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Determination of size limits of membrane separation in vesicle chromatography by fractionation of polydisperse dextran

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ABSTRACT

A recently described vesicular chromatographic packing material (VP), consisting of purified plant cell walls with vesicular and cellular morphology, was characterized with respect to the minimum Stokes' diameter necessary for complete exclusion (size limit of exclusion = SLE) and the maximum Stokes' diameter permitting permeation into the whole stationary liquid volume (size limit of permeation = SLP). Using vesicle chromatography, the size fractionation of a polydisperse dextran preparation with a defined size distribution was carried out to determine the percentages of completely excluded (P_{ex}) and completely permeable (P_{perm}) dextran molecules. SLE may be derived from P_{ex} and SLP from P_{perm} taking into account the molecular size range of the fractionated polydisperse dextran sample. Values determined for the size limits of the vesicle membrane with the help of dextran 35 (calibrated on Sephadex G-200) were nearly equal to those determined with the help of dextran 15 (calibrated on Sephadex G-75). The SLP of the standard VP is 5.6 nm. However, negatively charged proteins with a Stokes' diameter slightly below the SLP (pepsin and ovalbumin) are excluded from the VP. The method may be applied in controlling the separation limits of VPs with altered ultrafiltration properties.

INTRODUCTION

The solid structure of the vesicular packing material (VP) [1] commercially available as Permselekt or Vesipor is the purified cell wall framework developed by cell clusters grown in a higher plant suspension culture. It consists of a cellulose–pectin (cellin) matrix [2] with a mechanically and chemically unaltered structure. The separation mechanism in vesicle chromatography (VC) is membrane permeation (dialysis) between the mobile phase and the stationary liquid phase which is kept in vesicles (cells, microcapsules). The cell walls act as dialysis membranes with a sharp separation limit. Chromatography with the VP permits a rapid separation of two size groups of macromolecules on short columns. The absence of an extended fractionation range in VC, which is the main difference from gel permeation chromatography (GPC), is an advantage for size fractionations in a narrow molecular size range. VP consisting of unaltered cellin walls separates excluded macromolecules (*e.g.*, proteins of $>50\,000$ dalton) from slightly smaller molecules and other permeable molecules [1]. We have found (unpublished work) that the separation limits of the cellin membranes may be increased by depolymerization of the protopectin matrix with different methods.

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In this paper, we describe a procedure for determining the the size limits of separation by VC. Such a method is necessary for controlling the preparation of VPs with different separation properties.

PRINCIPLE OF METHOD

In VC, a mixture of macromolecules with a continuous and broad distribution of molecular size, *e.g.*, polydisperse dextran, may be separated into two main fractions, a permeable and an unpermeable (excluded) fraction [1]. The method is based on such a separation using a dextran standard of defined molecular size distribution.

A polydisperse dextran standard is fractionated by GPC on a suitable gel, the bed having been calibrated by proteins of defined Stokes' diameter. GPC results in a broad, continuous elution profile of the fractionated dextran. Using a calibrated gel column, the dependence of dextran concentration in the eluate on the elution volume, V_e, is transformed into a calibration graph giving the dependence of the Stokes' diameter on the percentage of dextran eluted, P_e. Both the excluded percentage, P_{ex}, obtained from VC of the standard dextran and the percentage P_e obtained from GPC are related to the sum of monodisperse fractions with Stokes' diameter, d_s, larger than a certain size. If $P_e = P_{ex}$, then d_s, coordinated to P_e, is also the Stokes' diameter of the smallest dextran molecules excluded by the VP. Therefore, once P_{ex} has been determined experimentally, d_s may be read off the calibration graph. The value determined in this way is designated as the size limit of exclusion (SLE) of the VP. Similarly, the permeable percentage P_{perm} obtained from VC and the non-eluted percentage $(100-P_e)$ obtained from GPC refer to the sum of dextran fractions with molecular size smaller than a certain value. If P_{perm} is equated to $100-P_e$, the Stokes' diameter allied to P_e is the diameter of the largest permeable dextran molecules in the permeable fraction obtained by VC. This Stokes' diameter is designated as the size limit of permeation (SLP) of the VP. The interval between these two limits is the size range of dextran molecules eluted after the excluded fraction and before the permeable fraction (fractionation range).

EXPERIMENTAL

Dextran 15 and 35 were supplied by Serva (Germany). Both preparations were used from the same package for all experiments. Blue Dextran 2000, Dextran T 250 and Sephadex G-75 and G-200 were supplied by Pharmacia (Sweden).

The chromatographic system consisted of a peristaltic pump from Unipan (Poland) and a Perkin-Elmer Model 141M recording polarimeter (volume of microcuvette 1 ml, light path 10 cm) for recording dextran concentration. Peaks of polyethylene glycol and proteins were recorded by means of an RIDK differential refractometer (Czechoslovakia). Permselekt vesicular packing material as ethanol-moist or dry material was washed with distilled water and dispersed in the elution buffer.

Glass columns of 2.9 cm I.D. with end frits (polyamide cloth, mesh width 80 μm, supported by a polyethylene sieve plate) were packed using a slurry containing about 10 mg/ml of dry packing material. After the liquid above the sedimented material had been absorbed, the chromatographic bed was allowed to shrink to about 80% of its original sedimentation volume by slow draining with mild suction (2–3

kPa). The chromatographic bed was covered with a polyamide cloth and a polyethylene sieve plate, which was fixed by a tight-fitting rubber ring. Column contained 23 to 27 mg of the dry packing material per millilitre of bed volume. Between chromatographic experiments, the columns were equilibrated with a stabilizing medium (0.5% KH_2PO_4–0.05% NaN_3). Before use they were saturated with the elution buffer (0.05 *M* phosphate buffer containing 0.05% NaN_3). Glass columns for GPC were packed by the usual procedure.

Planimetry of the chromatograms was carried out gravimetrically using suitable transparent paper. The excluded percentage was determined as twice the percentage of the dextran sample that was eluted up to the maximum of the first (excluded) peak and the permeable percentage as twice the dextran percentage after the second peak maximum. Elution volumes and K_{av} values (the fraction of the gel volume that is available for the sample) were determined as described by Laurent and Killander [3].

RESULTS AND DISCUSSION

For the investigation of the molecular size distribution, dextran elution profiles representing more than 2000 theoretical plates were considered. Ranges of the elution volume, V_e, which are characterized by small variations in concentration within the peak variance of a monodisperse molecule were selected for use in calibration graphs (region a–b in Fig. 1). With respect to calibration of the Sephadex beds (G-75 and

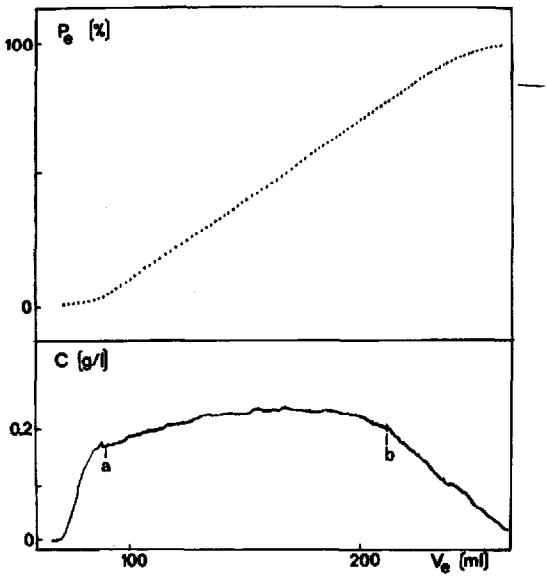
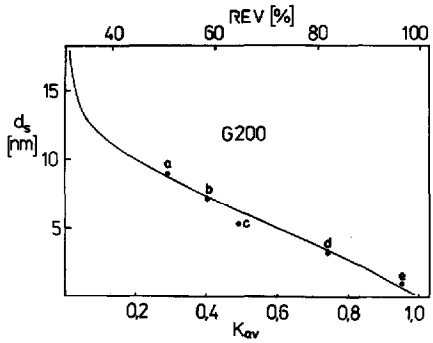


Fig. 1. GPC of dextran 35. Lower curve: trace for dextran 35 obtained by means of a Sephadex G-200 column (bed length 27.0 cm, bed volume 263 ml, particle size 40–120 μm, exclusion volume 84 ml). Sample (dissolved in 2 ml of eluent buffer), 40 mg; a–b, section of the elution curve with an approximately symmectrical influence of monodisperse neighbour fractions on the recorded dextran concentration. Trace recorded with a Perkin-Elmer Model 141 M polarimeter; wavelength = 436 nm. Upper curve: percentage of eluted dextran 35 derived by integration of the lower trace.

G-200) used for dextran fractionation, we did not find significant deviations of protein K_{av} values from the originally published curves [3]. Fig. 2 shows the corresponding curve from ref. 3, respresented by the line, together with the positions of the standard proteins determined with the applied column. In consequence, the calibration graphs (dependence of Stokes' diameter on eluted dextran percentage, Fig. 3) were derived from the dextran elution profile (Fig. 1) and the data in ref. 3. The investigation was carried out using two polydisperse dextrans (dextran 15 and 35) which were calibrated on different Sephadex gels. The size group fractionation of the two dextrans on the VP demonstrates the sharpness of separation ((Fig. 4).

Columns much larger than necessary for complete separation of the permeable from the excluded fraction were used in order to consider the possible influence of bed length and separation time. We have argued in a preceeding paper [1] that the small but significant share of a polydisperse sample eluting between the peaks may be an expression of non-uniformity of the cells (vesicles) with respect to their individual separation limit. If every cell in the chromatographic bed of the VP had an ultrafiltration membrane (cell wall) with a sharp transition from the highly permeable to the completely impermeable state at a certain molecular size (separation limit) but the cells varied to some extent with respect to the value of this limit, then molecules of a size within the statistical variance of cellular separation limits would have an intermediate elution volume between the exclusion volume and the total bed volume (statistical explanation of the fractionation range). Alternatively, permeable molecules with a size near the separation limit might be eluted before the second peak as their permeability is too low to reach the diffusional equilibrium (kinetic explanation). The results shown in Fig. 4 and Table I favour the statistical explanation, as both SLE and SLP were not clearly dependent on column length and separation time. As shown



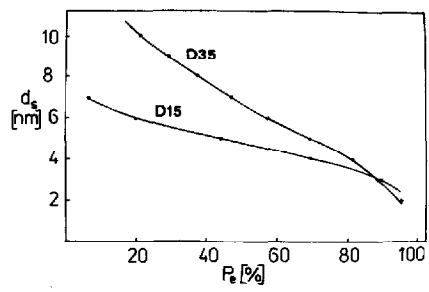


Fig. 2. Dependence of Stokes' diameter on K_{av} and relative elution volume (REV) as determined by chromatography of proteins on the Sephadex G-200 column used for fractionation of dextran 35. The positions of proteins used for calibration [(a) yeast alcohol dehydrogenase; (b) human serum albumin; (c) ovalbumin; (d) bovine cytochrome *c*; (e) sucrose] fit the original curve published for Sephadex G-200 [3]. Column as in Fig. 1.

Fig. 3. Calibration graphs used for the determination of the separation limits SLE and SLP from the percentage of excluded and permeable dextran. The curves represent the dependence of the molecular Stokes' diameter of the dextran fraction eluted from the GPC column at a certain volume V_e on the percentage of the sample eluted up to this volume. The curve for dextran 15 results from its fractionation by Sephadex G-75 and that for dextran 35 is based on fractionation by Sephadex G-200.

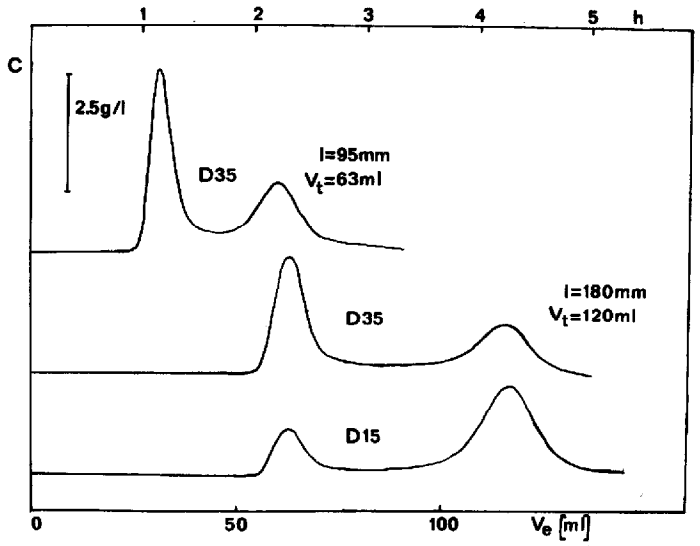


Fig. 4. Size group fractionation of two polydisperse dextran materials by VC on Permselekt. A 50-mg amount of dextran 35 or 15, dissolved in 2 ml of eluent solution, was fractionated on a column packed with the VP Permselekt (unaltered material). Beds of different lengths and volumes (as indicated) were eluted with 0.05 *M* PBS (pH 6.5). Column I.D., 29 mm. Detection as in Fig. 1.

previously [1], the elution volume of monodisperse samples (proteins) tested so far is either in the range 40–50% or 90–100% of the bed volume. It is interesting, however, that proteins with Stokes' diameters below the SLP determined for dextran may be excluded, presumably because of the increase in their apparent hydrodynamic molecular size by electrical interaction with the negatively charged matrix [4] (Fig. 5).

Although the cellin walls carry negative charges in high concentration (about 0.7 mequiv./g dry material), P_{ex} and P_{perm} for the neutral dextran molecules were found to be almost independent of the buffer pH (Table II).

Advantages of the described method for the determination of SLP and SLE are its simplicity and rapidity. As the size distribution of suitable polydisperse dextran

TABLE I

SIZE LIMITS OF EXCLUSION AND PERMEATION DETERMINED WITH THE HELP OF TWO CALIBRATED DEXTRAN MATERIALS

Details as described in the legend of Fig. 1.

Fractionated material	Column length (cm)	Elution time (h)	P_{perm} (%)	P_{ex} (%)	SLE (nm)	SLP (nm)
Dextran 35	9.5	2.5	39.7	49.3	6.8	5.7
	18	5	36.7	49.7	6.8	5.5
Dextran 15	9.5	2.5	67.4	22.3	6.0	5.5
	18	5	70.9	15.1	6.4	5.6

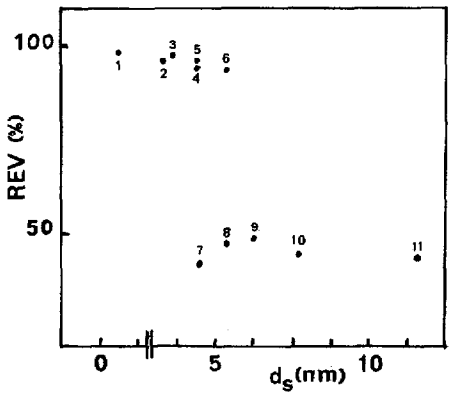


Fig. 5. Relative elution volumes (REV) of different proteins and polyethylene glycol preparations determined by VC with Permselekt columns as a function of their Stokes' diameter. 1 = Myoglobin; 2 = ribonuclease A; 3 = trypsin; 4 = chymotrypsinogen; 5 = polyethylene glycol 4000; 6 = polyethylene glycol 6000; 7 = pepsin; 8 = ovalbumin; 9 = peroxidase; 10 = human serum albumin; 11 = phycocyanin. Stokes' diameters of proteins were taken from ref. 3. Polyethylene glycol fractions of narrow size dispersion (Merck-Schuchardt) were analyzed by GPC on calibrated Sephadex G-75 for their mean Stokes' diameter.

samples is known (Fig. 2), only one size fractionation of such a sample by VC is sufficient for the determination of SLE and SLP. The SLE and SLP define the completely excluded and completely permeable fractions of a neutral hydrocolloid in terms of the minimum and maximum Stokes' diameter, respectively. Both dextran 15 and 35 are suitable materials for the determination of these limits if the VP has not been strongly altered in comparison with that consisting of the native cellin wall.

The reliability of the method is demonstrated by the nearly identical results on separation limits obtained with two dextran samples, the molecular size range of which was determined independently on different Sephadex gels (Table I). Dextran 15 is especially suitable for the size range of Stokes' diameter between 4 and 6 nm (Fig. 3). Dextran 35 (and other dextran materials with even larger mean molecular sizes)

TABLE II

EXCLUDED AND PERMEABLE PERCENTAGES OF DEXTRAN 35 AT DIFFERENT pH VALUES

The dextran was fractionated on the same bed with the pH of the elution buffer varied. Column length 9 cm; for other details, see legend of Fig. 1.

pH	P_{perm} (%)	P_{ex} (%)
5.5	38.1	50.0
6.5	41.9	48.5
7.0	40.9	51.8
5.5	41.8	51.6
6.5	42.3	49.0
7.0	41.3	48.3
5.5	40.3	52.1

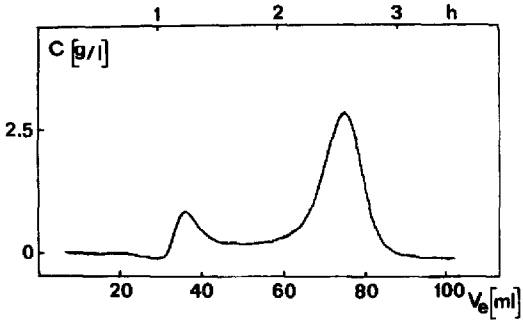


Fig. 6. Size group fractionation of dextran 35 on a column (29 mm I.D., bed length 116 nm) packed with VP Permselekt that had been treated before use with 2% sodium carbonate (3 days at room temperature). Details as in Fig. 4. The determined SLP and SLE are 8.1 and 11.5 nm, respectively.

would be preferred if the separation limits of the vesicle membrane have been increased.

As an example of application, the method was used to determine the separation limits of an alkali-treated VP. It is possible to increase the separation limits of the VP without changing the character of the fractionation (Fig. 6). The preparation of VPs with different but defined separation limits is important for their application in the preparative size fractionation of proteins, nucleic acids and other polymers. Papers on the variability and stability of the separation limits and the application of VPs with increased separation limits are in preparation.

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