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Note

High-speed aqueous gel-permeation chromatography of proteins

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Aqueous gel-permeation chromatography (GPC) on Sephadex has been used extensively for the separation and molecular-weight determination of proteins. However, as Sephadex is not mechanically stable under pressure, the flow-rate cannot be increased by applying pressure, and hence the analysis time is long. Much effort has therefore been devoted to developing column packings that can be operated at high speed under pressure, yield high resolution and do not adsorb proteins; in consequence, several new column packings have been introduced[1–3], one of which, TSK-GEL (Type-PW column) has recently become commercially available. This packing consists of microspheres of a hydrophilic polymer, and the Type-PW columns can be operated at high pressure and have a large number of theoretical plates. Moreover, several grades of columns with different pore sizes are available, so that a wide molecular-weight range of water-soluble substances can be separated. Some results for the high-performance aqueous GPC of polymers and oligomers with TSK-GEL (Type-PW) have been reported[4,5]; in this paper, investigations on the separation of proteins are described.

EXPERIMENTAL

A commercial liquid chromatograph, HLC-802UR (Toyo Soda) was used in GPC measurements. Eluates were monitored at 220 nm with a Uvigraph LC-10 variable-wavelength UV detector (Toyo Soda). Two grades of columns, G3000PW and G4000PW, were used, the molecular-weight exclusion limits for which were, respectively, 60,000 and 700,000 for dextran. Measurements were carried out at 25° with the column (60 × 0.76 cm I.D.) operated at a flow-rate of 0.9 ml/min; the pressure drop was 90 kg/cm^2 for G3000PW (particle diameter, 5–8 μm). For G4000-PW (particle diameter, 8–10 μm), the flow-rate was 1.0 ml/min and the pressure drop was 60 kg/cm^2. Proteins were eluted from the column in less than 15 min at these flow-rates with an eluent of 0.1 *M* phosphate buffer (pH 7.0) containing 0.5 *M* sodium chloride. The injection volume was 0.1 ml, and the sample concentration was 0.25–1 mg/ml. The samples were commercial proteins and oligopeptides (see Table I).

TABLE I

SAMPLE COMPOUNDS

Sample No.	*Protein or oligopeptide*	*Source**	*Molecular weight*
1	Fibrinogen (human)	A	341,000
2	γ-Globulin (fraction II from bovine plasma)	B	156,000
3	Bovine serum albumin	A	67,000
4	Haemoglobin (human)	C	64,500
5	Ovalbumin	A	43,000
6	Peroxidase (horseradish)	D	40,200
7	β-Lactoglobulin	C	35,000
8	Chymotrypsinogen A (bovine pancreas)	E	25,700
9	α-Chymotrypsin (bovine pancreas)	F	25,000
10	Myoglobin (equine skeletal muscle)	D	16,900
11	Ribonuclease (bovine pancreas)	D	13,700
12	Cytochrome *c*	C	12,400
13	Glycyl-glycyl-glycyl-glycine	G	246
14	Glycyl-glycine	G	132

* A, Wako (Osaka, Japan); B, Nakarai (Kyoto, Japan); C, Miles Labs. (Elkhart, Ind., U.S.A.);); D, Sigma (St. Louis, Mo., U.S.A.); E, Schwarz/Mann (Orangeburg, N.Y., U.S.A.); F, P-L Biochemicals (Milwaukee, Wisc., U.S.A.); G, Tokyo Chemical Industry (Tokyo, Japan).

RESULTS AND DISCUSSION

Figs. 1 and 2 show the elution curves of proteins obtained with G3000PW and G4000PW, respectively. Multiple peaks were observed in the elution curves of many proteins, and in the curve of γ-globulin, a minor peak appeared a little before the major peak. The major and minor peaks presumably correspond to, respectively, γ_1-globulin and γ_2-globulin[6]. A minor peak emerges before the major peak also for bovine serum albumin; this minor peak is presumed to be bovine serum albumin dimer[7]. Although each peak was not identified, Figs. 1 and 2 indicate that many commercial proteins contain several impurities. With a few exceptions, however, the major peaks are very narrow and symmetrical. The major peaks of fibrinogen and γ-globulin are broad and asymmetrical, the front half of the fibrinogen peak and the back half of the γ-globulin peak being the broader. The major peak of haemoglobin is a trifle broad, but symmetrical; on the other hand, the major peak of β-lactoglobulin, although narrow, "tails" appreciably. Because this is the typical shape of a peak when adsorption occurs, we suggest that β-lactoglobulin is adsorbed on the gel. The plot of molecular weight against elution volume for β-lactoglobulin, however, lies on the same line as other proteins (as will be shown later), so that adsorption of β-lactoglobulin must be very slight. It is probable from the shapes of the major peaks that peak-broadening with fibrinogen, γ-globulin and haemoglobin was due not to adsorption but to molecular-weight heterogeneities of the components of the major peaks. The sharp front and "tailing" in the major peak of fibrinogen (measured with G3000PW) is presumably attributable to the high-molecular-weight components of the fibrinogen eluted in the void volume of the column.

In Fig. 3, molecular weights are plotted against elution volumes for fibrinogen, bovine serum albumin, ovalbumin, peroxidase, β-lactoglobulin, myoglobin, cyto-

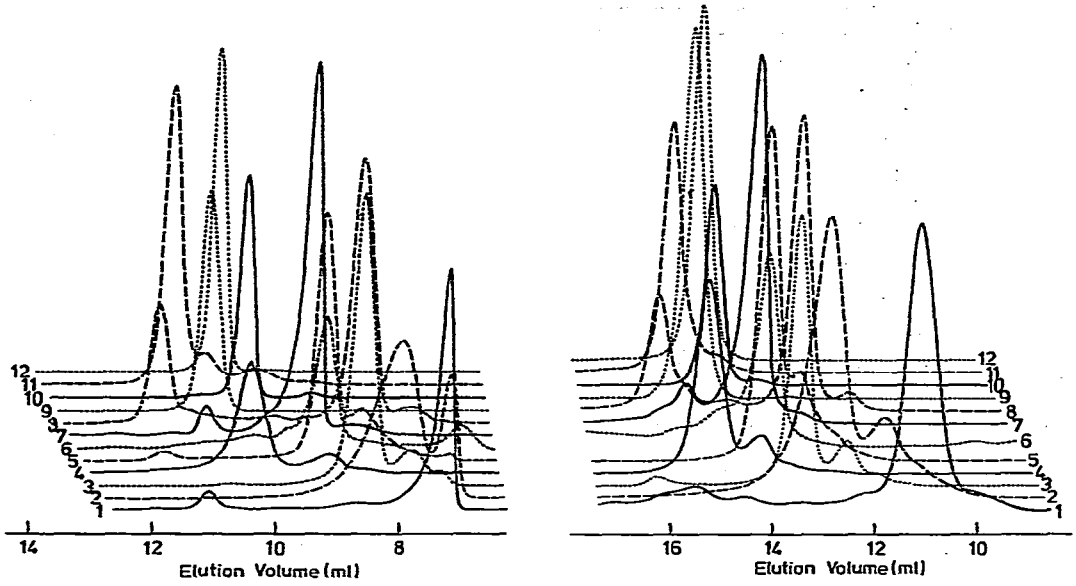

Fig. 1. Elution curves of proteins measured with G3000PW. The numbers on the curves are the sample numbers in Table I.

Fig. 2. Elution curves of proteins measured with G4000PW. The numbers on the curves are the sample numbers in Table I.

chrome *c*, glycyl-glycyl-glycyl-glycine and glycyl-glycine. The point for the minor peak of bovine serum albumin is also included in the plot, this component being considered as a dimer. The plots for both G3000PW and G4000PW lie, respectively, on a line, indicating that the proteins are separated on the basis of molecular weight. Further, the separation range of G3000PW is from oligopeptides to a molecular weight of *ca.* 200,000. With G4000PW, although the void volume was not determined, the exclusion limit in protein molecular weight will be above 10^6, as the exclusion limit of G4000PW is one order of magnitude larger than that of G3000PW for dextran. The plots for γ-globulin, haemoglobin, chymotrypsinogen A, α-chymotrypsin and ribonuclease deviate from the lines shown in Fig. 3, in which the elution positions of these proteins are denoted by arrows. The molecular weights corresponding to these positions on the lines are summarized in Table II. The molecular weights evaluated for each protein from the results with G3000PW and with G4000PW are in good agreement, but the values are different from those listed in Table I. As γ-globulin is generally heterogeneous in molecular weight, the molecular weight determined by any given method is an average and thus will not necessarily correspond to the peak position on the elution curve. It has been reported that haemoglobin eluted late from a column of polyacrylamide gel, and this is probably because haemoglobin, which consists of four sub-units, dissociated depending on the concentration or pH of the solution[7,8]. The molecular weight of haemoglobin evaluated from Fig. 3 is 15,000, which is approximately one quarter of the generally accepted value. It is therefore reasonable to suppose that dissociation into sub-units occurs before injection or during separation. The values for α-chymotrypsin and ribonuclease in Table II are

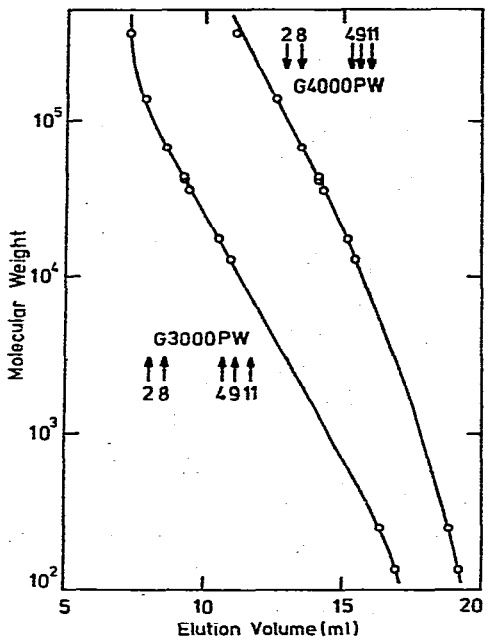

Fig. 3. Plots of molecular weight against peak elution volume. The arrows denote the peak positions of γ-globulin (2), haemoglobin (4), chymotrypsinogen-A (8), α-chymotrypsin (9) and ribonuclease (11).

smaller than those in Table I; on the other hand, the value for chymotrypsinogen A in Table II is larger than the value in Table I. These discrepancies cannot be successfully interpreted. No report exists on the anomalous behaviour of these proteins, and moreover, adsorption can be ruled out because the peaks of these proteins are sharp and almost symmetrical (see Figs. 1 and 2).

An application of this work is shown in Fig. 4; this is an elution curve for human plasma measured with G4000PW under the same conditions as for proteins (except for the sample concentration); 0.1 ml of a 1% solution of human plasma was injected. This curve shows that all the components were within the separation range of G4000PW; thus, G4000PW is suitable for the separation of proteins in human plasma. Multiple peaks were observed, the largest being, probably, albumin (the

TABLE II

MOLECULAR WEIGHTS ESTIMATED FROM FIG. 3 FOR PROTEINS THAT BEHAVED ABNORMALLY IN GPC

Protein	*Molecular weight on*	
	G3000PW	*G4000PW*
γ-Globulin	110,000	104,000
Haemoglobin	15,000	15,000
Chymotrypsinogen A	69,000	68,000
α-Chymotrypsin	11,000	11,000
Ribonuclease	7300	7300

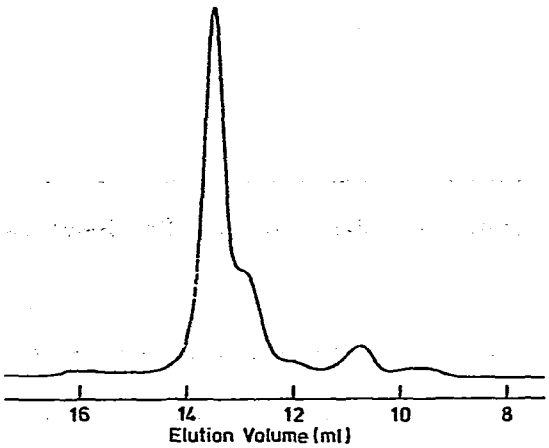

Fig. 4. Elution curve of human plasma measured with G4000PW.

elution volume of this peak is 13.5 ml and almost identical with that of bovine serum albumin).

From these results, it can be concluded that the rapid separation (and molecular-weight estimation) of proteins having wide range of molecular weights are possible by using TSK-GEL (type PW).

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