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# High-performance gel permeation chromatography of water-soluble β-1,3-glucans

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## ABSTRACT

High-performance gel permeation chromatography of water-soluble β-1,3-glucans was carried out on Separon HEMA-S as the sorbent in 0.1 *M* sodium chloride solution as the mobile phase at 25°C. This system was calibrated with two sets of standards. The molecular-weight distributions of the fractions of the sodium salt of carboxymethyl-β-1,3-glucan (CMG-Na), schizophyllan and lentinan and of the same samples treated with dimethyl sulphoxide were determined. The results obtained were almost identical. The only difference was found in the case of schizophyllan owing to melting of its higher order structure.

## INTRODUCTION

Glucans are natural biopolymers, polysaccharides with biological effects on the living body[1–3]. The water-soluble β-1,3-glucans, schizophyllan[4] and lentinan, are produced commercially in the injection form.

Studies of the behaviour of water-soluble β-1,3-glucans and/or their derivatives in solution are very sparse. Although the melting and degradation of the higher order structure of schizophyllan at various treating conditions have been mentioned[5–9], systematic high-performance gel permeation chromatographic (HPGPC) studies in this field have not yet been reported. However, checks of polymer stability during long-term storage either in powdered form or in solution are important. Moreover, sterilization of the polymer injection form by heating or irradiation may influence the

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molecular-weight distribution of β-1,3-glucans. HPGPC could be very effective in revealing such changes.

In previous work, β-1,3-glucan from cell walls of the yeast *Saccharomyces cerevisiae* was used for the preparation of a water-soluble derivative, the sodium salt of carboxymethyl-(1→6)-β-D-gluco-(1→3)-β-D-glucan (CMG-Na)[10]. CMG-Na was fractionated[11] and characterized by combined methods of gel permeation chromatography, light scattering and capillary viscometry[12].

In this study we studied the properties of CMG-Na samples by HPGPC. We determined the molecular-weight distribution and compared the behaviour of natural polymeric samples with that of samples treated with dimethyl sulphoxide (DMSO). Commercial samples of lentinan and schizophyllan were treated under the same conditions.

## EXPERIMENTAL

### *Materials*

TSK standards (Toyo Soda, Tokyo, Japan) of poly(ethylene oxide) (PEO) having molecular weights of $2.1 \cdot 10^4$ (SE-2), $4.5 \cdot 10^4$ (SE-5), $8.5 \cdot 10^4$ (SE-8) were used. Hydroxyethyl-starches were a gift from Dr. Kirsti Granath (Pharmacia, Uppsala, Sweden (weight-average molecular weight, $\bar{M}_w = 5.38 \cdot 10^4$, $1.28 \cdot 10^5$, $1.95 \cdot 10^5$, $3.98 \cdot 10^5$). Other chemicals used were of the analytical-reagent grade, except DMSO (spectroscopic grade). Lentinan (1 mg of lyophilizate) and schizophyllan (20 mg in 2 ml; clinical grade) were purchased from Taito (Kobe, Japan). Fractions of the sodium salt of carboxymethyl-(1→6)-β-D-gluco-(1→3)-β-D-glucan (II, $4A_2$, 8 and III) were prepared by stepwise precipitation using acetone[12].

### *High-performance gel permeation chromatography*

The HPGPC experiments were performed with a high-pressure pump (HPP 5001, Laboratorní přístroje, Prague, Czechoslovakia), an eight-port switching valve equipped with two 100-μl loops (Model PK 1, Vývojové dílny, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), two stainless-steel columns (250 × 8 mm I.D.) packed with Separon HEMA-S 1000 and Separon HEMA-S 300 (mean particle size 10 μm) connected in series (Tessek, Prague, Czechoslovakia) and a differential refractometric detector (RIDK 102, Laboratorní přístroje). Experiments were carried out at 25°C. The mobile phase was 0.1 *M* sodium chloride solution. The flow-rate of the eluent, "degassed" by purging with helium, was constant at 0.7 ml/min. The sample volume injected was 100 μl and the sample concentration was 0.5%.

### *Preparation of sample solutions for HPGPC*

The CMG-Na fractions, schizophyllan and lentinan were dissolved in the mobile phase.

All samples (2.5 mg each) were treated with DMSO (0.5 ml). The samples were dissolved in DMSO overnight, then evaporated under nitrogen at 50°C. The last evaporation step took *ca.* 4 h. Each solid polymer was dissolved in the mobile phase (0.5 ml) prior to HPGPC analysis.

## RESULTS AND DISCUSSION

The equipment for HPGPC was calibrated with two sets of standards, *viz.*, PEOs and hydroxyethyl-starches. Because the high-molecular-weight PEO standards showed strong hydrophobic interaction effects with the sorbent, only low-molecular-weight materials were used. The $\bar{M}_w$ value reported by the producer was taken as $M$ (peak). The dependences of the elution volume ($V_e$) on the logarithm of molecular weight ($M$) for both reference polymers are linear (Fig. 1) in the molecular-weight range measured:

for hydroxyethyl starch:

$$\ln M = 19.3663 - 0.03417V_e \qquad (r = 0.9989)$$

for PEO:

$$\ln M = 15.4639 - 0.01874V_e \qquad (r = 0.9987)$$

where $V_e$ is in counts.

Correction for the broadening effect was made mathematically[13] by solving Tung's integral equation[14]. The instrumental spreading function was approximated by the Gaussian curve with a resolution factor $h$. The values of $h$ were determined by the procedure proposed by Balke and Hamielec[15], using their relationship:

$$\bar{M}_w/\bar{M}_n = \bar{M}_w^*/\bar{M}_n^* \exp(-D_2^2/2h)$$

where $\bar{M}_w^*$ *and* $\bar{M}_n^*$ are the weight- and number-average molecular weights calculated from the uncorrected molecular-weight distribution curve and $D_2$ is the slope of the calibration graphs described by the equation $\ln M = D_1 - D_2V_e$.

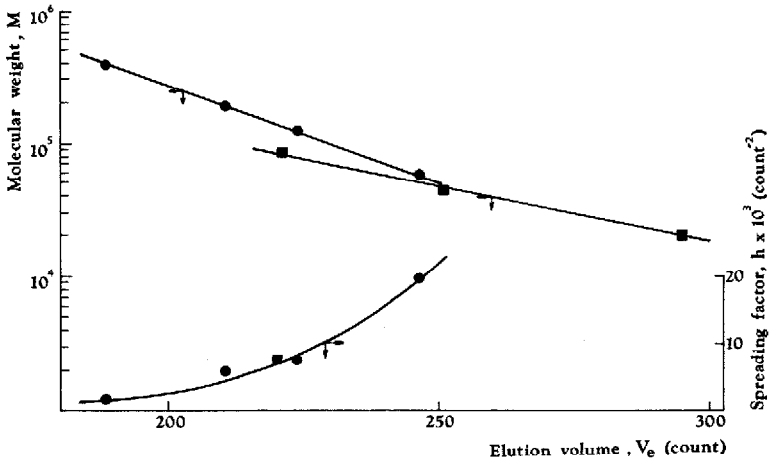


Fig. 1. Calibration of the HPGPC equipment. ● = Hydroxyethyl-starch; ■ = PEO.

TABLE I

CALIBRATION DATA OF THE HPGPC INSTRUMENT RESOLUTION

| *Specification of reference samples* | | *Calculated values* | | | |
|---|---|---|---|---|---|
| $\bar{M}_w$ | $\bar{M}_w/\bar{M}_n$ | $\bar{M}_w^*/\bar{M}_n^*$ | $h \times 10^3$ (counts$^{-2}$) | $\bar{M}_w$ | $\bar{M}_w/\bar{M}_n$ |
| *Hydroxyethyl-starch:* | | | | | |
| $3.98 \cdot 10^5$ | 1.276 | 1.832 | 1.61 | $3.94 \cdot 10^5$ | 1.391 |
| $1.95 \cdot 10^5$ | 1.204 | 1.334 | 5.69 | $2.08 \cdot 10^5$ | 1.209 |
| $1.28 \cdot 10^5$ | 1.196 | 1.294 | 7.41 | $1.47 \cdot 10^5$ | 1.239 |
| $5.38 \cdot 10^4$ | 1.338 | 1.378 | 19.81 | $7.60 \cdot 10^4$ | 1.351 |
| *Poly(ethylene oxide):* | | | | | |
| $8.50 \cdot 10^4$ | 1.06 | 1.085 | 7.53 | $8.36 \cdot 10^4$ | 1.069 |
| $4.50 \cdot 10^4$ | 1.07 | 1.203 | 1.50 | $4.83 \cdot 10^4$ | 1.201 |
| $2.10 \cdot 10^4$ | 1.12 | 1.117 | Negative | $2.18 \cdot 10^4$ | 1.114 |

The relationship $h = f(V_e)$ was fitted by a polynomial of the second order:

$$h = 0.1755 - 1.8547 \cdot 10^{-3} V_e + 4.9537 \cdot 10^{-6} V_e^2 \qquad (r = 0.9929)$$

for samples of hydroxyethyl-starch and PEO, where $h$ is in counts$^{-2}$. The iterative program of Chang and Huang[16] was used for calculation of both uncorrected and corrected averages of the molecular weight of all samples.

Table I contains the calibration data for the HPGPC instrument for "imperfect" resolution. On comparing the values of $\bar{M}_w^*/\bar{M}_n^*$ for each reference polymer given in Table I with the value of $\bar{M}_w/\bar{M}_n$ calculated from the corrected molecular-weight distribution curve, it is evident that the intensity of the sample broadening during HPGPC is not so high and both values are almost identical, as is typical in high-performance chromatography. The method for the calculation of the corrected

TABLE II

HPGPC DISTRIBUTION ANALYSIS OF POLYMERIC SAMPLES

Distribution analysis was done by using the calibration dependence $M = f(V_e)$ for hydroxyethyl-starch (see also Fig. 1).

| *Sample* | *Natural polymeric sample* | | *Sample treated with DMSO* | |
|---|---|---|---|---|
| | $\bar{M}_w$ | $\bar{M}_w/\bar{M}_n$ | $\bar{M}_w$ | $\bar{M}_w/\bar{M}_n$ |
| Schizophyllan | $6.24 \cdot 10^5$ | 1.307 | See Fig. 2 | |
| Lentinan | $2.66 \cdot 10^5$ | 3.705 | $2.05 \cdot 10^5$ | 3.226 |
| CMG-Na fraction: | | | | |
| II | $7.63 \cdot 10^5$ | 1.712 | $6.41 \cdot 10^5$ | 1.395 |
| $4A_2$ | $7.04 \cdot 10^5$ | 1.163 | $6.59 \cdot 10^5$ | 1.231 |
| 8 | $5.28 \cdot 10^5$ | 1.631 | $5.10 \cdot 10^5$ | 1.640 |
| III | $1.32 \cdot 10^5$ | 2.441 | $9.71 \cdot 10^4$ | 1.727 |

molecular-weight distribution gives real values of $\bar{M}_w$ and $\bar{M}_n$ for the calibration standards (Table I).

With regard to the ionic character of the CMG-Na samples, 0.1 $M$ sodium chloride solution was used as the mobile phase[17]. In contrast to the work of Kato *et al.*[18], the universal calibration graph is not valid for the standards used here, probably owing to their hydrophobic interaction with the Separon HEMA-S sorbent. Therefore, the β-1,3-glucan chromatograms were further treated by using the set of calibration data $M = f(V_e)$ valid for hydroxyethyl-starch. This is why the calculated molecular weight averages of these samples given in Table II should be taken as relative.

The β-1,3-glucan schizophyllan is considered to be exceptional in that in water its macromolecules adopt a higher order structure of a triple helix. From the crystallographic data, the same triple-helical conformation may be suggested for lentinan in the solid state. A number of papers[5–9] had shown that under denaturing conditions (alkaline pH, heating, admixing of DMSO with an aqueous solution of schizophyllan), the higher order structure of schizophyllan is probably unfolded.

We have studied the CMG-Na fractions, lentinan and schizophyllan after treatment with DMSO at higher temperature (50°C). Schizophyllan dissolves very well in DMSO and other β-1,3-glucan fractions of CMG-Na swell. Such treatment caused a total melting effect of schizophyllan, evident from the disappearance of the sample signal at low $V_e$ (Fig. 2), whereas the other β-1,3-glucans, i.e., lentinan and the CMG-Na fractions, were not been affected by the DMSO treatment (Table II). Consequently, the undirected molecular-weight distribution analysis neither excludes nor supports the triple-helical structure of the CMG-Na fractions and of lentinan in aqueous solution.

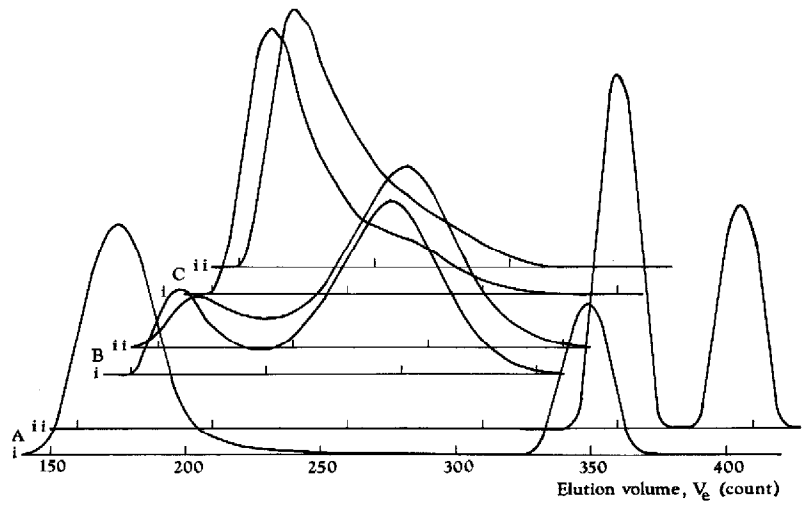


Fig. 2. Normalized chromatograms of (A) schizophyllan, (B) lentinan and (C) carboxymethylglucan II fraction (i) before and (ii) after treatment with DMSO.

## CONCLUSIONS

HPGPC on Separon HEMA-S is a suitable method for the determination of the molecular-weight distribution of water-soluble polymers ($\beta$-1,3-D-glucans) and for the study of its changes under various conditions, such as heat treatment, sterilization of injection solutions and long-term storage. In this way, the degradation of these compounds can be monitored.

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