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Chemically modified exopolysaccharide pullulans: physico-chemical characteristics of ionic derivatives

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The paper presents some physico-chemical characteristics of dilute solutions of exopolysaccharide pullulan and its carboxymethyl, sulfoethyl and sulfopropyl ionic derivatives.

On-line size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) was used for establishing their weight average molecular weight (M_w), number average molecular weight (M_n), polydispersity index ($I_p = M_w/M_n$) and radius of gyration R_g.

INTRODUCTION

Pullulan, a water soluble exopolysaccharide produced by *Aerobacidium pullulans*, has a linear flexible macromolecular chain, formed from α-1,4 linked glucose units which are included in α-1,6 linked maltotriose units (Brant & Burton, 1981).

The properties of pullulan make it an excellent adhesive, thickener, viscosity stabilizer and coating agent for many applications. Because of its oxygen impermeability, non-toxic and non-irritating properties, pullulan is used in biotechnical, pharmaceutical and other industries. By introducing functional groups onto the pullulan chain, its performance can be improved, extending its area of application.

A very important series of references on pullulan derivatives can be found in the patent literature. This study reports the syntheses of the following pullulan derivatives: etherified (Fujita *et al.*, 1978; Nishyima *et al.*, 1979), cyanoethylated (Murase *et al.*, 1983), carboxylated (Tsuji *et al.*, 1976) trimethylsilylated (Kirlich *et al.*, 1968), cationized (Onishi, 1985), sulfated (Carpov *et al.*, 1984), acetylated (Hijiya & Shiosaka, 1974a), esterified with aliphatic or aromatic acids (Hijiya & Shiosaka, 1974*b, c*).

By comparison with the extensive data existing in the literature, concerning solution behaviour of synthetic polymers or polysaccharide (cellulose, dextran) derivatives, studies on the physico-chemical characterization of pullulan derivatives have received scant attention. Viscoelastic properties of pullulan have been studied in correlation with fermentation conditions (Le Duy *et al.*, 1987); recently some more extensive studies were reported on hydrophobized pullulans (Kato *et al.*, 1982; Akiyoshi *et al.*, 1991, 1992, 1993).

The ionic derivatives of pullulan are interesting both as polyelectrolytes and as precursors for other chemical modifications. It is, therefore, important to correlate their physico-chemical characteristics with synthesis conditions.

The aim of this paper is to present some physico-chemical characteristics in dilute solution of pullulan and some of its ionic derivatives: weakly acidic-carboxymethyl and strongly acidic-sulfoethyl and sulfopropyl pullulan. This study has been conducted by using size exclusion chromatography coupled with multi-angle laser light scattering (SEC/MALLS).

MATERIALS AND METHODS

Materials

Pullulan: PF-20, called PULL-P, was purchased from Hayashibara Biochemical Laboratory (Okoyama, Japan). Sodium monochloracetate 90% (NaClAc), propane sultone and sodium hydroxide were of commercial origin. Sodium chloroethane sulfonate ($NaClSO_3$) was synthesized in the laboratory.

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Methods

Synthesis

Carboxymethylpullulans (CMP) were obtained by reacting PULL-P with sodium monochloracetate (British Patent, 1975); sulfoalkylation involved the use of propanesultone for the preparation of sulfopropyl derivatives (SPP) (Jermstad & Ratjora, 1972) and, in the case of sulfoethylation (SEP) of sodium chloroethane sulfonate (Mocanu *et al.*, 1983).

Five grams of PULL-P was dissolved at 20°C in 20 ml aqueous NaOH solution (which contains the stoichiometric amount of NaOH against the reagent used). Three volumes of a cosolvent (toluene, acetone or isopropylalcohol (see Table 1) were added) under stirring and the reaction vessel was placed in a preheated bath; the necessary amount of reagent was added and the reaction was continued under heating and stirring for 6 h. The bath temperature was 70°C for the preparation of SEP and CMP, and 50°C for SPP.

On completion of the reaction, the products obtained were purified by dialysis against water, then dried using a Mini Spray Buchi-190 Dryer.

Chemical characterization of the products

The degrees of substitution with carboxymethyl groups were determined by conductimetric titrations (Eyler *et al.*, 1947), (Conductimeter Radiometer type CDM-2, Copenhagen, Denmark). The degrees of substitution with sulfoethyl and sulfopropyl groups were determined by sulfur content analyses (Schöniger, 1955; Schöniger, 1956) and by potentiometric titration (Radelkis OP-506 pH-meter, Budapest, Romania). The results obtained with the two methods were in good agreement.

Preparation of solutions

The powdered pullulan derivatives were dispersed at about 0.2% w/w in 0.1 M NaCl and gently stirred for 4 h under ambient temperature conditions. Solutions were clarified through a 0.45 μm sterile filter unit (Millex HA). Water came from a Milli-Q water reagent system.

Size exclusion chromatography and multi-angle laser light scattering

Absolute determination of molecular weight and size distributions was performed by coupling on-line a size exclusion chromatography (SEC), a multi-angle laser light scattering photometer (MALLS) and a differential refractive index detector (DRI). 0.1 M $LiNO_3$ eluent was filtered through Millipore GS 0.22 μm, carefully degassed (ERC-313), eluted at a 0.7 ml/min flow rate (Kontron HPLC pump-420) and clarified through a 0.45 μm filter unit. The injected volume was 400 μl.

The SEC system consisted of a PWH (guardcolumn) as a protection and two serial TSK G4000PW and G6000PW columns (Toyo Soda). The column packing is a polyether gel, rich in hydroxyl groups, specially designed for the separation of polysaccharides (4.10^4–8.10^6 g/mol).

The MALLS photometer, a DAWN-F from Wyatt Technology Incorporation (Santa Barbara, CA, USA) fitted with a K5 flow cell and a He–Ne laser ($\lambda = 632.8$ nm) was installed on-line between the columns and the DRI detector (Shimadzu RID-6A).

The DAWN-F measures the angular distribution and intensity of light scattered simultaneously at fifteen angles for each elution volume (V_i) of very low concentration (C_i) measured on-line by the DRI detector.

The Astra V-3.0 software package was used for calculating the molecular weight (M_i) and the radius of gyration (R_{gi}) from to the extrapolation of the light scattered to zero-angle at each 'slice' according to the following relation:

$$\left(\frac{KC}{\Delta R_\theta}\right)_i = \frac{1}{M_i}\cdot\left(1 + \frac{16\pi^2 n_0}{3\lambda_0^2}.(R_g^2)_i.\sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C_i + \dots \tag{1}$$

where

$$K = \frac{2\pi^2 n_0}{\lambda_0^4 N_a}\cdot\left(\frac{dn}{dc}\right) \tag{2}$$

Table 1. Synthesis conditions and characteristics of ionic pullulan derivatives

Samples	Reagents	Molar ratio reagent/glucopyranosic unit	Cosolvents	T (°C)	DS[a]	Functional groups
SEP-12	$NaClEtSO_3$	1.25/1	Toluene	70	0.12	Sulfoethyl
SEP-44		3.75/1		70	0.44	strongly acidic
SPP-22	Propane sultone	0.32/1	Acetone	50	0.22	Sulfopropyl
SPP-60		2.5/1		50	0.60	strongly acidic
SPP-77		3.5/1		50	0.77	
CMP-14	NaClAc	0.6/1	Isopropyl	70	0.14	Carboxymethyl
CMP-51		2/1	alcohol	70	0.51	weakly acidic
CMP-99		4/1		70	0.99	

[a] DS, degree of substitution.

Table 2. Macromolecular characterization of pullulan precursor and its ionic derivatives from SEC/MALLS data in 0.1 M $LiNO_3$

Samples	M_w (g/mol) (+/−10,000)	M_n (g/mol) (+/− 10,000)	I_p (+/− 0.1)	R_g (nm) (+/− 3)
PULL-P	345,000	170,000	2.03	24
SEP-12	235,000	188,000	1.25	24
SEP-44	170,000	136,000	1.25	23
SPP-22	290,000	232,000	1.25	27
SPP-60	280,000	224,000	1.25	26
SPP-77	260,000	208,000	1.25	25
CMP-14	280,000	224,000	1.25	28
CMP-51	245,000	196,000	1.25	26
CMP-99	125,000	100,000	1.25	19

M_w, weight average molecular weight; M_n, number average molecular weight; I_p, polydispersity index; R_g, radius of gyration.

with dn/dc = 0.147 for the whole of pullulan derivatives.

$2A_2C_i$ is insignificant compared to the rest of equation (1), therefore M_i and R_{gi} are obtained from the y-intercept to zero-angle and from the slope of KC/R_θ vs $\sin^2(\theta/2)$, respectively.

Light scattering measurements allow the molecular weight and size distribution as well as the number, weight and z-average molecular weights to be calculated.

The results as a whole were analysed using the EASI V-7.0 software package programme.

RESULTS AND DISCUSSION

Conditions for synthesis and chemical characteristics of the pullulan derivatives are presented in the Table 1. In each case the role of the cosolvent was to diminish the occurrence of the competitive aqueous basic hydrolysis of the reagent.

The data presented in Table 1 show the greatest reactivity of propane sultone in the mildest reaction conditions. The propane sultone cycle is easy cleaved by the PULL-O^- anion (which is formed in the presence of NaOH), thus giving derivatives with rather high degree of substitution (DS).

The sodium chloroethane sulfonate is the least reactive agent. To obtain approximately the same DS it is necessary to use larger quantities of reagent, because the competitive reaction with the more nucleophilic OH^- anion is dominant over that with the less nucleophilic PULL-O^- anion.

The carboxymethylation reaction needs also an excess of reagent because of the competitive hydrolysis of sodium chloracetate. To this excess of reagent there is a greater quantity of NaOH.

The physico-chemical characteristics of PULL-P and its ionic derivatives in dilute solution are presented in Table 2. All the samples present a very low angular dependency of the scattered light in relation to their small molecular size. An example of the scattering envelope is given for PULL-P in Fig. 1.

Two different methods can be used for the extrapolation of the data. The Debye method uses a fit of R_θ/KC vs $\sin^2(\theta/2)$ for each slice, while the Zimm method uses a fit of KC/R_θ vs $\sin^2(\theta/2)$. Then the desired polynomial order can be adjusted according to the angular dependency of the studied polymer. This is particularly important for large molecular size. For smaller molecular size, a first order fit is generally sufficient and both methods are expected to perform equally well.

This is exactly what we observed for PULL-P and its derivatives, as shown in Fig. 2. Moreover, the data have been fully confirmed by low-angle laser light scattering (LALLS) with the Chromatix KMX-6.

Figure 3 shows the dependence of the molecular weight and the radius of gyration on the elution volume. From the molecular weight dependence of the radius of gyration, interesting information can be obtained concerning the macromolecular conformation of pullulan and its derivatives. As indicated by data

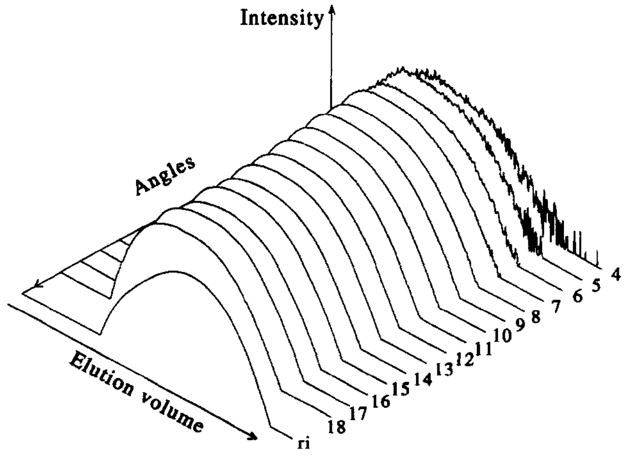

Fig. 1. Scattering envelope for PULL-P in 0.1 M $LiNO_3$ between 21.7° (detector 4) and 158.3° (detector 18).

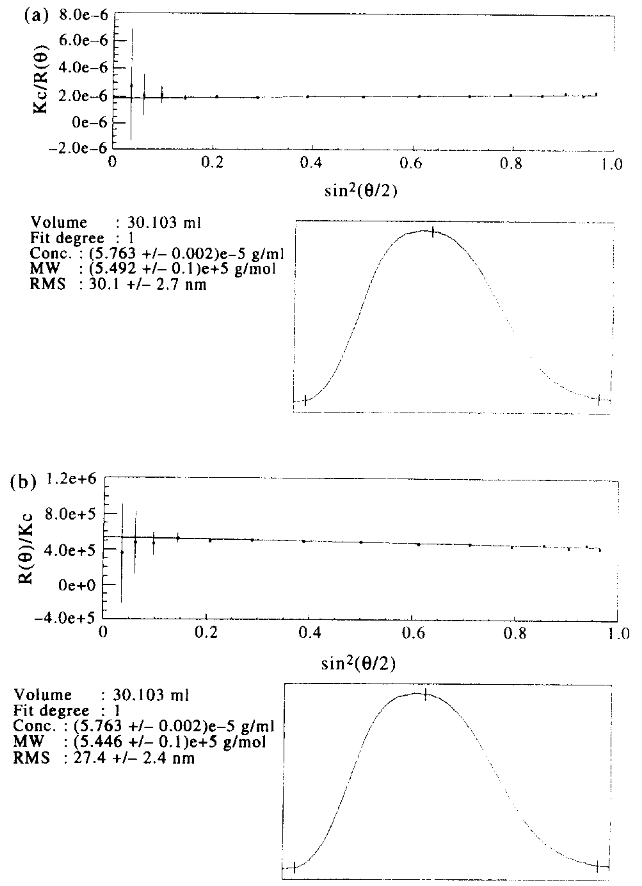

Fig. 2. Angular dependency of light scattered of PULL-P for the same slice of C_i: (a) Zimm method; and (b) Debye method.

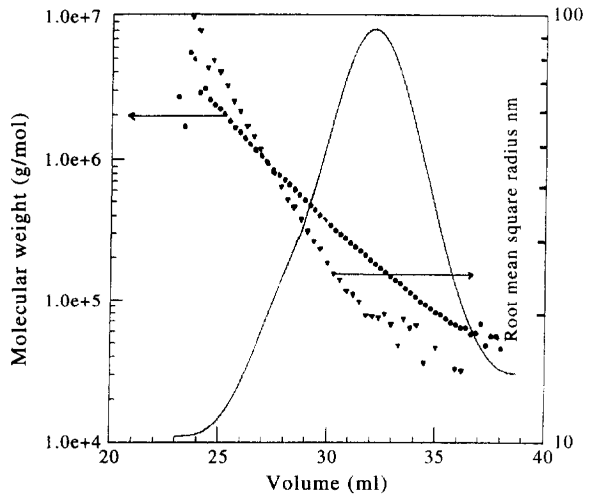

Fig. 3. Distribution of molecular weight (●) and radius of gyration (▼) on the elution volume for PULL-P.

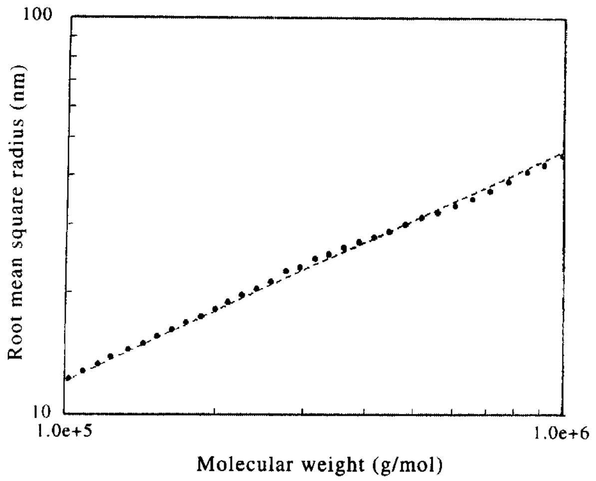

Fig. 4. Double logarithmic plot of R_g vs M_w for PULL-P (exponent $x = 0.58$).

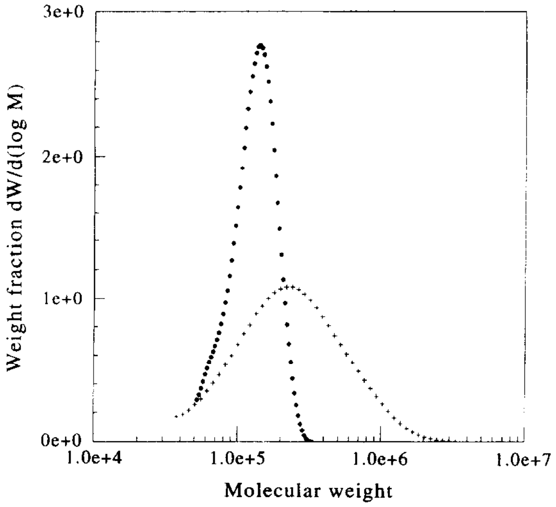

Fig. 5. Differential molecular weight distribution of PULL-P (+); and of CMP-99 (●).

reported in Fig. 4, R_g scales with M_w with an exponent, the value of which ($x = 0.58$) is found to be consistent with literature data for a flexible coil conformation (Kato *et al.*, 1984).

As appears from data reported in Table 2, ionic derivatives of pullulan have a molecular weight distribution narrower than that of the precursor (PULL-P). This is probably the consequence of the purifying operations (precipitation and dialysis) subsequent to the chemical modification reaction, which leads to elimination of lower molecular weight fractions.

The data reported in Fig. 5 clearly provide evidence for this explanation and show that the differential molecular weight distribution of the precursor is much larger than that of the carboxymethyl derivative (CMP-99).

Data presented in Table 2 show that the physicochemical parameters of ionic derivatives from PULL-P are a function of the reagent and the reaction conditions used for the modification. Sulfoethylation results in substantial degradation because of the more drastic reaction conditions (e.g. elevated pH and temperature). This is illustrated in Fig. 6 which shows the cumulative molecular weight distribution of SEP-12 and 44; the degradation is all the more important as the DS is large.

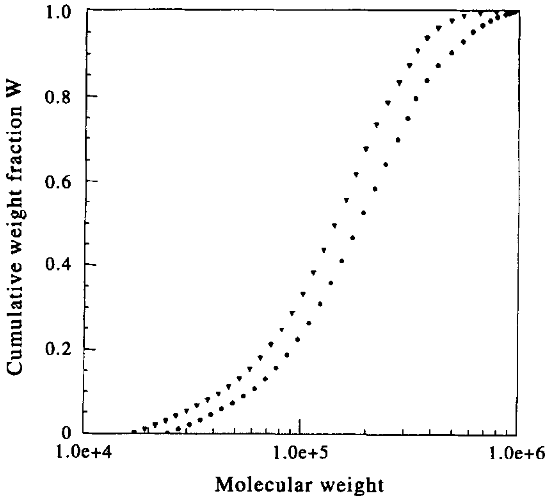

Fig. 6. Cumulative molecular weight distribution of sulfoethylpullulan: SEP-12 (●); and SEP-44 (▼).

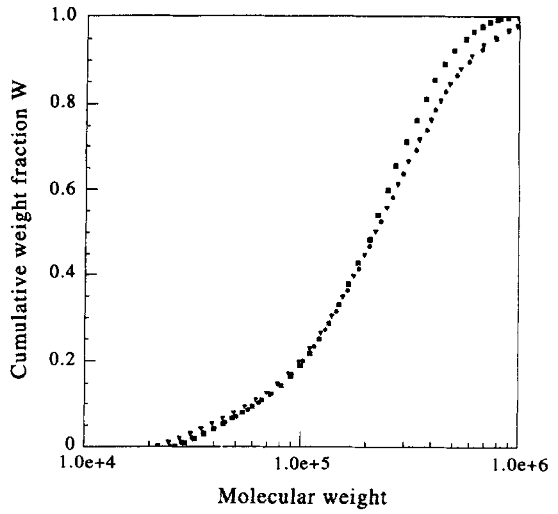

Fig. 7. Cumulative molecular weight distribution of sulfopropylpullulan: SPP-22 (●); SPP-60 (▼); and SPP-77 (■).

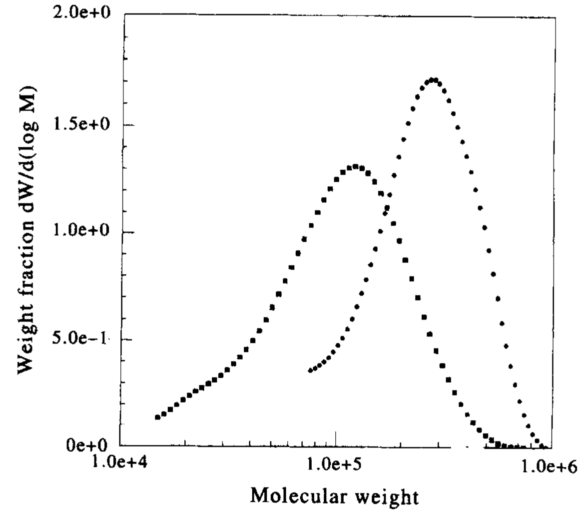

Fig. 8. Differential molecular weight distribution of carboxymethylpullulan; CMP-14 (●); and CMP-99 (■).

This can be explained by the higher quantity of NaOH according to the higher molar ratio reagent/glucopyranosic unit needed for obtaining larger DS.

On the other hand, as expected, sulfopropylation is less degradative; this is in relation to the high reactivity of propane sultone and the softer conditions of the chemical reaction (Fig. 7). For weakly acidic carboxymethyl pullulan, the more aggressive conditions of the chemical modification explain the degradation evidenced by light scattering, particularly for the derivatives of higher DS, this is illustrated by the differential molecular weight distributions presented in Fig. 8. However, more recent work has shown that no degradation occurred when the chemical modification was performed under milder conditions.

CONCLUSION

In conclusion, the reported data clearly show the value of size exclusion chromatography coupled to on-line multi-angle laser light scattering for absolute molecular characterization of novel ionic polysaccharides derived from pullulan. These chemically modified pullulans are polyelectrolytes and can serve as new precursors for further modifications. Work is in progress on the hydrophobization of carboxymethylpullulan; the objective is to prepare new polysaccharides for a variety of applications.

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