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Dye-complexed bacterial lipopolysaccharide as a void volume marker for permeation chromatography

Permeation chromatography, also commonly termed gel permeation, molecular sieve and exclusion chromatography, is an extensively employed method for the separation, purification and analysis of mixtures of biological materials. The use of agarose gels and controlled-pore glass (CPG) having an exclusion limit above a molecular weight of 2×10^6 daltons presents a problem in accurately determining the void volume (V_0) of the column. Blue Dextran 2000 and tobacco mosaic virus[1] have been employed for this purpose. CAMERON[2] reported the use of a bacterial lipopolysaccharide (LPS) as a V_0 marker for agarose columns with an exclusion limit of 1.5×10^8 but its use is subject to several limitations. The LPS marker is not visible while on the column and is not readily detected spectrophotometrically, necessitating fraction collecting and analysis for carbohydrate content. The attachment of a chromophore to the bacterial LPS permits visualization of the V_0 marker on the column, which provides the investigator with information regarding column properties. In addition, the V_0 marker can be monitored continuously in the eluent spectrophotometrically in both the ultraviolet and visible regions.

Material

Dye-complexed LPS. LPS was prepared from *Salmonella typhimurium* by the phenol–water method as described by LÜDERITZ *et al.*[3] and the trichloroacetic acid method of BOIVIN and MESROBEANU[4]. Commercial LPS preparations were also obtained from Difco Laboratories (Detroit, Mich.). LPS preparations were suspended at a concentration of 0.5% in 0.05 *M* tris(hydroxymethyl)aminomethane (Tris-HCl), pH 10.0 and Procion Scarlet MGS dye (ICI, Stamford, Conn.) was added in the ratio of eight parts dye to one part LPS. The solution was mixed thoroughly and incubated for 1–2 h at 75° with occasional agitation. After cooling, the mixture was centrifuged at 80,000 × *g* for 75 min. The free dye was discarded in the supernatant and the pellet was washed twice with distilled water. The final pellet was resuspended in a minimal amount of water or Tris-HCl buffer, 0.05 *M*, pH 7.2. The uncombined dye could also be removed from the solution after the initial centrifugation by dialysis against water or buffer. Microbial contamination was prevented by heating the dye-complexed LPS at 100° for 15 min and storage in a sterile container at 4° or by the addition of 0.02% sodium azide.

Column matrices. Columns were prepared using spherical beads of agarose obtained from Bio-Rad Laboratories (Richmond, Calif.), agarose (Sepharose) and dextran beads (Sephadex) from Pharmacia Fine Chemicals (Piscataway, N.J.), porous glass beads (Porasil) from Waters Associates (Framingham, Mass.) and controlledpore glass (CPG) from Corning Glass Works (Corning, N.Y.). The specific matrices employed are listed in Table I. Porasil B, D and F were 100–150 mesh and Porasil E was 75–125 mesh. All the surface-treated CPG employed was 100–200 mesh (75–125 μ) particle size with a pore diameter distribution reported by the manufacturer to vary not more than 10% from the average. Polyethylene glycol 20,000 was employed as described by HAWK *et al.*[5] to decrease the surface charge of the CPG for use with biological materials.

TABLE I

ELUTION VOLUMES OF DYE-COMPLEXED LPS FROM *Salmonella typhimurium* ON VARIOUS COLUMN MATRICES

Matrix		*Molecular weight exclusion*[a]	$V_t - V_0$ *(ml)*	*Dye-complexed LPS*		*Blue Dextran 2000*		
				V_e *(ml)*	*Detector (nm)*	V_e *(ml)*	*Detector (nm)*	$K_{av.}$
BioGel								
A-150		150×10^6	102.0	64.0	260	73.0	260	0.09
Sepharose								
2B		20×10^6	107.0	50.0	260	50.0	260	0.00
Sephadex								
G-25		5×10^3	52.0	35.0	260	35.0	260	0.00
G-200		2×10^5	127.0	61.0	260	61.0	260	0.00
Porasil								
Type	APD (Å)							
B	100–200	2.5×10^5	32.0	28.5	260	28.5	260	0.00
D	400–800	1×10^6	34.0	30.0	260	30.0	260	0.00
F	1500	2×10^6	51.0	49.0	RI[b]	58.0	RI[b]	0.09
F	1500	2×10^6	72.0	56.0	510	77.0	650	0.29
Ex	—	4×10^6	27.0	26.8	510	44.5	650	0.66
Treated CPG								
Type	APD (Å)							
10–75	81	3.8×10^4	26.5	33.5	260	33.5	260	0.00
10–370	363	2.7×10^5	29.0	29.5	260	29.5	260	0.00
10–700	693	6.6×10^5	26.5	25.5	260	25.8	260	0.01
10–1250	1195	1.2×10^6	26.3	30.0	260	30.2	260	0.01
10–2000	1915	2.2×10^6	27.0	28.0	260	28.2	260	0.01
10–2000	1915	2.2×10^6	176.0	181.0	RI[b]	194.0	RI[b]	0.07
10–2000	1915	2.2×10^6	180.0	189.0	510	309.0	650	0.67
10–2000	2795	12×10^6	26.0	26.5	260	30.0	260	0.13
10–2000	2795	12×10^6	26.0	26.5	RI[b]	30.0	RI[b]	0.13
10–2000	2795	12×10^6	26.0	26.5	510	45.5	650	0.73

[a] As determined with dextrans or globular proteins by the manufacturer.
[b] Refractive index monitor.

Columns and buffers. Both 1.27- and 2.54-cm-diameter columns were used, which ranged from 30–85 cm in length. The glass columns were fitted with flow adaptors for ascending and descending chromatography and were packed in accordance with the instructions of the manufacturers. A variety of buffers were employed: Tris-HCl, 0.05 *M* pH 7.2; 0.1 *M* pH 8.0; distilled water; 0.02% sodium azide; 1% sodium deoxycholate pH 8.1; and 0.05 *M* sodium chloride. A pressure-regulated gravity elution system was employed with the "soft" gels (via a Mariotte flask) and a piston pump was used to deliver 5–6 ml/min with the rigid glass matrices.

Methods

Samples of 2–10 mg of dye-complexed LPS or Blue Dextran 2000 (Pharmacia) in a volume of 0.5 to 2.0 ml (less than 1% of the bed volume) were applied to the column and eluted with 0.05 *M* Tris-HCl, pH 7.2 unless otherwise noted. The column effluents were monitored continuously with a recording spectrophotometer or a differential refractometer. The dye-complexed LPS was monitored at 260 or 510 nm

and Blue Dextran at 260 or 650 nm. The column V_t (total volume) was experimentally determined with bacitracin and monitored at 280 nm.

Results and discussion

The utility of dye-complexed LPS as a void volume marker is demonstrated in Table I where the leading peak of each column run is reported. On A-150, having a molecular weight exclusion of 150×10^6, the dye-complexed LPS eluted as a single peak at 64 ml while the small leading peak of Blue Dextran eluted at 73 ml and the major peak at 142 ml. The dye-complexed LPS eluted at the same V_e (elution volume) as Blue Dextran on Sepharose 2B, Sephadex G-25 and G-200. Both V_0 markers eluted at the same V_e on Porasil B and D but a significant difference was observed on Porasil F and Ex, where dye-complexed LPS eluted first. On surface-treated CPG with an average pore diameter (APD) of 81, 363 and 1195 Å, the V_e was identical for both V_0 markers. However, on a 1915 Å (APD) glass matrix column the dye-complexed LPS eluted before Blue Dextran. The manufacturer of Blue Dextran reports that it absorbs at both 260 and 650 nm[6]. On the 1915 Å (APD) glass column monitored by refractive index the dye-complexed LPS eluted before the Blue Dextran. On a similar column where the dye-complexed LPS was monitored at 510 nm and Blue Dextran at 650 nm, the LPS eluted at 189 ml and Blue Dextran at 309 ml since the 260 nm absorbing peak was not recorded. The results obtained on the 2795 Å (APD) glass were similar to those observed with the 1915 Å (APD) glass column. The elution profiles of dye-complexed LPS and Blue Dextran are shown in Fig. 1, where the column was monitored by refractive index. Attempts to use dye-complexed LPS on

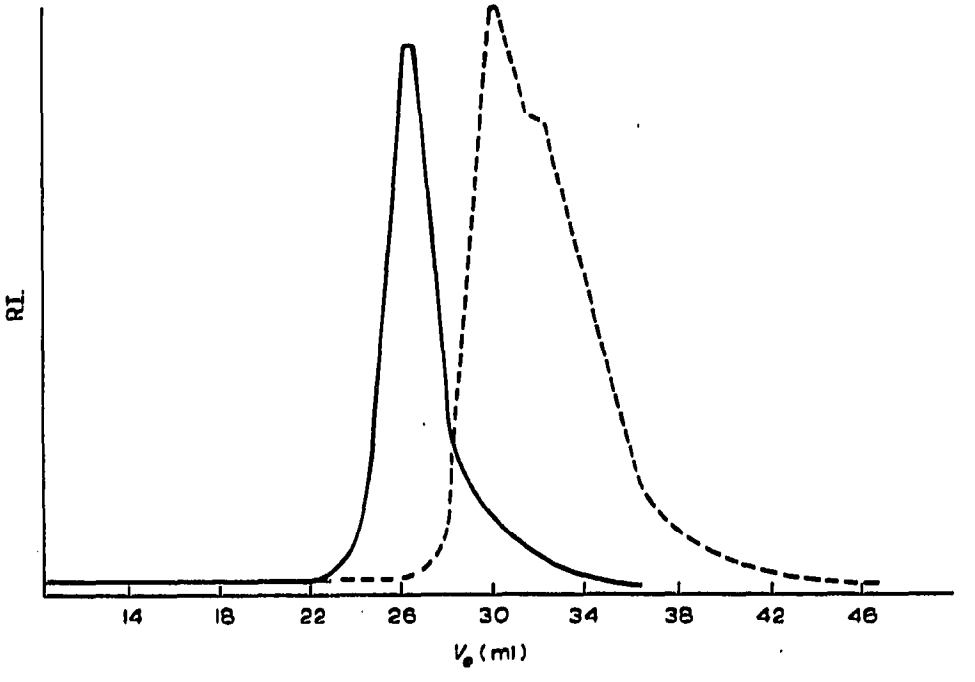

Fig. 1. Elution patterns of dye-complexed LPS (———) and Blue Dextran 2000 (— — —) on surface-treated CPG 10–2000, average pore diameter 2795 Å. The column dimensions were 1.27 × 41.0 cm and the eluent, 0.05 *M* Tris-HCl, pH 7.2, was monitored continuously with a recording differential refractometer.

the Bio-Gel P matrices (Bio-Rad) resulted in a non-specific attachment of the V_0 marker to the column matrix.

The $K_{av.}$ value[1] is commonly employed to characterize solute migrations.

$$K_{av.} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

Thus, for excluded molecules which are incapable of entering the pores of a matrix the $K_{av.} = 0$. The assumption was made that the V_e of the dye-complexed LPS represented the V_0 of the column and the $K_{av.}$ values were calculated for Blue Dextran. Consideration of the $K_{av.}$ value for Blue Dextran on the 2795 Å (APD) glass column clearly indicates that the experimental V_0 marker eluted earlier and also demonstrates the variation in observed elution volume as a function of the method employed for detection. At 260 nm and with refractive index the Blue Dextran elutes with a $K_{av.}$ value of 0.13 while at 650 nm a $K_{av.}$ value of 0.73 was observed.

The use of dye-complexed LPS is of particular value on matrices with a molecular weight exclusion limit of 2×10^6 or greater. The use of Blue Dextran with such matrices will not always give an accurate measurement of the V_0, resulting in incorrect $K_{av.}$ values and incorrect estimates of size. On matrices with an exclusion limit below 2×10^6, Blue Dextran is as useful as dye-complexed LPS for determining the V_0, and, when monitored at 260 nm or by refractive index it is useful on agarose matrices with an exclusion limit up to 20×10^6. However, porous glass matrices with an exclusion limit of 2.2×10^6 show some differences in the V_e of the markers, indicating that some caution is necessary when employing Blue Dextran on large pore size glass columns.

To determine the stability of the dye-complexed LPS in various buffers, it was chromatographed on a 1990 Å (APD) glass column utilizing the buffers listed earlier. In all instances the experimental V_0 marker eluted as a single peak. Also, a dye-complexed LPS preparation was stored at 4° and an aliquot was chromatographed every month for sixteen months on a 1915 Å (APD) glass matrix and the elution pattern remained unchanged.

The dye-complexed LPS is chemically stable and can be stored lyophilized or in solution. If collected after use it can be reconcentrated by dialysis or centrifugation and re-used.

LPS is toxic and although dye-complexed LPS has been found in this laboratory to have lost some of its biological activity, as with other toxic materials, appropriate precautions should be observed in handling.

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