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Fractionation of Poly(γ-b-enzyl-L-glutamate) by Gel-Permeation Chromatography

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ABSTRACT: Gel-permeation chromatography (GPC) was utilized for fractionating poly(γ-benzyl-L-glutamate) (PBLG) polymerized from *N*-carboxylanhydrides. The resolving power of this GPC column was checked by several monodisperse materials such as Gramicidin D, carbobenzoxy-L-Leu-L-Leu-L-Val-L-Phe-methyl ester, diketopiperazine, and 1,2,4-trichlorobenzene (TCB). The fractionation efficiency was analyzed by comparison of the molecular-weight distribution of fractionated PBLG and that of unfractionated PBLG, which was determined by the relation between molecular weights of PBLG samples and their elution volumes. On the basis of these results we conclude that GPC offers a useful method of obtaining the monodisperse PBLG whose M_w/M_n ratio is less than 1.1.

KEY WORDS Poly(γ-benzyl-L-glutamate) / GPC / Polydisperse / Molecular Weight Distribution /

In studies of the helix—coil transition of polypeptides, the polydispersity of somples sometimes makes it difficult to build a definite conclusion. In particular, the helix—coil transitions of low-molecular-weight polypeptides depend very much on the degree of polymerization, owing to the cooperative nature of the transition. So we often misinterpret the experimental results of helix-coil transitions of polypeptides when we use polydisperse samples. A typical example of this situation is seen in the controversy about the interconversion rate of the helix—coil transition.[1–5] Thus highly monodisperse polypeptides are required to simplify the interpretation of experimental results and to find the real picture of cooperative phenomena which are often hidden under the polydispersity in molecular weight.[6]

To obtain a monodisperse polypeptide the solid phase peptide synthesis is best. But its technical difficulties and the amount of labor involved made us choose the fractionation method as the best alternative.

Up to now various fractionation methods have been applied to polydisperse PBLG. Some of them are the fractional extractions,[7,8] fractional precipitations[9,10] and the chromatography with use of glass beads.[11] These methods are good for fractionating a large amount of sample, but all have weak points due to cumbersome procedures.

In this paper we report a simple method to obtain a fairly monodisperse PBLG, using gel-permeation chromatography.

EXPERIMENTAL

Materials

PBLG was polymerized in dry *N,N*-dimethylformamide (DMF) or dioxane with *N*-hexylamine or sodium methoxide as an initiator from *N*-carboxylanhydrides (NCA's) synthesized by the Blout and Karlson method.[12] To determine the resolution of the column we made use of several monodisperse samples.

Diketopiperazine of γ-benzyl-L-glutamate and γ-benzyl-D-glutamate was formed from the dipeptide, which was synthesized by the Heitz and Spach procedure.[13] Carbobenzoxy-L-Leu-L-Leu-L-Val-L-Phe-methyl ester was kindly supplied from Prof. M. Tsuboi and Dr. T. Akimoto, Faculty of Pharmacology, University of Tokyo. Gramicidin D was kindly supplied from Prof. S. Ishii, Faculty of Pharmacology, University

Table I. Molecular weights for various PBLG samples

Lot no.	M_w (viscosity)	M_w (sedimentation)	Initiator	Solvent	
7006	1.9×10^5		Triethylamine	Dioxane	unfractionated
7005	1.04×10^5		NaOMe	〃	〃
7003	7.4×10^4		〃	〃	〃
7102-3		1.8×10^4	Hexylamine	DMF	fractionated from PBLG 7102
7101I-4		9.0×10^3	〃	〃	fractionated from PBLG 7101
7101II-3		6.4×10^3	〃	〃	〃

of Hokkaido. 1,2,4-trichrolobenzene was purchased from Wako Pure Chemical Industries.

Gel and Column

A vinylacetate copolymer gel sold by Merck under the trade name Mercogel OR-PVA 20000 was used. The usefulness of this gel for separation of polymers in organic solvents has already been reported by Heitz, *et al.*[14] This gel has a mesh size of 270—600 mesh (20—55μ). Special grade DMF purchased from Wako Pure Chemical Industries was used as an elution solvent without further purification. DMF was thought to be the best organic solvent as the elution solvent for the following reasons. (1) It dissolves PBLG in the helix form without aggregation of polymers; (2) Its viscosity is smaller than other good solvents such as *m*-cresol; (3) It is inactive to both PBLG and granules; (4) Although organic acids dissolve PBLG in the random coil form, they are not desirable, because they attack PBLG and granules over a long time duration.

The experiments were performed with a gel column measuring 2.2×95 cm. The gel was swollen in DMF for one day and fine granules were removed by stirring the gel and decanting off the turbid supernatant solvent. The gel swollen by DMF was poured into the column, packed without pressure, and washed with DMF for three days. The flow rate of the effluent was 6.3 ± 0.05 m*l*/hr; the concentration of PBLG in the effluent, whose volume was measured in 2.0 m*l* increments (counts), was checked by an UV spectrometer (Shimazu DR-40). All of the experiments were carried out at an initial concentration of 0.5% or less in 1-m*l* DMF in a temperature-controlled room (20 ± 1°C).

Molecular Weights of PBLG Samples

Six samples were used to find the relation between the molecular weight of PBLG and its elution volume.

Three of these were of relatively high molecular weight and their weight-average molecular weights were estimated by the intrinsic viscosity in dichroloacetic acid (DCA), using the relation determined by Doty, Bradbury, and Holtzer.[8] The other three were of low molecular weight and their weight-average molecular weights were obtained by the sedimentation equilibrium method on a Beckman—Spinco model-E ultracentrifuge. A standard 12-mm cell was used for solutions with an initial concentration of 1 g/d*l* in DMF. Measurements were made at 16°C for PBLG 7101II-3, 13°C for PBLG 7101I-4, and 12°C for PBLG 7102-3. The value of 0.782 m*l*/g was assumed for the partial specific volume of PBLG in DMF at 12°C and 13°C and 0.783 m*l*/g at 16°C, as reported by Deloze *et al.*[15] The apparent molecular weight of PBLG at the concentration of 1 g/d*l* was corrected for the concentration, assuming that the second virial coefficient of PBLG has the same value of 2.5×10^{-4} c.g.s. in the range of molecular weight (6×10^3—1.8×10^4) as reported by Fujita, *et al.*[16] So the accuracy of the weight-average molecular weight determined by sedimentation is within $\pm5\%$, which was comparable with the percentage of the concentration correction to the molecular weight. The weight-average molecular weights of the PBLG samples are listed in Table I.

RESULTS AND DISCUSSION

Reproducibility

The reproducibility of column constants must be checked through the course of the experiments, because the packed gel in the column usually

suffers some aging effect during the long duration of the experiments. PBLG 7003 and Gramicidin D were used as standard samples in our experiments.

For three chromatograms of PBLG 7003, the results are as follows. Elution volumes at the peak position: mean 108.1 m*l* (max 108.5 m*l*, min 107.8 m*l*) and half widths of chromatograms: mean 10.4 m*l* (max 10.8 m*l*, min 10.0 m*l*).

For five chromatograms of Gramicidin D, the results are as follows. Elution volumes at the peak position: mean 192.3 m*l* (max 193.5 m*l*, min 191.5 m*l*) and half widths of chromatograms: mean 7.0 m*l* (max 7.2 m*l*, min 6.8 m*l*).

All of the experiments were performed only in three weeks. Within statistics the measurements do not seem to be influenced by the aging effect. Therefore the above uncertainty in the values within $\pm 0.5\%$ for the elution volume and within $\pm 4\%$ for the half width of the chromatogram comes only from the error in the count-volume and the fluctuation in the temperature condition ($\pm 1°C$). Consequently the accuracy of our experiments of GPC is of the same order of magnitude as that of the results of the reproducibility test.

Logarithmic Calibration Plot of Molecular Weights

The $\log M_w$ is plotted *vs.* V_e in Figure 1.

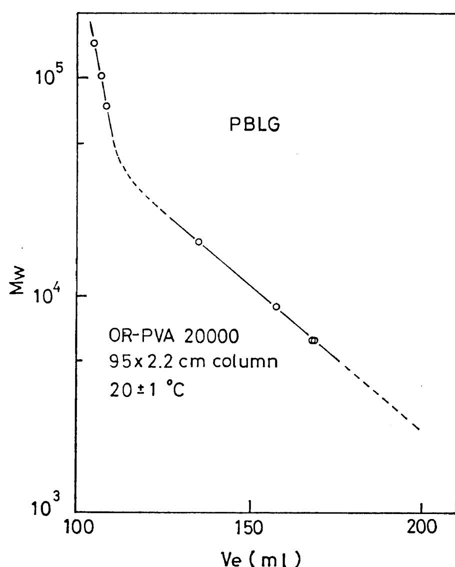

Figure 1. GPC calibration: PBLG/DMF.

From this plot it is found that the exclusion limit of the OR-PVA 20000 gel for PBLG in a helix form is about 4.5×10^4, which corresponds to a void volume of 115 m*l*. The void volume 115 m*l* is a reasonable value for this column, because it is one third of the bed volume of the column (350 m*l*), as expected for a column packed closely with identical spherical particles.

The linear relationship between $\log [\eta] M_w$ and V_e was empirically established by Grubisic, *et al.*,[17] but it may be possible to assume the log of M_w relates linearly to V_e in the narrow range of molecular weight of homologous polymers. As shown in Figure 1, three data points at the part of low molecular weight lie on the same straight line within the experimental error. Of course this plot must be corrected for the molecular weight distribution of each sample, but the measurements were performed for fractionated ones and all of them showed narrow chromatograms with half widths of about 15 m*l*, so this plot was thought to be good enough to check the molecular weight and its distribution for the PBLG sample.

Resolution of GPC and Molecular-Weight Distributions of PBLG Samples

The resolving power of this GPC system was checked by using the following samples: 1,2,4-trichlorobenzene ($M_w = 181.5$), diketopiperazine ($M_w = 440$), carbobenzoxy-L-Leu-L-Leu-L-Val-L-Phe-OMe ($M_w = 638$), and Gramicidin D ($M_w = 1850$). These samples were selected for the following reasons. (1) They are soluble in DMF without forming aggregates. (2) They are monodisperse in molecular weight. (3) Their concentrations in effluents are easily checked by UV spectrometry. All the chromatograms of these samples showed Gaussian-like curves, so their adsorption to gel should be negligible. We made use of the ratio of the elution volume at the peak and half width ΔV_e of each chromatograms as the measure of the resolution of this column. The results are shown in Figure 2.

From the plate theory of liquid chromatography the ratio $\Delta V_e / V_e$ is expected to become constant for samples having the same physicochemical properties under the same conditions.[18] Although the experiments were made for different materials, the main reason for the vari-

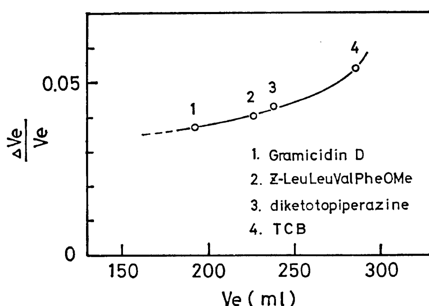

Figure 2. Variation of the ratio $\Delta V_e/V_e$ with respect to the elution volume.

ance in $\Delta V_e/V_e$ among the four samples is thought to arise from the small flow rate of the elution and consequently to be an effect of the diffusion of molecules. We can estimate the effect of the diffusion. The void volume of this GPC column was about 110 m*l*, as shown in the preceding section, so the elution rate of 6.3 m*l*/hr means only a small velocity of 17 μ/sec for the solvent. This value is not so large compared with the diffusion velocity of small molecules (for example, that of TCB is about 35 μ/sec in benzene at 25°C). So the broadening effect due to the translational diffusion of molecules must be explicitly estimated in our GPC system.

When we estimate the diffusion broadening, it is sufficient for us to notice the fact that the chromatogram of a completely monodisperse material becomes the convolution of two curves: one expected from the plate theory and the other from the diffusion theory. Since the two curves are approximately Gaussian, the chromatogram is also approximated by a Gaussian curve whose the half width is a simple sum of that of each Gaussian curve.

$$\Delta V_e = \Delta V_p + \Delta V_d \qquad (1)$$

Here ΔV_e is the total half width, ΔV_p is the half width expected from the plate theory and proportional to the elution volume V_e, and ΔV_d is the diffusion broadening. Eq 1 is rewritten as eq 2

$$\Delta V_e = aV_e + 3.332(Dt)^{1/2} \qquad (2)$$

Here a is a constant characteristic of the column and D is the translational diffusion constant of polymers. The second term can be rewritten as a function of V_e as follows. Time t is proportional to V_e owing to the constancy of the flow rate and D is inversely proportional to the molecular weight raised to a fractional power. The fractional exponent varies its value from 1/2 to about 4/5 depending on the conformations of molecules.[19] So if we assume a logarithmic relation of molecular weight to the elution volume, the second term in eq 2 relates to V_e as eq 3. Finally we can get ΔV_e only as a function of V_e as shown in eq 4.

$$(Dt)^{1/2} \propto V_e^{1/2} \exp(AV_e) \qquad (3)$$

$$\Delta V_e = aV_e + bV_e^{1/2} \exp(AV_e) \qquad (4)$$

It is possible to assume that b and A of four samples do not differ so greatly from one another, although they depend on molecular conformations in general. To separate the constant a which is characteristic of the resolution of the column, we plot $\log(\Delta V_e/V_e - a)V_e^{1/2}$ *vs.* V_e, assuming the constancy of b and A, as shown in Figure 3. From Figure 3 one finds that the plot becomes linear at $a = 0.027 \pm 0.003$. When we take 0.027 as the value of a, HETP of this column becomes 170μ and the theoretical plate number becomes 5.5×10^3. These values are thought to show the high efficiency of this GPC column.

If this plot is adapted to PBLG and if the extraporation is allowed to the higher-molecular-weight region, we obtain the value of 5.4 m*l* for the half width of the chromatogram of the completely monodisperse PBLG with the elution

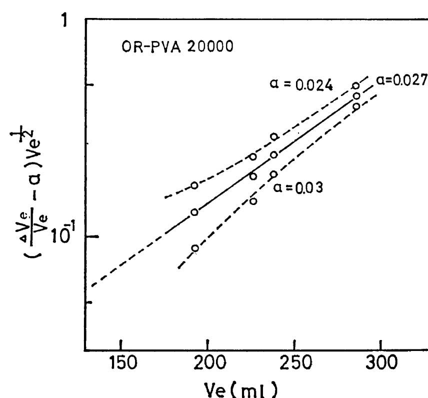

Figure 3. Plots of $(\Delta V_e/V_e - a)$ *vs.* V_e for four samples.

volume of 160 ml, which corresponds to $M_w = 8.2 \times 10^3$.

Next, we show the molecular weight distribution curves of unfractionated and fractionated PBLG, which were determined by use of the calibration plot of the molecular weight in Figure 1. These curves do not show real molecular-weight distributions, because they have not corrected for the broadening effect. Especially in this case the polynomial expansion method must be applied for the correction,[20] as the unfractionated and fractionated PBLG have shown relatively narrow distributions. But such a procedure is not fruitful, in spite of the labor involved, because of the inaccuracy of the calibration plot, the working hypothesis needed to derive the chromatogram of the completely monodisperse PBLG, and some other experimental uncertainties. Therefore, we show in Figure 4 only uncorrected molecular-weight distribution curves of two samples, along with the apparent molecular-weight distribution curve expected for the completely monodisperse PBLG.

The estimated values of M_w/M_n from the uncorrected molecular-weight distributions are 1.16 for the unfractionated PBLG and 1.08 for the fractionated PBLG. Although these values are tentative ones, we can say that the change of the value from 1.16 to 1.08 indicates the usefulness of this GPC as a fractionation method of PBLG.

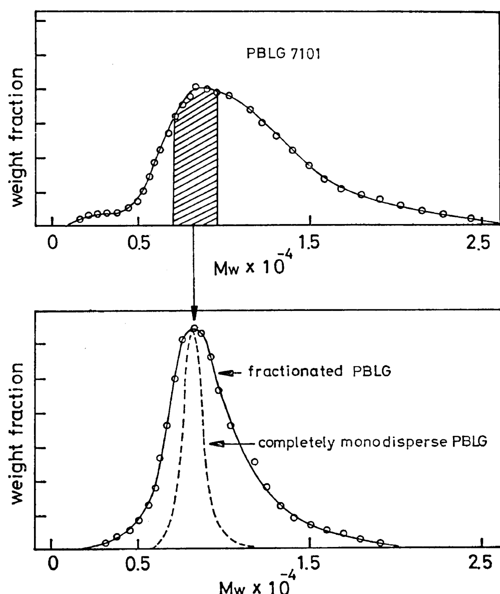

Figure 4. Molecular-weight distribution curves of unfractionated and fractionated PBLG and the completely monodisperse PBLG. Optically estimated polymer recoveries were 97% for fractionated PBLG and 95% for unfractionated PBLG.

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