Insight into the Distribution of Molecular Weights and Higher-Order Structure of Hyaluronans and some β-(1→3)-Glucans by Size Exclusion Chromatography

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The effects of high energy ultrasound and slightly raised temperature combined with the denaturing action of dimethylsulphoxide on the molecular weight and higher-order structure of hyaluronans and some β-(1→3)-glucans were studied by means of size exclusion chromatography (SEC) technique. Some experimental conditions connected with the (bio-)polymer sample preparation prior to its SEC analysis are overviewed in the light of informational relevance of studies where the action of a physical and/or chemical agent changes the hydrodynamic size of the m omolecule.

INTRODUCTION

One of the characteristic features of high molecular weight polymers is their polymolecularity, i.e. the fact that a given sample always consists of a certain mixture of polymer homologues. The most general qualitative—quantitative expression of polymolecularity is the determination of the molecular weight distribution of the (bio-)polymer analysed, e.g. the dependence of the weight fraction of a given polymer homologue on the corresponding molecular weight.

Experimental methods of molecular weight distribution determination of (homo)polymers can be substantially divided into twb categories.

The first (classical) category consists of procedures of polymer fractional precipitation and/or dissolution into a series of 'monomolecular' fractions with subsequent determination of the relevant parameters, i.e. the fraction weight and its corresponding molecular weight.

The second, historically younger category, is represented by techniques where the sample is 'divided' into molecularly more homogeneous fractions; these are not isolated but their frequency in the mixture is determined on the basis of changes of a defined physicochemical characteristic of the polymer accompanying the separation process. This category also includes the (high-performance) liquid chromatographic separation method — HPLC operating in the mode of size exclusion chromatography (SEC).

In this paper we are overviewing and commenting on results of HPLC/SEC characterizations of water soluble, electrically uncharged polymers (Mislovičová *et al.*, 1985), represented by schizophyllan and lentinan, as well as characterizations of polymer materials with a polyanionic nature, represented by salts of carboxymethylated glucans (Horváthová *et al.*, 1990, 1991b; Šoltés *et al.*, 1993) and hyaluronic acid or hyaluronans (Chabreček *et al.*, 1990a, 1992; Orviský *et al.*, 1990a, b, 1992; Šoltés *et al.*, 1989, 1992).

Besides a detailed analysis of the methodology of establishing the molecular weight of biopolymers, special attention was paid to the determination of molecular characteristics which document the existence of a certain higher-order structure of the samples of schizophyllan and lentinan, as well as of carboxymethylated glucans.

EXPERIMENTAL

Chemicals and biopolymers. NaCl, $NaNO_3$ and acetone were of p.a. purity degree. The dimethylsulphoxide (DMSO) used was of ultra violet (UV) spectrophotometry quality grade (Lachema, Brno, Czech Republic). Water was of HPLC quality (J. T. Baker Chemical Company, Phillipsburg, NJ, USA).

Reference samples of hydroxyethylated starch (HES; substitution degrees 0.64–0.67; see Table 1) were provided by Dr. Kirsti Granath (Pharmacia Fine Chemicals, Uppsala, Sweden).

Schizophyllan (Fig. 1; injection batch No. 131014) and lentinan (Fig. 2; injection batch No. A 1101) were provided by Dr P. J. Jacques (CIRMAP, Bruxelles, Belgium). Sodium salt of carboxymethylated derivative of glucan (CMG-Na; substitution degree 0.91) isolated from baker's yeast *(Saccharomyces cerevisiae)* was offered by Dr J. Šandula (Chemical Institute, Slovak Academy of Sciences, Bratislava, Slovak Republic). Pleuran—a carboxymethylated derivative of glucan isolated from oyster mushroom (*Pleurotus ostreatus*)—was a gift from Dr Ľ. Kuniak (Chemicotechnological Faculty,

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Table 1. Molecular characteristics of hydroxyethylated starch samples

Given[a]		Determined[b]	
M_w (Da)	M_w/M_n	M_w (Da)	M_w/M_n
9.37×10^5	≈1.5	7.75×10^5	1.48
3.98×10^5	1.28	4.22×10^5	1.28
1.95×10^5	1.20	2.14×10^5	1.25
1.28×10^5	1.20	1.37×10^5	1.23
5.38×10^4	1.34	6.35×10^4	1.25

[a] K. Granath (Pharmacia Fire Chemicals, Uppsala, Sweden) Personal Communication.
[b] Chabreček *et al.*, 1990b.

Slovak Technical University, Bratislava, Slovak Republic). High molecular weight hyaluronan (Fig. 3) was produced by MOVIS, Holíč, Slovak Republic.

CMG–Na fractionation. The CMG–Na sample (1 g) was dissolved in 250 mL of 0.5% aqueous NaCl solution overnight. The insoluble part was separated by centrifugation. Acetone, 220 mL was added to the opalescent sol. The precipitated gel (pre-fraction 9.3%) was separated by centrifugation. A further 60 mL of acetone were added to the supernatant solution and thus Fraction II was precipitated (yield 73.1%).

Fraction II prepared by this procedure was completely soluble both in water and in aqueous solutions of inorganic salts. Its intrinsic viscosity number determined in aqueous NaCl solution (0.1 mol/L) at 25°C was 96.6 mL/g (Horváthová *et al.*, 1989, 1991a; Mislovičová *et al.*, 1992) One part of Fraction II was further fractioned by precipitation (Mislovičová *et al.*, 1992) to prepare subfractions $4A_2$, 4B, and 8 (see Table 2).

Denaturation of glucans by DMSO. The sample—2 mg of glucan (schizophyllan, lentinan, pleuran, Fraction II or its subfraction)—was dissolved in 0.2 mL H_2O overnight. Subsequently 1 mL of DMSO was added to the aqueous solution. The solvent mixture was evaporated at 50°C in a stream of nitrogen to dryness over about 4 h.

The solid evaporate was dissolved overnight in 1 mL of the mobile phase used for HPLC (see legend to Fig. 4) Another set of native glucan samples (2 mg each) was dissolved in the eluent (1 mL) using a similar procedure. Before HPLC analysis the solutions were clarified by centrifugation.

Hyaluronan degradation by ultrasound. The sample—5 mg of native high molecular weight hyaluronan — dissolved in 10 mL of the HPLC eluent was exposed to high energy ultrasound (150 W; 20 kHz) generated by a UGA 20452 device (Tesla, Vráble, Slovak Republic). The molecular characteristics of hyaluronan degraded for 5, 15, 30, 45 or 75 min were determined by HPLC (Chabreček *et al.*, 1990b, 1191a; Orviský *et al.*, 1993).

RESULTS

Figure 4 illustrates the original chromatographic records of a sample pair—native pleuran and pleuran denatured by DMSO. As is evident from Table 2, in the denatured pleuran sample, the use of dimethylsulphoxide or its 'chaotropic' effect resulted in a 12% relative decrease in the parameter studied. A decrease of 16% was observed in the case of Fraction II, and of 23% in the case of lentinan, while the schizophyllan sample exhibited a very large degree of denaturation.

Figure 5 represents normalized chromatographic profiles of native hyaluronan as well as samples degraded by the action of ultrasound. The depolymerizing effect of high energy ultrasound is accompanied by a significant decrease in molecular weight with a simultaneous increase in the polymolecularity parameter M_w/M_n of hyaluronan (see also Table 3).

DISCUSSION

Glucans, glycosaminoglycans, and other polysaccharides

High molecular weight compounds including biopolymers (macrobiomolecules) are a significant component of living organisms (plants and animals), with glucans as the most frequently occurring substance. These homopolysaccharides with α- and β-, (1→3)-, (1→4)-, and (1→6)-glycosidic bonds are either naturally crystalline compounds or can be easily crystallized (Sarko *et al.*, 1983), suggesting a high degree of stereoregularity of their macromolecular chains (see Figs. 1 and 2).

Several β-(1→3)-glucans isolated from mushrooms or yeasts exert a usually immunomodulating effect when administered to mammals including man (Chihara *et al.*, 1982; Di Luzio, 1983, 1985; Maeda, 1989). Of these biologically active natural substances the greatest attention has been paid to water soluble polysaccharides, especially to schizophyllan—an extracellular product of the mushroom *Schizophyllum commune* Fries (Norisuye *et al.*, 1980), and to lentinan isolated from the mushroom *Lentinus edodes* (Berk.) Sing. (Chihara *et al.*, 1970).

In the solid crystalline phase schizophyllan forms a stereoregular associate, which is characterized by a higher-order structure of triple helical type. For lentinan in the solid phase both triple and single helical conformations were reported (Adachi *et al.*, 1990).

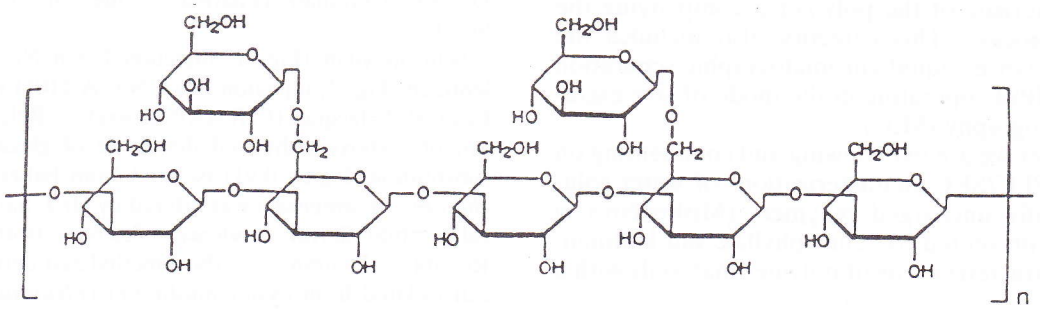

Figure 1. Chemical structure of schizophyllan.

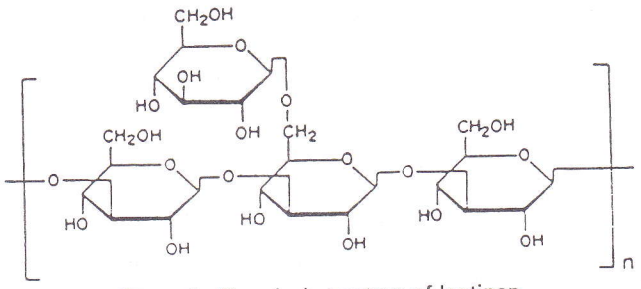

Figure 2. Chemical structure of lentinan.

Glucans isolated from baker's yeast or the oyster mushroom are water insoluble compounds, which considerably limits their clinical applicability. On the other hand, however, their carboxymethylated derivates—the polyanions CMG-Na and pleuran may play a significant role as they are easily soluble in water.

Hyaluronic acid (HA) is a non-branched glycosaminoglycan (muco-)polysaccharide (Fig. 3). For medicinal purposes, high molecular weight ultrapure hyaluronans are recovered from rooster comb tissue, human umbilical cord, and from several other animal tissues (Balázs, 1979).

HA is a polyanion with the mean pK_a value of 3.21 (Ryabina *et al.*, 1987). Owing to their specific rheological characteristics, aqueous solutions of high molecular weight hyaluronans are administered intra-articularly into the patient's knee-joint. Viscoelastic gel solutions of hyaluronans with $M_w > 1 \times 10^6$ Da serve as artificial aqueous humour during ophthalmo-surgical interventions (Balázs, 1979; Healon®, 1985; Van Brunt, 1986).

Dissolution, denaturation, and degradation of biopolymers

In the solid phase, many polysaccharides as well as other biopolymers form amorphous aggregates, associates bearing crystallinities, and true crystals stabilized by intra- and intermolecular hydrogen bonds, often with the contribution of incorporated water molecules (Park and Chakrabarti, 1978; Sarko *et al.*, 1983; Turner *et al.*, 1988) In triplet organization, for example, three macromolecular chains with parallel orientation are wound together, with the resulting associate 'physically crosslinked' with many hydrogen bonds. Such multiply helically-wound structures provide compact, highly dense, water insoluble, however strongly hydrated structural elements building cell walls and polysaccharide reserves (Sarko *et al.*, 1983).

Dissolution of such substances in water often leads only to a more or less extensive swelling of the biopolymer, i.e. only to the formation of a gel/colloid consistence, which is an aggregate of many macromolecules. Determination of the molecular weight of substances

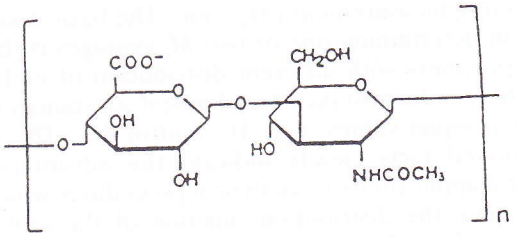

Figure 3. Chemical structure of hyaluronan.

Table 2. DMSO induced changes of molecular weight parameters of glucans[a]

Glucan sample	$[(M_{wN} - M_{wD})/M_{wN}] \times 10^2$ (%)	M_{wN} (Da)
Schizophyllan	→ 100	6.24×10^5
Lentinan	23	2.66×10^5
Pleuran	12	7.62×10^5
Fraction II	16	7.63×10^5
$4A_2$	6.4	7.04×10^5
8	3.4	5.28×10^5

[a] M_{wN} and M_{wD} represent weight average of molecular weights of native (N) and denatured (D) glucan sample. The HPLC device was calibrated by reference HES samples.

with such a consistence (dispersion, sol) is therefore irrelevant.

In the light of the above mentioned facts alkalization with the addition of e.g. NaOH, which disrupts the physical crosslinks, is recommended for the preparation of molecularly true aqueous polysaccharide solutions (Saitô *et al.*, 1979; Sarko *et al.*, 1983). Another procedure is the use of polar organic solvents (DMSO, 4-methylmorpholine-*N*-oxide (James *et al.*, 1990), cadoxen, Schweitzer's agent, etc.) alone or with water, as well as application of 'physical agents' such as increased temperature, microwave irradiation, action of ultrasound, etc.

However, such procedures of biopolymer dissolution usually lead to destruction of the higher-order structure of the sample, i.e. to irreversible denaturation or even

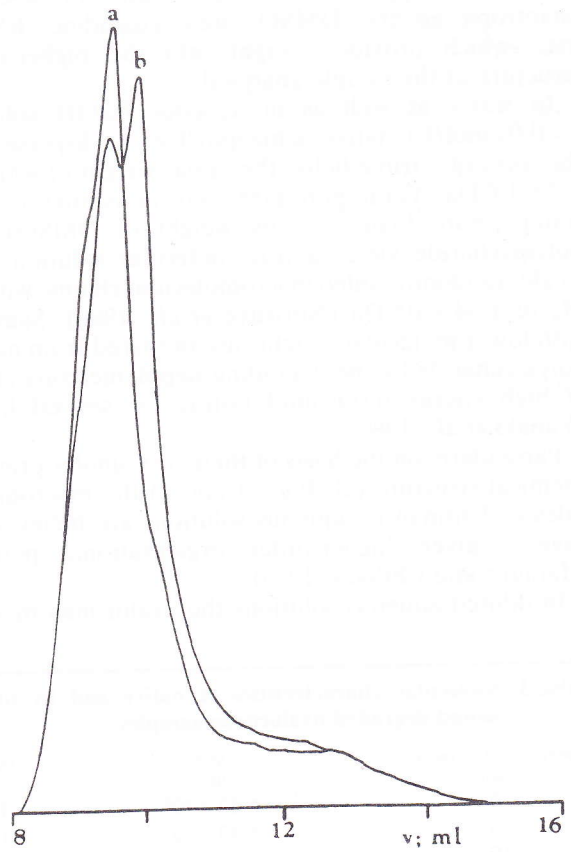

Figure 4. Chromatogram of pleuran: (a) native and (b) sample denatured by DMSO. Column, 8 mm × 25 cm, two connected in series; Sorbent, Separon HEMA-BIO 1000 and HEMA-BIO 300, 10 μm; Eluent, Aqueous $NaNO_3$ solution (0.1 mol/L; Elution rate, 0.4 mL/min; Injected sample volume, 100 μL; Detector, Differential refractometer RIDK 101.

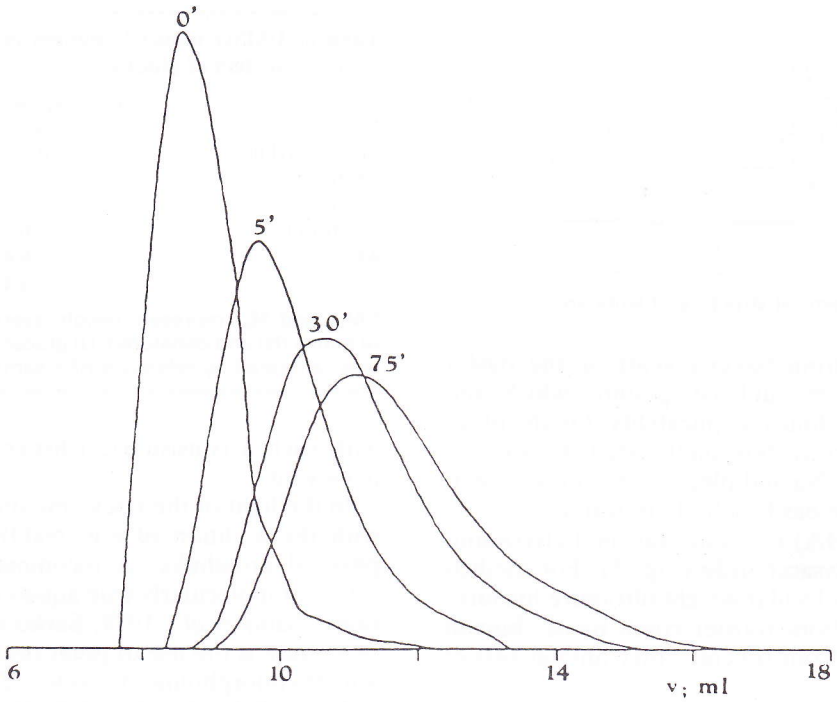

Figure 5. Normalized chromatograms of hyaluronan: native sample (0') and samples degraded by high energy ultrasound (lasting 5, 30, and 75 mins.)

degradation/depolymerization of the biopolymer treated. On the other hand, in some studies of biopolymer solution characteristics (e.g. measurement of optical rotation, viscosity, formation of adducts, etc.) it is the very application of denaturants or so-called chaotropic agents—DMSO, urea, guanidine, KSCN, etc.—which provides insight into the higher-order structure of the sample analysed.

In water as well as in aqueous NaOH solution (<0.01 mol/L) native schizophyllan is 'dispersed' in the form of a triple helix, the molar weight of which is 5.7×10^6 Da. Yet in pure DMSO or in its mixture with water (more than 87% by weight of DMSO) this polysaccharide yields a true molecular solution with single, randomly coiled macromolecular chains, with an M_w of 1.64×10^6 Da (Norisuye *et al.*, 1980). Samples with lower molecular weights are prepared from native polysaccharide by the degrading/depolymerizing effect of high energy ultrasound lasting for several hours (Yanaki *et al.*, 1980).

Particularly on the basis of their very similar primary chemical structure (cf. Figs. 1 and 2) the macromolecules of lentinan in aqueous solutions are believed to have a given higher-order organisational pattern (Hamuro and Chihara, 1985).

In diluted aqueous solutions the hyaluronan macromolecules are in the form of single-strand randomly coiled chains (Cleland, 1977). At higher concentrations the chains associate (aggregate) owing to the intermolecular interactions involved (Beaty *et al.*, 1985).

Samples of HA or hyaluronans with lower molecular weights can be prepared from a native high molecular weight biopolymer by the action of the enzyme hyaluronidase (EC 3.2.1.35) (Beaty *et al.*, 1985; Iwata, 1987; Turner *et al.*, 1988), by acidic or alkaline hydrolysis (Cleland, 1977, 1984), by the degrading effect of increased temperature (Beaty *et al.*, 1985; Bothner *et al.*, 1988), and also by high energy ultrasound (see also Fig. 5 and Table 3) (Chabreček *et al.*, 1990b, 1991a,b; Orviský *et al.*, 1993).

Table 3. Molecular characteristics of native and by ultrasound degraded hyaluronan samples

Depolymerization period (min)	M_w[a] (Da)	M_w/M_n
0	1.32×10^6	1.29
5	6.49×10^5	1.44
15	4.53×10^5	1.62
30	3.30×10^5	1.88
45	2.80×10^5	2.10
75	2.21×10^5	2.36

[a] The HPLC device was calibrated by reference HES samples.

Molecular characteristics of polymers

One of the most important molecular characteristics of a given compound is its molecular weight. In the case of polymers, the sample is molecularly characterized by the distribution function of molecular weights. Parameters of this function are its moments of molecular weight averages defined by means of these moments: M_n, numerical; M_v, viscosity; M_w, weight; M_z, so called *z*-average, etc., where $M_n < M_v < M_w < M_z$.

For determination of polymer molecular weight averages (M_i), several special analytical methods can be used, e.g. osmometry (M_n), viscometry (M_v), light scattering measurement (M_w), etc. The basic disadvantage in determining one or two M_i averages is the fact that polymers with different distribution of molecular weights can be mistaken for identical substances owing to their equal values, e.g. M_v, and/or M_w. The above mentioned facts clearly indicate the advantage and general applicability of analytical procedures which can determine the distribution function of the molecular weights of a polymer.

Size exclusion chromatography

Size exclusion chromatography (SEC), also called gel filtration, gel (permeation) chromatography, etc. (Berek *et al.*, 1983), is currently the most frequently used method for determination of the distribution as well as of individual defined averages of polymer molecular weights.

In the case of an 'ideal' SEC the separation of macromolecules is determined by the following mechanisms and processes: size exclusion; limited diffusion; hydrodynamic effects; so called secondary exclusion. In a real experimental setting, however, many other mostly undesirable secondary processes (effects) come into play, such as polar—adsorptive or repulsive—interactions between the chemical groups on the surface of the chromatographic sorbent and polar or polarizable functional groups of the analysed biopolymer sample, viscosity effects, etc.

The present setting of experimental conditions (see e.g. the legend to Fig. 4)—i.e. the sorbent type, eluent, concentration of the sample analysed, etc. — can be classified as being optimal for standard SEC analysis of electrically uncharged and polyanionic biopolymers (Barth, 1987; Polymer Notes, 1987; Dark, 1988).

Calibration of the equipment

The detector unit of a common HPLC/SEC device usually monitors the instantaneous concentration of the eluted fraction of the polymer sample. The evaluated distribution function of the polymer molecular weights should, however, represent the dependence of the amount—the fraction weight—of the particular polymer homologue on the corresponding molecular weight. That is why it is necessary to transform the values of the elution volumes (v) to values of the molecular weights (M) using the formerly established $v=f(M)$ dependence, i.e. the calibration curve. Such calibration dependences can be described by different equations (Benoit *et al.*, 1966); Gemeiner *et al.*, 1987), but practically the most frequently used is the $\log M = D_1 - D_2 v$ equation (Anderson and Stoddart, 1966), which in the semilogarithmic scale represents a certain SEC separation interval by a straight line.

At direct calibration, the elution volumes of the standard reference unimodal samples with the narrowest available molecular weight distribution width are measured. The elution volume of the peak of the chromatographic record is taken as v; the value of M_p (HPLC 1991), $(M_w \times M_n)^{1/2}$ or M_w is substituted for$M$, since the difference between the latter two values and M_p is practically negligible ($M_n \leq M_p \leq M_w$; M_p represents the molecular weight of the maximum of the differential distribution function of the polymer molecular weights).

Transformation of the chromatogram into the distribution function of polymer molecular weight

The direct consequence of broadening of the chromatographic zone is that the injected 'monodisperse' sample at the column exit is detected as a typical bell-shaped wave (kernel), also designated as the function of instrumental spreading—$G(v, y)$. The normalized chromatographic record of a polydisperse polymer, $g^*(v)$, is therefore a superposition of the whole set of mutually overlapping waves of individual polymer homologues. Tung's integral equation mathematically describes this fact (Tung, 1966) as follows

$$g^*(v) = \int_{-\infty}^{\infty} G(v, y) \cdot w(y) \cdot dy$$

where $w(y)$ represents the chromatogram corrected for the broadening of the polymer homologue zones.

The procedure of correcting the SEC data with subsequent determination of the distribution of polymer molecular weights can therefore be formulated as follows: based on the given normalized chromatogram $g^*(v)$ and the instrumental spreading function the corrected chromatogram is calculated and by using the calibration function $v=f(M)$ it is transformed into the distribution function of molecular weights of the analysed polymer (Šoltés and Hudec, 1982; Šoltés and Berek, 1983).

In most cases we approximated the function of instrumental spreading by using the symmetrical (Gaussian) curve

$$G(v, y) = (h/\pi)^{1/2} \cdot \exp[-h(v-y)^2]$$

with the resolution factor $h = 1/2\sigma^2$, where y represents the elution volume of the peak of the $G(v, y)$ curve characterized by the variance σ. The calculations of the corrected SEC data and of the subsequent differential molecular weight distribution of the polymer sample, as well as of the defined averages of molecular weights M_w and M_n were made using the calculation method according to Chang and Huang (1969). The values of the resolution factor h were determined using the technique of sequential iterations (Kotaka, 1976, 1977).

Application of the method

Calibration of a device is a fundamental and inevitable condition for using the SEC method in the distribution analysis of polymer molecular weights. The determined calibration dependences are $\log M$ vs. v as well as h vs. v.

Since for the determination of the so-called absolute molecular characteristics of the (bio-)polymer analysed it is mandatory to determine the dependence $v=f(M)$ valid for the type of polymer examined, the only values that can be considered absolute are those determined (recalculated) for the HES samples (see Table 1). The molecular characteristics of various glucans or hyaluronan samples shown in Tables 2 and 3, determined by using the calibrating dependence valid for the HES samples, represent relative values. The parameters determined by such a calibrating procedure were therefore further applied only in the evaluation of the degradation/depolymerization of the high molecular weight hyaluronan, and in the mutual comparison of the pairs of native and denatured glucan samples.

Based on these studies, it is possible to schematically represent (in extended, linearized, form) the higher-

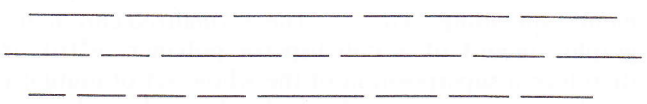

Figure 6. Scheme of the higher-order structure (in extended, linearized, form) of the schizophyllan injection sample analysed.

order structure of schizophyllan in the injection sample analysed as shown in Fig. 6, i.e. as an associate of three mutually 'helically wound' but degraded polymer chains, which—after denaturation—give a mixture of corresponding 'oligosaccharides' characterized by a relatively small M_{wD} value.

The lentinan injection sample is clearly represented by a bi-component polymer in which only the part with the higher molecular weight is denaturable (Horváthová *et al.*, 1991). This finding, original to our laboratory, along with the fact that the purified lentinan was obtained by fractional precipitation from 6% aqueous NaOH solution (approximately 1.6 mol/L) (Chihara *et al.*, 1970), implies the conclusion that the injection sample analysed consisted of a mixture of non-denatured and denatured polysaccharides.

Similarly is the pleuran sample analysed a multi-component mixture (cf. Fig. 4). The same holds for Fraction II (Horváthová *et al.*, 1991b) since its subfractions $4A_2$ and 8 are characterized by significantly different values of the parameter studied, i.e. 6.4 or only 3.4%, compared to 16% determined for Fraction II (cf. Table 2).

CONCLUSION

The liquid chromatographic separation method (SEC) is currently the most frequently applied analytical technique for determination of the distribution function as well as of defined averages of molecular weights of (bio-)polymers (Barth, 1987). Its speed, accuracy, and reproducibility makes it rank first among the methods in this field.

The SEC method also permits the observation of the effects of various physical and/or chemical agents which change the effective size of macromolecules. This type of research is illustrated by the evaluation of the effect of slightly raised temperature (50°C) combined with the denaturing, chaotropic effect of DMSO on the higher-order structure of the selected glucans and also by studying the degradation/depolymerization effect of high energy ultrasound on the native hylauronan sample.

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