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Note

High-speed aqueous gel permeation chromatography of proteins

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Since the introduction of gel filtration by Porath and Flodin[1], the method using a cross-linked dextran or a cross-linked polyacrylamide gel column, has been utilized in the field of biological science as one of the essential techniques for separation and molecular-weight estimation of water-soluble macromolecules.

The estimation of the molecular weight of macromolecules is also made by gel filtration together with the other techniques such as analytical ultracentrifuge, light scattering, or electrophoresis. For this purpose, the classical gel filtration method using a soft-gel column does not need sophisticated and expensive instruments nor special techniques; however, the matrix of these gels are quite soft and weak so that the gels do not withstand the high pressure required for high-speed analysis. Therefore, very low flow-rates should be applied to the column and it takes a few days to completely analyse proteins. Moreover, this tedious method also needs a considerable amount of sample for the detection of the peak; usually milligram levels of proteins are required.

Several attempts have been made for the high-speed liquid chromatography of proteins by gel permeation chromatography (GPC) in aqueous media. However, most of the results do not seem to be completely satisfactory due to the low column efficiency and the adsorption of the solute on the packings. To prevent the adsorption of protein molecules, controlled-pore glass beads substituted with glycerol were developed and their applications have been reported[2–5]. The developments of aqueous GPC are reviewed by Cooper and Van Derveer[6]. Recently, Hashimoto *et al.* described[7] the separations of proteins on TSK-GEL PW-type columns. In the present study, we report on the chromatographic properties of another type of TSK-GEL column which uses different gel materials than the PW-type gel.

MATERIALS AND METHODS

Chromatography was carried out on a chromatograph consisting of a Trirotar pump, loop type sample injector, and UV detector of UVIDEC 100-II (JASCO, Tokyo, Japan). TSK-GELs (8–12 μm) were packed in a stainless-steel tube (600 × 7.5 mm) (Tokyo Soda, Tokyo, Japan).

Proteins were dissolved in water to a concentration of 2 mg/ml. Blue dextran 2000 and alanine were used as the marker compounds for totally excluding and totally permeating molecules, respectively.

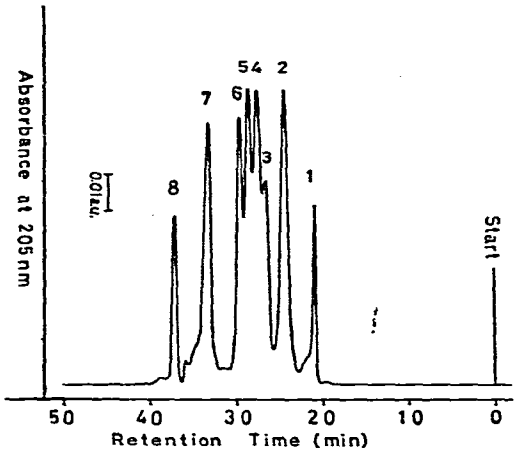

Fig. 1. Chromatogram of proteins on a TSK-GEL SW 2000 column. Elution buffer, pH 6.5 0.01 *M* phosphate + 0.2 *M* sodium sulfate. 1 = Aldolase; 2 = ovalbumin; 3 = lactoglobulin; 4 = trypsin inhibitor; 5 = chymotrypsinogen; 6 = ribonuclease A; 7 = insulin; 8 = alanine.

RESULTS AND DISCUSSION

A typical chromatogram of proteins is shown in Fig. 1. Samples of 2–4 μg of seven proteins with different molecular weights from aldolase to insulin and 40 μg of alanine, were mixed and chromatographed on a TSK-GEL 2000 SW column. The eluent used was 0.01 *M* phosphate buffer containing 0.2 *M* sodium sulfate (pH 6.5) which was pumped at a flow-rate of 0.5 ml/min. Absorbance of the effluent was monitored at 205 nm. Eight compounds were eluted in order of the molecular weights. Fig. 2 shows the chromatograms of six compounds separated on a TSK-GEL 3000 SW column, using different flow-rates.

The relationship between eluent flow-rate and height equivalent of a theoretical

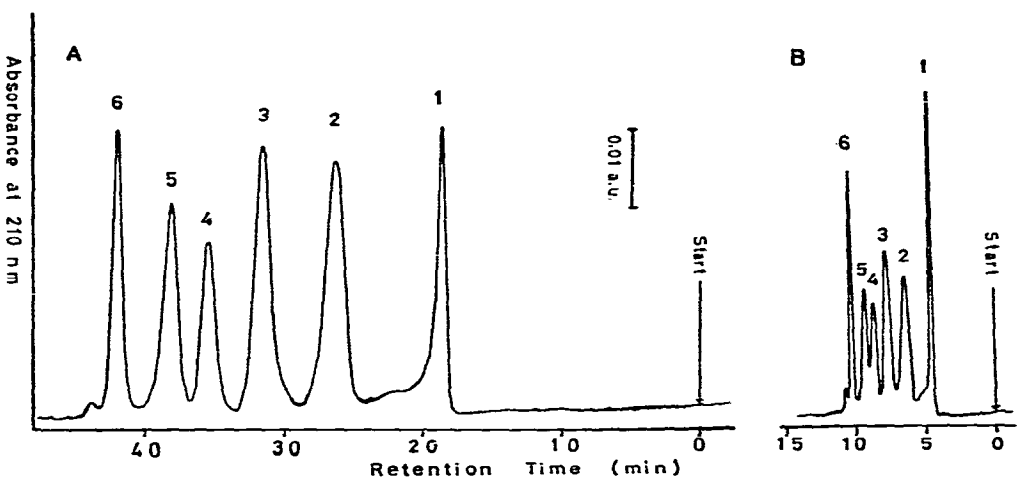

Fig. 2. Chromatograms of proteins on a TSK-GEL SW 3000 column. Elution buffer, pH 6.5 0.01 *M* phosphate + 0.2 *M* sodium sulfate. Flow-rate, (A) 0.5 ml/min, (B) 2.0 ml/min.

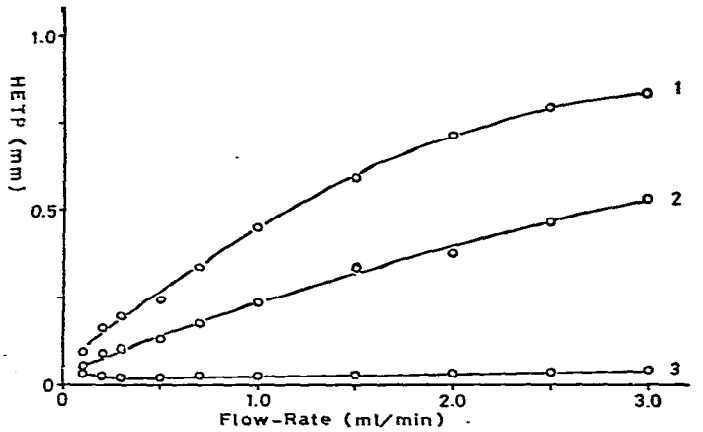

Fig. 3. Effect of flow-rate on the plate height for a TSK-GEL 2000 SW column. 1 = Ovalbumin; 2 = chymotrypsinogen; 3 = alanine.

plate (HETP) is shown in Fig. 3 for a TSK-GEL 2000 SW column at flow-rates ranging from 0.1 ml/min (0.04 mm/sec) to 3.0 ml/min (1.14 mm/sec). HETP for macromolecules is greatly increased with flow-rate due to the small diffusion coefficients as usually observed in the GPC results. On the other hand, HETP of small molecules, such as alanine, depend little on flow-rate and showed a minimum value at about 0.3 ml/min. The value increased at both lower and higher flow-rates. The maximum plate number 48,500/m for this column was obtained for the peak of totally permeating alanine eluted at the flow-rate of 0.3 ml/min. Fig. 4 shows the results from the TSK-GEL 3000 SW column. For macromolecules, a plate number of 8700/m for aldolase (mol. wt. 154,000), and 20,000/m for chymotrypsinogen (mol. wt. 24,500), were obtained at the flow-rate of 0.1 ml/min. The maximum of 30,000 plates/m for this column was obtained with the alanine peak eluted at 0.3 ml/min.

To examine the effects of the flow-rate on the retention volume, aldolase, chymotrypsinogen and alanine were chromatographed at various flow-rates. It was

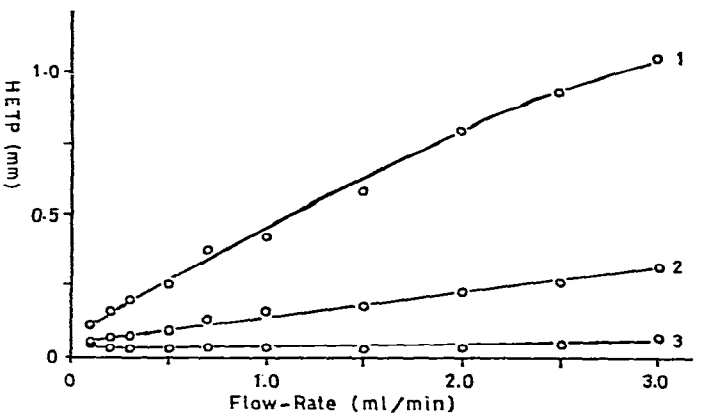

Fig. 4. Effect of flow-rate on the plate height for a TSK-GEL 3000 SW column. 1 = Aldolase; 2 = chymotrypsinogen; 3 = alanine.

found that the retention volume of the compounds was not dependent on the flow-rate (Fig. 5). Therefore, a high flow-rate can be applied to determine the retention volume of the compounds at the sacrifice of resolution. For example, by eluting at 2 ml/min, the analysis of proteins was completed within 11 min as shown in Fig. 2B.

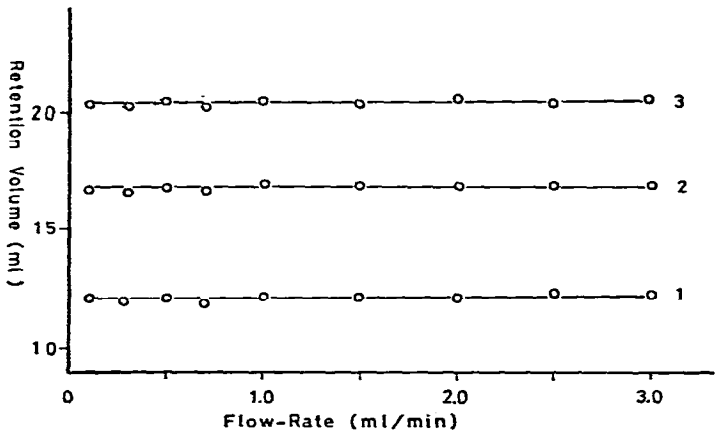

Fig. 5. Effect of flow-rate on the retention volume for a TSK-GEL 3000 SW column. 1 = Aldolase; 2 = chymotrypsinogen; 3 = alanine.

On the TSK-GEL 3000 SW column, a linear relationship between logarithm of molecular weight of proteins and retention volume was obtained for 16 proteins, their molecular weights ranging from 480,000 (ferritin) to 6000 (insulin). As shown in Fig. 6, ferritin eluted from the column after blue dextran 2000. This means that the exclusion limit of this column is somewhat larger than 480,000 daltons. Fig. 6 also gives the protein calibration curve for the TSK-GEL 2000 SW column with the same set of proteins as authentic samples. Ferritin, catalase, aldolase, and bovine serum albumin eluted at the void volume, and ovalbumin and the rest of the smaller compounds were retained on the column, so that the exclusion limit of this column for protein molecules was about 60,000 daltons. Good linearity between the logarithm of molecular weight and retention volume was also obtained on this column for various proteins from ovalbumin to insulin. Lysozyme, however, eluted slower than the expected retention volume on the basis of its molecular weight; when no salt was added to the buffer solution, this compound did not elute from the column. The adsorption of solutes on the column was reduced by the addition of sodium sulfate or sodium chloride. With higher salt concentrations than 0.2 M, the adsorption of lysozyme and smaller aromatic compounds were still observed. Table I shows the distribution coefficient, K, for several amino acids and nucleic acid-related compounds. The K value was obtained from retention volume of the solute, V_r, retention volume of totally excluded blue dextran 2000, V_0, and retention volume of totally permeating alanine, V_t, as $K = (V_r - V_0)/(V_t - V_0)$. These results show that basic amino acids are not adsorbed on the gel, but aromatic amino acids are adsorbed, and nucleic bases are also adsorbed strongly on the gel. The extent of adsorption of nucleic acid-related compounds is in the order of base, nucleoside, and nucleotide. Purine derivatives are adsorbed stronger than pyrimidine derivatives.

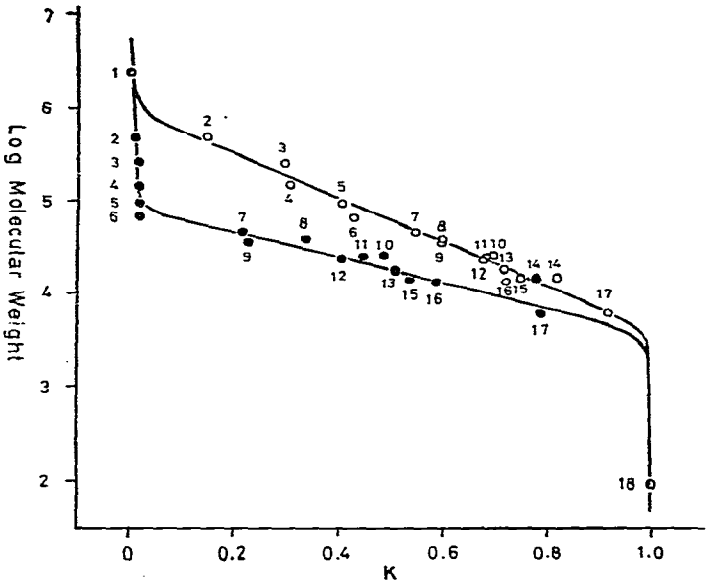

Fig. 6. Molecular weight calibration curves for TSK-GEL 2000 SW (●) and 3000 SW (○) columns. 1 = Blue dextran 2000 (MW 2000,000); 2 = ferritin (480,000); 3 = catalase (244,000); 4 = aldolase (145,000); 5 = transferrin (90,000); 6 = bovine serum albumin (65,000); 7 = ovalbumin (45,000); 8 = lactoglobulin (38 000); 9 = pepsin (33,500); 10 = chymotrypsinogen (24,500); 11 = trypsinogen (24,000); 12 = trypsin inhibitor (22,000); 13 = myoglobin (17,000); 14 = lysozyme (14,400); 15 = ribonuclease A (13,800); 16 = cytochrome *c* (13,000); 17 = insulin (6,000); 18 = alanine (89).

The high performance of these columns permits detection of very small amounts of protein. Even nanogram amounts can be detected satisfactorily by monitoring at a 200–220 nm range. Fig. 7 shows the chromatogram of the mixed solution of aldolase, chymotrypsinogen, and alanine. Between 5–10 ml of elution

TABLE I

ADSORPTION OF SMALL COMPOUNDS ON A TSK-GEL SW 3000 COLUMN

Elution buffer, same as for Fig. 2.

Compound	*K*	*Compound*	*K*
Blue dextran	0	Adenosine	1.37
Alanine	1.00	Cytidine	1.09
Phenylalanine	1.16	Guanosine	1.20
Tyrosine	1.09	Uridine	1.03
Tryptophan	1.54	Thymidine	1.11
Histidine	1.01	5′-AMP	1.11
Lysine	0.97	5′-CMP	0.99
Arginine	0.98	5′-GMP	1.01
Adenine	1.95	5′-UMP	1.00
Cytosine	1.21		
Guanine	1.36		
Uracil	1.21		
Thymine	1.24		

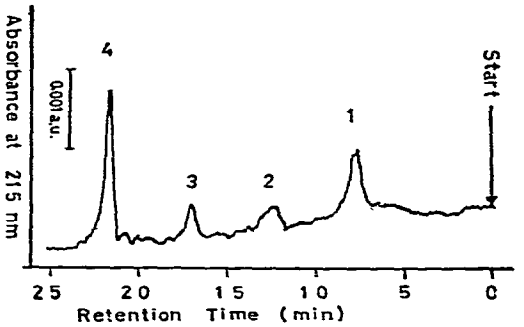

Fig. 7. Chromatogram of nanogram level proteins on a TSK-GEL 3000 SW column. 1 = Ghost peak; 2 = aldolase (20 ng); 3 = chymotrypsinogen (20 ng); 4 = alanine (120 ng).

volume usually a few ghost peaks appeared when detector sensitivity was high. One of these peaks has the same elution volume as the void volume; the other is less.

This work shows the excellent performance of the newly developed TSK-GEL SW-type columns on the hydrophilic GPC of proteins under high pressure. High-speed GPC in aqueous eluent with high resolution and high sensitivity should greatly aid in easing the increasing demands made in the fields of biological and hydrophilic polymer sciences.

REFERENCES

1 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657.
2 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
3 C. Persiani, P. Cubor and K. French, *J. Chromatogr. Sci.*, 14 (1976) 417.
4 H. D. Crone and R. M. Dowson, *J. Chromatogr.*, 129 (1976) 91.
5 R. J. Blagrove and M. J. Frenkel, *J. Chromatogr.*, 132 (1977) 399.
6 A. R. Cooper and D. S. Van Derveer, *J. Liquid Chromatogr.*, 1 (1978) 693.
7 T. Hashimoto, H. Sasaki, M. Aiura and Y. Kato, *J. Chromatogr.*, 160 (1978) 301.