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Average Molecular Weight and Molecular Weight Distribution of Agarose and Agarose-Type Polysaccharides

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ABSTRACT

A procedure to determine the absolute weight-average molecular weight and molecular weight distribution of agarose and agarose-type polysaccharides by aqueous size-exclusion chromatography coupled with low-angle laser light scattering is described. The molecular weights of the majority of the commercial samples investigated were between 80 000 and 140 000 with a polydispersity lower than 1·7. In contrast, most of the laboratory-extracted agarose-type polysaccharides had lower molecular weights.

INTRODUCTION

Agarose and agarose-type polysaccharides are gel-forming cell-wall polysaccharides extracted with water from certain members of the marine red algae. They are composed of alternating β (1 → 4) D-galactose and α (1 → 3) L-galactose repeating units with the latter 4-*O*-linked sugar usually occurring as the 3,6-anhydride form or, at times, as L-galactose-6-sulfate which is believed to be the biological precursor of the anhydro-galactose residue (Araki, 1966). Agar consists of a family of agarose-type

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polysaccharides that differ in the level of substitution by methoxyl, sulfate and pyruvate groups (Duckworth & Yaphe, 1971; Lahaye *et al.*, 1986). The molecule having the lowest degree of substitution is referred to as agarose and has the highest gelling potential of all the agarose-type polysaccharides (Kennedy *et al.*, 1984). Agar usually forms soft gels that are mainly used in the preparation of microbiological media or in the food industry whereas agarose, forming brittle gels, has primarily biochemical applications (McLachlan, 1985). The gelling properties of this polysaccharide family not only depend on the chemical characteristics of the molecules but also probably on the weight-average molecular weight and the molecular weight distribution. The elastic modulus and the yield stress in particular of polysaccharide gels are known to be related to the molecular weight of polysaccharides (Mitchell, 1980).

In recent years, considerable progress has been made in size exclusion chromatography in relation to the molecular weight of polysaccharides. A low-angle laser light-scattering system (LALLS) connected on-line to the chromatographic system allows the monitoring of the molecular weight and this system has been used with success for numerous polysaccharides such as dextran (Van Dijk *et al.*, 1987), guar (Vijayendran & Bone, 1984) or carrageenans (Lecacheux *et al.*, 1985).

We now describe the weight-average molecular weight and molecular weight distribution of several commercial agarose and agarose-type polysaccharides extracted from *Gracilaria* and *Gelidium* spp., using high-performance aqueous size-exclusion chromatography (SEC) coupled to an LALLS.

MATERIALS AND METHODS

Agaroses available from different companies (Table 1) were studied. In order to obtain samples of lower molecular weight, sample A was hydrolyzed by acidic treatment (agarose concentration 10 g/liter, HCl 10^{-2} N, 1 h at 60°C) and is referred to as sample M in Table 2.

Agarose-type polysaccharides were extracted from *Gracilaria* sp. and modified by alkali according to the methods described by Lahaye *et al.* (1986). Fractions from *Gracilaria pseudoverrucosa* and *Gracilaria crassissima* were those studied by Lahaye and Yaphe (1988) and Lahaye *et al.* (1988), respectively. Agarose-type polysaccharides extracted from *Gracilaria compressa* were described by Lahaye *et al.* (1986). *Gelidium robustum* agarose was extracted and alkali treated according to the procedure described by Craigie and Leigh (1978).

TABLE 1
List of Commercial Agaroses Tested

Sample	*Company*	*Country*	*Reference*
A	FMC	USA	291402
B	FMC	USA	92364
C	Colab Laboratories	USA	
D	IBF	France	A 37
E	IBF	France	FF 2743
F	Maknur Laboratories	Canada	984108
G	Sigma	USA	VII
H	Sigma	USA	VI
I	Litex	Denmark	LSL
J	Litex	Denmark	HSB
K	Oxoid	England	LII
L	Biorad	USA	12689

TABLE 2
Moisture Content (%), Intrinsic Viscosity ($[\eta]$), Weight-average Molecular Weight $\overline{M_w}$ and Polydispersity (P) of Commercial Agaroses

Sample	*(%)*	*$[\eta]$ (ml/g)*	$\overline{M_w}$	*P*
A	16·6	302	134 000	1·82
B	12·2	326	139 000	1·51
C	18·1	294	112 000	1·45
D	10·9	127	35 700	1·70
E	14·2	254	104 000	1·49
F	14·7	212	78 000	1·68
G	11·7	251	92 000	1·41
H	15·8	277	102 000	1·32
I	18·0	256	97 000	1·66
J	17·6	363	144 000	1·59
K	23·6	292	111 000	1·39
L	18·6	170	48 700	1·67
M	16·6	72	17 000	2·30

The SEC–LALLS measurements were carried out on a high pressure liquid chromatograph (HPLC) consisting of the following components: a Waters 6000 pump, a Waters U6K injector, one or two columns Shodex B804 and/or B805, a Chromatix KMX-6 LALLS photometer and a Jobin Yvon Iota differential refractometer. Usually, a 0·22 μm filter (Millipore or Sartorius) was placed between the end of the columns and

the LALLS cell. The columns were thermostated at 45°C and eluted with aqueous 0·1 M $NaNO_3$ at a flow rate of 1 ml min^{-1}. Samples were dissolved in the same solvent at 1 g ml^{-1} and injected (200 μl) hot (90–95°C). General operation and cleaning conditions of the LALLS flow cell were as described by Van Dijk *et al.* (1987). No axial dispersion correction (Hamielec *et al.*, 1981) or second virial correction (Martin, 1982) were used.

The refractive index increment ($dn/dc = 0{\cdot}14$) at 633 nm was determined from the refractometric signals ($\lambda = 940$ nm) of the agarose samples. It was assumed that the dn/dc depended on λ like dextran that was chosen as reference and for which the dn/dc is well known at both wavelengths (Domard & Rinaudo, 1984). The moisture contents of every sample were determined by thermogravimetry using a Setaram G 70 thermobalance.

The intrinsic viscosities $[\eta]$ were determined at 35°C in 0·75 M NaSCN using a Ubbelhode viscometer.

RESULTS AND DISCUSSION

To our knowledge, there is no report in the literature on chromatographic experiments of agarose and the reason may be related to the gelling properties of the polysaccharide. The agarose thermoreversible gel results from the rigid helical conformation adopted by the molecule when the solution conditions (temperature, ionic strength, solvent) are no longer favorable to the coil state (Arnott *et al.*, 1974). Thus, in order to chromatograph this polysaccharide, a solvent and/or a temperature which prevent the formation of the ordered conformation is required. Furthermore, since agarose cannot be considered as a neutral polysaccharide due to the low concentrations of pyruvate or sulfate groups always present, a salted eluent has to be used to avoid electrostatic interactions between the column matrix and the chromatographed molecules (Rochas *et al.*, 1908a).

We have verified that agarose does not adopt a helical conformation between 18 and 80°C (Fig. 1) and does not gel at concentrations up to 50 g $liter^{-1}$ in DMSO with or without salt ($NaNO_3$, NaSCN). Indeed, in contrast to aqueous solutions of agarose, no variation in optical rotation, due to the conformational change of the molecule, is observed on cooling DMSO solutions of agarose. This characteristic is also found for agarose in mixture of up to 25% of water and DMSO. Furthermore, ^{1}H or ^{13}C NMR spectra of agarose are always obtained with a good resolution between 18 and 80°C and polymer concentrations up to 50 g $liter^{-1}$.

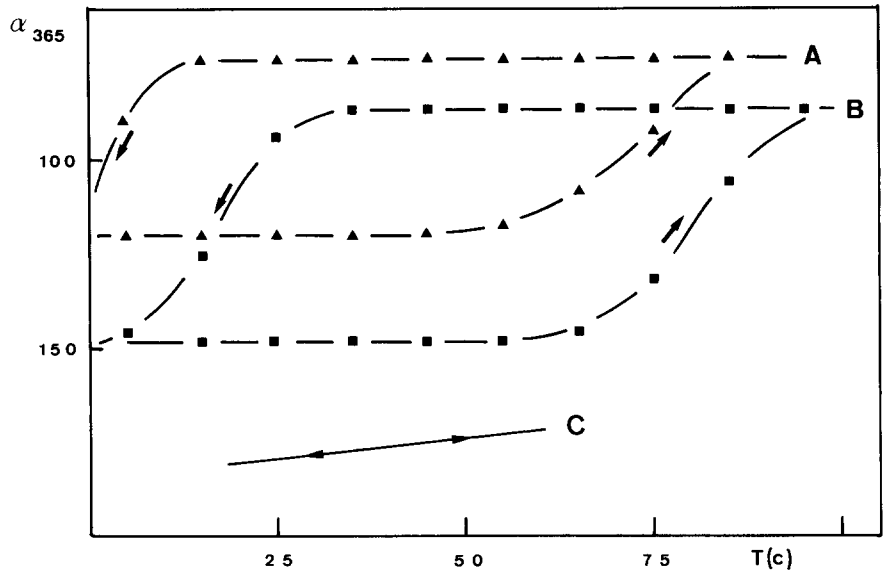

Fig. 1. Temperature dependence of optical rotation of agarose solution (0·5 g/liter) in various solvents. A, 0·75 N NaSCN; B, H_2O or 0·1 N $NaNO_3$; C, DMSO.

The latter observations, already reported for other polysaccharides such as xanthan (Morris *et al.*, 1977), carrageenan (Rochas *et al.*, 1980b) and the extracellular polysaccharides from *Arthrobacter* sp. (Darke *et al.*, 1978), prove that the conformation of agarose in DMSO is not a rigid rod but rather, a coil. Unfortunately, DMSO is incompatible with the packing of Shodex columns but this solvent could be used with other rigid packing such as pore glass.

An alternative is to use aqueous sodium thiocyanate. Because of the lyotropic effect of NaSCN (Hermans, 1949), this salt shifts the coil to helix transition of agarose to lower temperatures (Fig. 1) and we verified that the higher the salt concentration, the greater the shift. A gelling temperature of agarose under room temperature is obtained with an aqueous solution of 0·75 M NaSCN. Unfortunately, the use of this corrosive solvent for long periods of time is incompatible with the Shodex columns. Nevertheless, aqueous 0·75 M NaSCN at 35°C was used for viscosimetric experiments.

A third alternative is to work at high temperatures. After preliminary experiments, we chose the chromatographic conditions described in the Materials and Methods section. At injection (90–95°C) and during chromatography (45°C), it is impossible for agarose to adopt a helical conformation and thus form a gel. However, because the two on-line detectors at the end of the columns are at room temperature, it is

possible for agarose to adopt the helical conformation if the eluent temperature decreases to room temperature. Fortunately, at an agarose concentration of 1 g ml^{-1} and a flow rate of 1 ml min^{-1}, the cooling of the eluent was insufficient to allow the agarose conformational transition to occur. Indeed, the same molecular weight was obtained when different solvents were used: 0·1 M $NaNO_3$; 0·1, 0·5, or 0·75 M NaSCN, for which the conformational ordering and consequently the aggregation are completely different at room temperature (Fig. 1). The absence of gelation was also proved by the low molecular weight observed for the different agarose samples (about 100 000) and by the reproducibility of the experiments.

The molecular weight of every commercial sample of agarose or laboratory-extracted agarose-type polysaccharides is low (Tables 2 and 3), when compared to the values for closely related galactans such as

TABLE 3

Weight-average Molecular Weight of Agarose-type Polysaccharides Extracted from *Gracilaria pseudoverrucosa* Collected in January and September, *Gracilaria compressa*, *Gracilaria crassissima* and *Gelidium robustum* with (+) and without (−) Treatment with Alkali

Alga	*Alkali*	*Fraction*[a]	$\overline{M_W}$[b]
G. pseudoverrucosa	−	Cold water	40 000
January	−	40%	43 000
	−	0%	52 000
	+	Cold water	24 000
	+	60%	24 000
	+	40%	39 000
	+	0%	29 000
September	+	Cold water	40 000
	+	60%	38 000
	+	40%	51 000
	+	0%	34 000
G. compressa	−	60%	52 000
	−	40%	65 000
	+	Whole extract	50 000
G. crassissima	−	40%	124 000
	+	Cold water	89 000
	+	40%	100 000
G. robustum	+	Whole extract	75 000

[a]The percentages refer to the level of ethanol in the boiling ethanol–water mixtures used to extract the polysaccharide.

[b]The very small fraction of material excluded from the column as shown on Fig. 2 is not taken into account for the determination of $\overline{M_W}$.

kappa- or iota-carrageenan (Lecacheux *et al.*, 1985). The majority of the investigated samples have weight-average molecular weights between 80 000 and 140 000. These values are in good agreement with the only one reported in the literature (Hikson & Polson, 1968) from sedimentation and diffusion experiments. The polydispersity (Table 2) of every commercial agarose sample is low which is also in agreement with the observation of Hikson and Polson. This is a marked difference from carrageenan for which the polydispersity is equal to or larger than 4.

The good correlation ($r = 0{\cdot}995$) between the intrinsic viscosity and the weight-average molecular weight allows the determination of a Mark-Houwink equation for agarose:

$$[\eta] = 0{\cdot}07\ M^{0{\cdot}72}$$

where $[\eta]$ is ml g^{-1}.

The agarose intrinsic viscosity (Table 2) is very high for the molecular weight observed and for an almost neutral polysaccharide. Therefore, agarose is not a compact coil when it is in its disordered state and parts of the chain may be quite rigid.

The results from different fractions of laboratory-extracted agarose-type polysaccharides were significantly different (Table 3). The extraction procedures and alkali treatment of these fractions were described elsewhere (Lahaye *et al.*, 1986, 1988) and the effects of seasons on the chemical structure and gel strength of *G. pseudoverrucosa* agar were also reported (Lahaye & Yaphe, 1988). The weight-average molecular weight of the different extracts from *G. compressa* and *G. pseudoverrucosa* are notably lower than the values recorded for commercial samples of agarose. The procedure of fractionation may partly be responsible for these low molecular weights. Indeed, it is possible that the polysaccharides were hydrolyzed during the freeze-drying and/or the dialysis step (Goring, 1954). Another degradation process, probably involving a free radical and described by Wedlock *et al.* (1987) for alginates, may also occur on freeze-drying.

The extraction sequence of agarose-type polysaccharides from algae — water at room temperature (referred to as cold water), boiling 80%, 60%, 40%, 20% ethanol, boiling water and water at 121°C — is primarily based on chemical structure differences of the polysaccharides and not on their molecular weight. Indeed, if the latter physical parameter was the main determinant in the extraction, we should have obtained fractions of polysaccharides of increasing molecular weights — but we did not (Table 3).

As expected, alkali treatment which acts in removing sulfate ester groups, leads to the decrease in the molecular weight of the agaroses

(Table 3). It is also possible that such a treatment slightly degraded the molecules.

Every sample of agarose-type polysaccharide extracted in the laboratory contained a small fraction of high-molecular-weight material that may be floridean starch (Fig. 2). The very low refractometric signal indicated that this material amounted to well below 0·1% of the injected sample. The bimodal distribution and the excluded fractions shown in Fig. 2 were never observed with the commercial samples.

The differences observed in molecular weight of agarose-type polysaccharides from various species of *Gracilaria* cannot solely be attributed to artifacts arising from the procedures of extraction and preparation but most probably represent species characteristics. Such differences in molecular weight may explain in part the well-known distinction between soft and brittle agar gels (McLachlan, 1985).

CONCLUSION

Aqueous SEC coupled with LALLS allows for the rapid and accurate determination of the molecular weight and molecular weight distribution

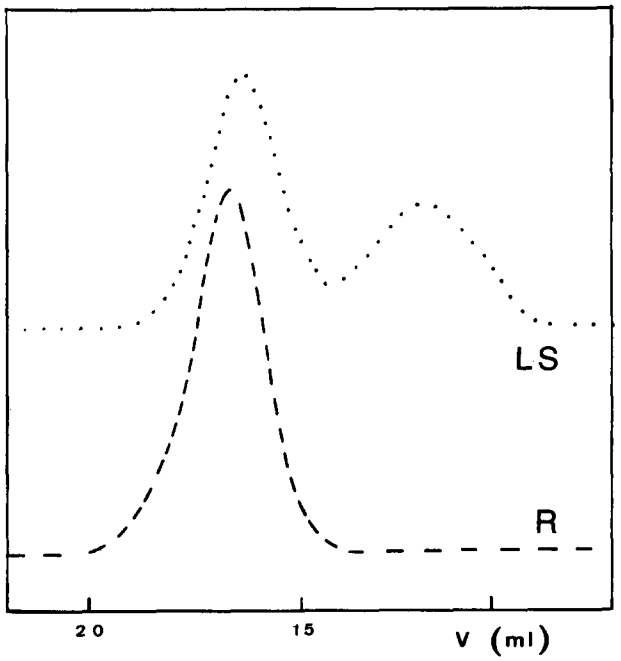

Fig. 2. Light scattering (LS) and refractometric (R) response for 0·1 mg injected of *Gracilaria pseudoverrucosa* (January, alkali-treated, 60% fraction).

of agaroses. Thus it is now possible to relate molecular weight, chemical structures and rheological properties of agaroses. Furthermore, due to the simplicity of the method, the viscosity measurements are a useful means of roughly determining the molecular weight of agaroses.

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