueous solutions of sodium sulfate at the reported ionic strength. Flow-rates as in Fig. 8.

the hyaluronan B in Fig. 9b indeed show a consistent tendency to increase their hydrodynamic size and to display a bimodal distribution as ionic strength increases.

4.6. Exopolysaccharides from A. pullulans

As outlined in the Introduction, pullulan is not the only polysaccharide that the *A. pullulans* may synthesize. Recent investigations [14] indicate that this highly versatile micro-organism produces different types of exopolysaccharides whose composition and molecular mass seem to depend on the type of nutrient and incubation time. In particular, when glucosamine is the carbon source, the exopolysaccharides synthesized by the *A. pullulans* appear to be mainly copolymers of glucose and mannose. Exopolysaccharides (EPS) in-laboratory obtained from the ATCC 42023 fungal strain by varying the nutrient, pH and growth time have been analyzed by flow FFF in water and in Na_2SO_4 aqueous solutions of increasing concentration. The analysis in water shown in Fig. 10 suggests that when the fungus is incubated for 2–3 days only as in the EPS_2 and EPS_3 samples, respectively, polymers with broader size distributions are obtained. Without taking into account possible electrostatic effects by charged species, this observation would be consistent with results evidencing the presence of a hydrolysing enzyme produced by the fungus and acting on the polysaccharides [14]. In such a system, chain degradation by hydrolysis is expected to be more extensive the longer the polymer is exposed to the enzyme activity, i.e. the longer the time before separation of the polysaccharides from the growth medium.

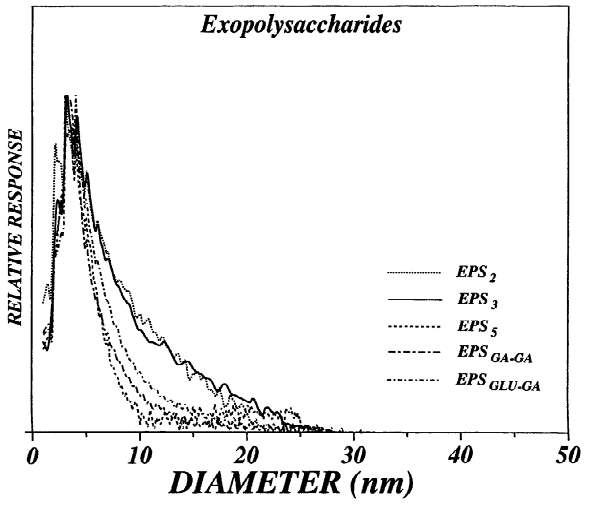

Fig. 10. Superimposition of the size distribution of mixtures of exopolysaccharides produced by *A. pullulans* under different fermentation conditions. The flow FFF experiments were all carried out using water as eluent at a channel flow-rate of 0.2 ml/min and crossflow-rate of 0.6 ml/min; 3 μg of sample were injected in all cases.

Analysis of some of these samples in solutions of increasing ionic strength indicates in all cases a noticeable increase in the size distribution in the presence of the simple electrolyte, but with different patterns depending on the incubation conditions. This behavior, manifest in Fig. 11a–c, and typical of the ionic polysaccharides as previously shown, suggests a polyionic character for at least some of the species present in the mixtures of the exopolysaccharides by *A. pullulans*. In particular the EPS_2 and EPS_3 in Fig. 11a,b seem to be affected by the electrolyte regardless of its concentration. Moreover, higher molecular mass species in these mixtures appear almost unaffected by the electrolyte solution as found for neutral polymers. The presence of a nonionic polysaccharide such as pullulan in these mixtures would agree with NMR studies [32,35] that show considerable amounts of the pullulan in mixtures obtained after a few days of incubation, whose content decreases with time, possibly by enzymatic degradation. By comparison, the mixture of $EPS_{GA\text{-}GA}$ in Fig. 11c would mostly contain charged species subjected to reduction in molecular size by increased salt concentration. In general, the polysaccharides present in these mixtures appear to have rather low molecular masses as shown by their distributions at higher ionic strength. Further investigations however are needed to fully characterize the polysaccharide production by *A. pullulans* under varying growth conditions.

5. Conclusions

The analysis of a large number of polysaccharides structurally and compositionally heterogeneous, shows the capability of the flow FFF technique of providing reliable elution patterns.

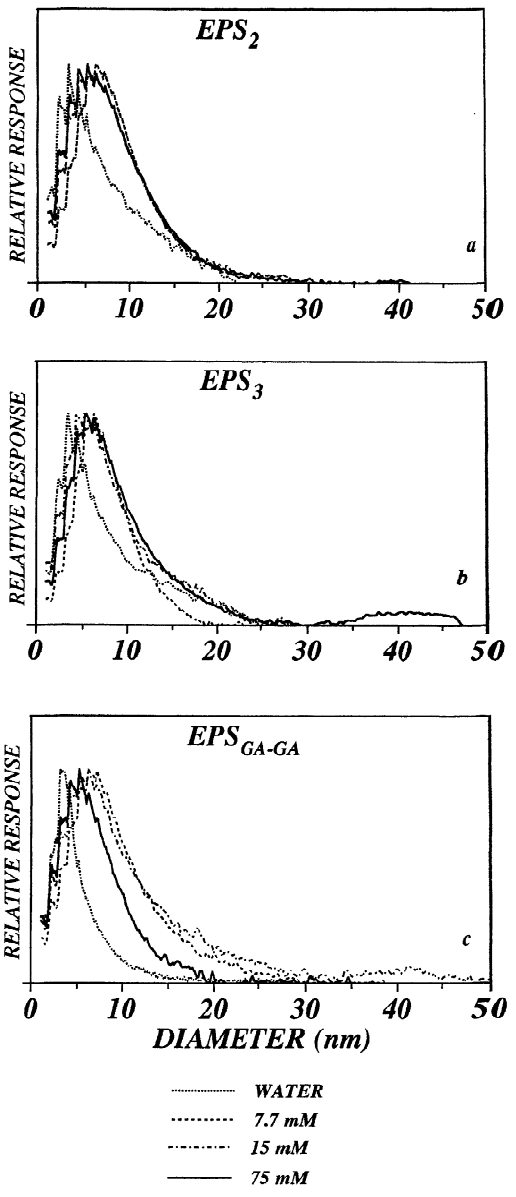

Fig. 11. (a) FlFFF derived size distributions of the mixture of exopolysaccharides obtained from the fungus *A. pullulans* grown using glucosamine as the carbon source in the nutrient. (b) Overlaid size distributions of a mixture of exopolysaccharides produced by *A. pullulans* grown for 3 days using glucosamine as culture nutrient. (c) Superimposed size distributions of the EPS extracted after 7 days of growth from the *A. pullulans* culture medium where the fungus nutrient was glucosamine. The carriers used for the FlFFF experiments were water and sodium sulfate solutions of the ionic strength shown in the legend. Flow conditions in all cases were: channel flow-rate = 0.2 ml/min and crossflow-rate = 0.6 ml/min.

The study of the microbial pullulan indicates that when this polysaccharide is produced under conditions which are not strictly controlled, unlike the standard samples exhibiting low polydispersity, a broad distribution range spanning more than one order of magnitude is observed. In all the samples however, pullulan discloses its neutral character in the modest response to the solvent ionic strength. In contrast, the dramatic effect of ionized groups on the retention of electrically charged polysaccharides is verified by the analysis of the C6 oxidized salep glucomannan and the native compound, a β-linked copolymer of glucose and mannose. While the native neutral form (of molecular size lower than that of the commercial pullulan) exhibits a retention behavior similar to that of the PF-20 pullulan sample in both water and aqueous Na_2SO_4 solutions of increasing ionic strength, the polyuronans obtained by oxidation of the salep, undergo severe exclusion effects in water. Under the experimental conditions used, these phenomena hamper the sample relaxation and thus retention. The remarkable broadening of the size distribution range of this ionic polysaccharide in electrolyte solutions, while confirming a moderate chain degradation undergone during oxidation, indicates that, when water is the flow FFF eluent, electrostatic effects on the retention of charged polysaccharides are more prominent than for the more flexible synthetic polyelectrolytes such as polystyrene sulfonate, which may be separated in a wide molecular mass range although in a poorly reproducible manner. This finding is confirmed by the behavior of all the other charged polysaccharides studied, namely the oxidized konjac glucomannan, the carboxymethylcellulose and the hyaluronan, which consistently display very low retention levels in water, remarkably increasing when a simple electrolyte is added to the solvent even in very low concentration. The two fractions of hyaluronic acid for instance, which do not exhibit their difference in molecular mass when water is the carrier liquid and both elute as unretained peaks, display distinct size distributions in a 15 m*M* ionic strength solution.

Mixtures of exopolysaccharides produced by the fungus *A. pullulans* under different growth conditions, appear from the flow FFF analysis in general as somewhat low molecular mass species having different physico-chemical properties, and size dis-

tributions dependent on the incubation conditions. For some of these samples, the presence of a hydrolyzing enzyme capable of degrading the pullulan molecular chain, considered responsible for this result, is confirmed by independent studies indicating a higher content of pullulan in the polysaccharides mixtures extracted after shorter incubation periods.

Evidences obtained from the study of the effect of the ionic strength on the retention of these mixtures are attributed to the presence also of ionic polysaccharides that contribute to the low retention in pure water. Retention time however increases in low ionic strength solutions as a consequence of the reduction of exclusion phenomena but decreases because of coil contraction as ionic strength increases.

6. Nomenclature

b	FFF channel breadth
d_h	particle hydrodynamic diameter
D	diffusion coefficient
k	Boltzmann constant
f	friction coefficient
F	force exerted by the field
ℓ	characteristic thickness of solute zone
L	channel length
M	polymer molecular mass
t^0	void time
t_r	retention time
T	absolute temperature
U	field-induced velocity
$\dot{V}$	volumetric channel flow-rate
$\dot{V}_c$	volumetric crossflow-rate
V^0	void volume
w	channel thickness

Greek letters

η	fluid viscosity
λ	retention parameter

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