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Size-exclusion chromatography study of the molecular-weight distribution of γ-irradiated pullulan

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Abstract

The polyelectrolyte behavior of γ-irradiated pullulan in aqueous solutions leads to secondary effects of adsorption on a Shodex OHPak KB 806 column gel during size-exclusion chromatography. Suppression of the polyelectrolyte properties of γ-irradiated pullulan is achieved by using a 0.05 M aqueous solution of NaH_2PO_4 (pH 4.95) as the mobile phase. Under these conditions, adequate molecular-weight distributions of γ-irradiated pullulan samples are obtained. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: γ-Irradiated pullulan; High-performance size-exclusion chromatography; Molecular-weight distribution

1. Introduction

Investigation of processes of the radiation–chemical destruction of polysaccharides is important for understanding mechanisms of ionizing radiation effects on biopolymers, and because of possible applications. The state of the art of the radiation chemistry of polysaccharides has recently been reviewed by Ershov [1]. Substantial progress in this field during the last few decades has stimulated the use of polysaccharide radiolysis in the pharmaceutical industry for production of blood-plasma substitutes [2,3].

It is well known that γ-irradiation results in a decrease in the molecular weight of polysaccharides and in the viscosity of their solutions [4]. Polysaccharide radiolysis is accompanied by chemical modification of the polymer molecules [5], in particular, by formation of carbonyl, carboxyl, and keto groups [6–8]. The newly formed groups in polysaccharide macromolecules change their physical properties [9], which can lead to polyelectrolyte behavior of these compounds in solutions, as has been earlier demonstrated for dextran [10].

High-performance size-exclusion chromatography (SEC) is known to be an efficient tool for investigation of molecular-weight parameters of polymers including polysaccharides and their derivatives. However, some secondary effects, such as ionic interactions between the stationary phase and the polymer can distort the actual size distribution of polymer molecules. These effects are especially pronounced in the case of polyelectrolyte molecules due to the electrostatic field surrounding the polyionic molecule [11].

Stringent requirements imposed on the quality of polysaccharide-based blood-plasma-substituting preparations lead to the necessity of developing methods for accurate evaluation

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of the molecular weights and molecular-weight distributions (MWDs) of polysaccharide substances. In the present work we develop an adequate method for measuring the MWD of γ-irradiated pullulan and investigate the effect of eluents on its chromatographic separation.

2. Experimental

The sample of irradiated pullulan was produced by γ-irradiating a native pullulan solution (Belmedpreparaty Pharm. Co.) with a dose of 50 kGr. Conditions of γ-irradiation are described elsewhere [7]. The intrinsic viscosity of the irradiated pullulan sample $[\eta]$ was equal to 0.235 dL g^{-1}. The weight-averaged molecular weight (M_w) of the irradiated pullulan sample was calculated by the Mark–Kuhn–Houwink equation with the constants $K = 4.73 \times 10^{-4}$ and $\alpha = 0.57$, which were evaluated earlier for polydisperse samples of γ-irradiated pullulan (P.T. Petrov, unpublished data) and had the value $M_w = 54,000$. This sample was separated by gel filtration into 40 fractions using a Sephadex-G200 (Pharmacia) column (120 × 5 cm i.d.). The fractions were collected and lyophilized and were the subjects of the present investigation.

Intrinsic viscosities $[\eta]$ of fractions in solvents used as eluents were measured on an Ubbelohde dilution viscosimeter at 25.0 ± 0.1 °C. In cases when the concentration dependence of the intrinsic viscosity could not be extrapolated to zero dilution, the intrinsic viscosity was calculated by Fedors' equation [12,13].

The change in the chemical structure of pullulan and the appearance of the newly formed functional groups lead to changes in the UV absorption spectra of the polysaccharide. UV absorption spectra of 1% (w/w) solutions of the original irradiated pullulan sample and its fractions in 0.025 M phosphate buffer (pH 6.89) were recorded on a Perkin–Elmer 409 spectrophotometer within the range 200–300 nm in a 10-mm quartz cell. The reference cell was filled with the same 0.025 M phosphate buffer.

The chromatographic analysis was carried out using a liquid chromatography system equipped with a Waters 410 differential refractometer. A Shodex OHPak KB 806 (300 × 8 mm i.d.) column with a void volume V_0 of 7.68 mL, as determined from the dextran T-2000 (Pharmacia) elution peak, and a total volume V_T of 11.533 mL, as determined from the D_2O retention peak, was used for separation. From the values V_0 and V_T, and the values of retention volume V_R, the equilibrium distribution coefficients $K_D = (V_R - V_0)/(V_T - V_0)$ [17] were calculated for each of the fractions collected. Calibration curve parameters were calculated using the GPC option of the MILLENNIUM^{32} software package (Waters) [14]. In experiments on investigation of MWDs, the injection volume V_i was 20 μL, the polysaccharide concentration was 0.1% (w/w), and the mobile phase flow rate was set to 0.80 mL min^{-1}. Milli-Q filtered distilled water (18.2 MΩ cm^{-1}, pH 5.5) and 0.05 M aq NaH_2PO_4 solution, pH 4.95 were used as eluents.

The order of injections of irradiated pullulan fractions and standards was followed as described in Ref. [15]. Intrinsic viscosity values of pullulan standards were calculated using the coefficients of the Mark–Kuhn–Houwink equation $K = 2.21 \times 10^{-4}$ and $\alpha = 0.66$ [16] for both eluents, since pH and the ionic strength do not affect hydrodynamic properties of a neutral polysaccharide within the range of molecular weights under investigation [17].

The universal calibration $\log(M_P[\eta])$ versus V_R, where M_P is the peak molecular weight obtained using P 82 pullulan standards (Shodex) under corresponding conditions, was used for the calculation of molecular weights (M) of fractions of the irradiated pullulan sample.

3. Results and discussion

Upon separation in an aqueous solution, elution curves of irradiated pullulan fractions had two or three peaks (Fig. 1). This can occur due to either nonequilibrated separation on the Sephadex column or effects of the secondary sorption on the gel of the Shodex OHPak KB 806 during high-performance

SEC. However, the viscosity distribution curve ($w_i[\eta]_i/\Sigma w_i$ versus $[\eta]$, where w_i and $[\eta]_i$ are weights and intrinsic viscosities of individual fractions) of the original sample was unimodal (Fig. 2).

Appearance of newly formed carboxyl and α-oxyketo groups in the structure of glucopyranose rings upon γ-radiolysis of an aqueous polysaccharide solution leads to a change in the hydrodynamic and conformational properties [9]. The presence of these groups in the macromolecular structure makes it possible to consider carboxy derivatives of neutral polysaccharides as weak polyelectrolytes. We hence concluded that the secondary sorption of modified pullulan macromolecules on the gel of the analytical column takes place upon elution of irradiated pullulan fractions in water due to the presence of charged functional groups in polymer molecules. This adverse effect precludes an adequate interpretation of a chromatogram, in particular, calculation of molecular weights and the MWD analysis.

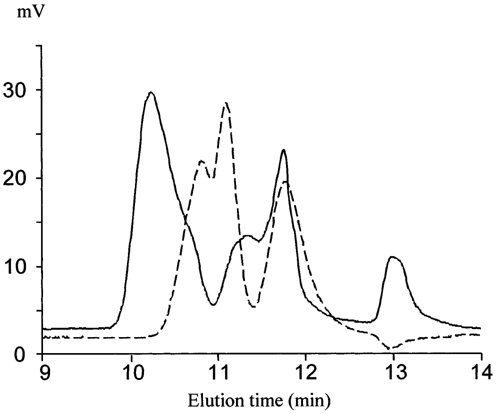

Fig. 1. Elution curves of aqueous solutions of fractions of γ-irradiated pullulan sample with molecular weights of 108,000 (–) and 10,300 (- - -). Shodex OHPak KB 806 column; flow rate 1 mL min^{-1}. Detector response (y-axis) expressed in millivolts.

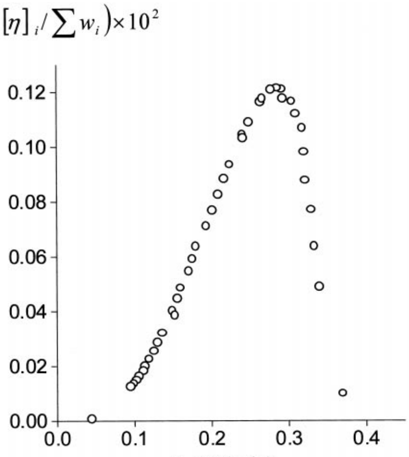

Fig. 2. Viscosity distribution of the original sample of γ-irradiated pullulan.

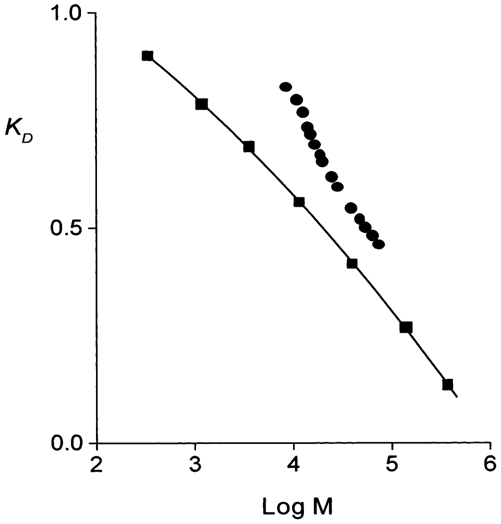

Fig. 3. Equilibrium distribution coefficient K_D as a function of log M for fractions of γ-irradiated pullulan sample (●) and pullulan standards (■). The solid curve approximating pullulan standards points was plotted by using a polynomial least-squares regression.

In order to provide screening of charges of the newly formed functional groups in irradiated pullulan, we varied the ionic strength and pH of the mobile phase by adjusting the concentration of NaH_2PO_4. The screening of the charge of a polymer chain in salt solutions results in a decrease in the hydrodynamic volume of macromolecules compared with that in aqueous solutions. In addition, salt-containing solvents inhibit polyelectrolyte behavior of the polymer and the secondary sorption of the polymer on the column gel. We have found that the 0.05 M NaH_2PO_4 solution (pH 4.95) meets the above requirements, and therefore this solution was chosen as the mobile phase.

The fractions of the irradiated pullulan sample were found to show different behavior upon SEC compared with pullulan standards, as follows from the dependences of the equilibrium distribution coefficient K_D on log M presented in Fig. 3, which makes it possible to conclude that compaction of radiation-modified pullulan molecules takes place.

It should also be noted that the difference between SEC retention times and coefficients K_D of fractions of the irradiated pullulan sample and pullulan standards increases with decreasing molecular weight (Fig. 3).

This can take place as a result of a dependence of the degree of modification on the molecular weight of a fraction of the irradiated polysaccharide. Indeed, earlier [6] we have shown for γ-irradiated dextran that fractions with a lower molecular weight have a higher degree of modification. This should also be expected for pullulan. In order to verify this assumption, we analyzed the dependence of UV absorption of fractions of the irradiated pullulan sample on their molecular weight. The UV absorption spectrum of irradiated pullulan is characterized by a wide band with a maximum in the region of 255–260 nm. The presence of the absorption maximum in this spectral range substantiates the appearance of α-oxyketo groups, and its intensity is proportional to their concentration [6]. The dependence of the optical density, D_{255}, at 255 ± 5 nm of fractions of the irradiated pullulan sample on their molecular weights has a complicated character (Fig. 4). Generally, the optical density, D_{255}, increases with decreasing molecular weight, which reflects peculiarities of the radiation-induced destruction of pullulan accompanied by formation of new functional groups. Appearance of the newly formed carboxyl, oxyketo, and carbonyl groups in the structure of polysaccharide molecules substantially affects the conformational properties of the polymer and thus can explain the increase in differences in the SEC retention times and K_D values for fractions of the irradiated pullulan sample and pullulan standards with decreasing molecular weight (Fig. 3).

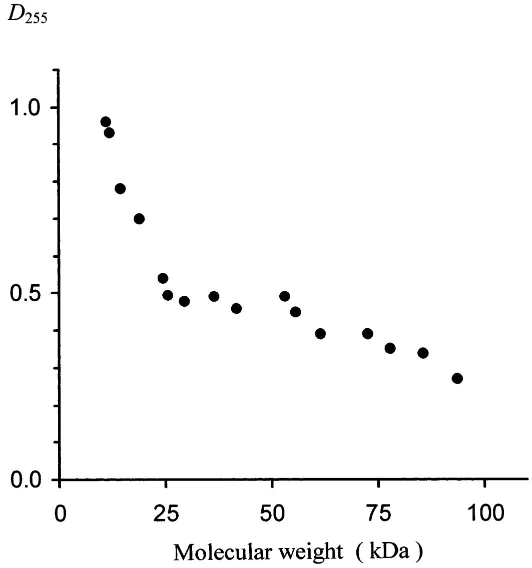

Fig. 4. Optical density of fractions of γ-irradiated pullulan sample measured at 255 ± 5 nm as a function of the molecular weight. Concentration 1% (w/w); solvent: 0.025 M phosphate buffer (pH 6.89).

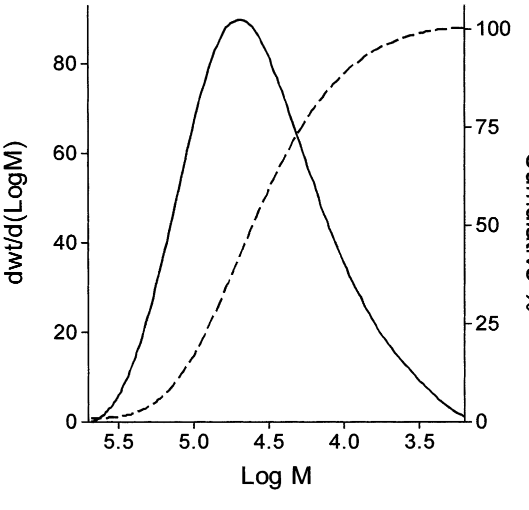

Fig. 5. MWD of the original sample of γ-irradiated pullulan ($M_W = 54{,}300$ and $M_W/M_N = 2.25$) calculated from its elution curve in 0.05 M aqueous solution of NaH_2PO_4.

The use of the 0.05 M NaH_2PO_4 (pH 4.95) as a mobile phase in SEC of γ-irradiated pullulan leads to screening of the residual charges of groups in the modified polymer, which makes it possible to measure MWDs of γ-irradiated pullulan within the entire range of molecular weights investigated. We illustrate this by the successful calculation of the MWD of the original (nonfractioned) sample of γ-irradiated pullulan from its elution curve in 0.05 M aqueous solution of NaH_2PO_4 (Fig. 5). The weight- (M_w) and number-averaged (M_n) molecular weights of the original sample calculated from the SEC data presented in Fig. 5 ($M_w = 54{,}300$ and $M_w/M_n = 2.25$) perfectly agree with those calculated from the molecular weights of its fractions measured under identical conditions ($M_w = 53{,}700$ and $M_w/M_n = 2.10$).

To summarize, we have found SEC conditions suitable for the measurement of the MWD of γ-irradiated pullulan. The polyelectrolyte behavior of radiation-modified pullulan that manifests itself in the elution process is inhibited by the use of a salt solution as the eluent. Elution in a salt solution prevents the secondary sorption of the polysaccharide on gel and/or charge–charge repulsions along the backbone of the polymer chain and provides an adequate interpretation of elution curves when investigating MWDs of γ-irradiated pullulan.

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