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HIGH-SPEED GEL FILTRATION OF GLYCOPOLYPEPTIDES IN 6 *M* GUANIDINE HYDROCHLORIDE

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SUMMARY

The hydrodynamic behaviour of reduced glycopolypeptides was studied by high-speed gel filtration in 6 *M* guanidine hydrochloride using a column packed with a rigid matrix, TSK-GEL G 3000 SW, in conjunction with a high-pressure liquid chromatograph. When the apparent molecular weight of each glycopolypeptide was calculated on the basis of a calibration graph constructed with simple polypeptides, no appreciable deviation from the actual molecular weight was observed in nine out of the eleven glycopolypeptides examined. Even though a carbohydrate-rich glycopolypeptide may occasionally yield an underestimated value, the high-speed gel filtration method appears to be highly useful for the rapid estimation of the molecular weights not only of simple polypeptides but also of glycopolypeptides.

INTRODUCTION

Since the pioneering work of Porath and Flodin[1] in 1959, gel filtration has gained wide acceptance as a useful method for the separation of biopolymers on the basis of differences in size. As a result of the development of both theoretical and experimental aspects, this method is now used extensively not only for preparative purposes but also for the analytical determinations of biopolymer systems[2].

As reduced simple proteins in concentrated guanidine hydrochloride solutions behave hydrodynamically as randomly coiled linear homopolymers whose radius of gyration is a simple function of the molecular weight[3], gel filtration in 6 *M* guanidine hydrochloride provides a reliable method for the estimation of the molecular weights of protein polypeptide chains[4,5]. However, its application has been limited so far because conventional gel filtration using soft gel matrices requires too long a time for the completion of a single run and has a low resolving power. As reported previously[6], these disadvantages could be substantially overcome by the use of a column packed with a rigid matrix developed recently (TSK SW-type gels)[7] in conjunction with a high-pressure liquid chromatograph. In this new high-speed gel filtration system in 6 *M* guanidine hydrochloride, the time required for a single run was only 50 min, and the resolving power achieved was excellent. Furthermore, sixteen different

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polypeptide chains of known molecular weight, prepared mostly from simple proteins, gave linear calibration graphs, indicating the usefulness of this system for the rapid estimation of the molecular weight of polypeptide chains constituting simple proteins[6].

With glycoproteins, however, reduction of disulphide bonds gives branched heteropolymers instead of linear homopolymers, so that the behaviour of reduced glycoproteins on gel filtration in 6 *M* guanidine hydrochloride would not be the same as that of simple proteins. Therefore, it seemed important to know how glycopolypeptides behave in the newly developed gel filtration system. Such investigations would be of particular value in view of the well known fact[8] that sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, a method which is most frequently used at present in estimating the molecular weights of protein polypeptide chains, gives erroneous results for glycopolypeptides. This paper describes the gel filtration behaviour of a number of well characterized glycopolypeptides compared with that of simple protein polypeptides.

EXPERIMENTAL

Materials

The glycopolypeptides used were prepared from the following purified preparations of glycoproteins. Transferrin, Taka-amylase A, fetuin, α_1-acid glycoprotein and ovomucoid were kindly donated by Dr. Ikuo Yamashina of Kyoto University. Chicken and Japanese quail ovoinhibitors[9] were generously provided by Dr. Ikunoshin Kato of Purdue University. Acid carboxypeptidase[10] purified from *Aspergillus niger* var. *macrosporus* was a gift from Drs. Izumi Kumagai and Makoto Yamasaki of Tokyo University. Bovine γ-globulin was obtained from Armour (Kankakee, IL, U.S.A.), and ovalbumin and bovine ribonuclease B were purchased from Sigma (St. Louis, MO, U.S.A.).

These glycoproteins were reduced and alkylated essentially as described previously[6]. Proteins dissolved in 6 *M* guanidine hydrochloride containing 1 *M* Tris buffer (pH 8.2) and 2 m*M* EDTA were reduced by dithiothreitol added in at least a 5 *M* excess of the amount required theoretically. After the reaction mixture had been allowed to stand at 22°C for 2 h, the resultant SH groups were carboxyamidomethylated by the addition of 2 molar equivalents of iodoacetamide (pH 8) with respect to dithiothreitol. After incubation for 20 min at 22°C, a small amount of dithiothreitol was added to stop the reaction, and the mixture was dialysed against 6 *M* guanidine hydrochloride containing 10 m*M* phosphate buffer (pH 6.5) and 1 m*M* EDTA.

The molecular weights of glycopolypeptides were calculated, in most instances, from the published data for amino acid sequences and carbohydrate compositions. For acid carboxypeptidase, the molecular weight of the reduced–carboxymethylated protein was found by sedimentation equilibrium to be half that of the native protein[10]; an average value of 64,000 was taken as the most reliable molecular weight for the glycopolypeptide. The molecular weight of Taka-amylase A adopted was the value determined recently by Takagi[11] by the low-angle light scattering technique in combination with high-speed gel filtration. The molecular weights and carbohydrate contents of all glycopolypeptides used are listed in Table I.

Standard simple polypeptides used for constructing a calibration graph were

bovine serum albumin, α-chymotrypsinogen A, L chain of bovine γ-globulin, soybean trypsin inhibitor, ribonuclease A, calf thymus histone H 4 and erabutoxin a. Their sources and molecular weights were reported previously[6].

High-speed gel filtration

A column (60 × 0.75 cm I.D.) of TSK-GEL G 3000 SW, packed with spherical silica gel chemically bonded with hydrophilic compounds, and a short pre-column (TSK-GEL G SWP, 5 × 0.75 cm I.D.) were obtained from Toyo Soda (Tokyo, Japan). Before use, the columns were thoroughly washed and equilibrated with a medium containing 6 *M* guanidine hydrochloride, 10 m*M* phosphate buffer and 1 m*M* EDTA, previously adjusted to pH 6.5. High-speed gel filtration was carried out at room temperature in a Hitachi 635 high-pressure liquid chromatograph equipped with a Hitachi 034 double-beam effluent monitor. The flow-rate was fixed at 0.5 ml/min, and the time required for a single run was approximately 50 min. The absorbance of the effluent at 280 nm was recorded continuously on a chart.

The elution volume of proteins, V_e, was determined from the location of the protein peak on the recorder chart, and the distribution coefficient, K_d, was calculated by the equation

$$K_d = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_0 is the void volume and V_t is the total available volume of the column. Blue Dextran 2000 and tritiated water (^3H_2O) were used to determine V_0 and V_t, respectively. To construct a calibration graph, $K_d^{1/3}$ was plotted against $M^{0.555}$, where M is the molecular weight, as described previously[6].

RESULTS AND DISCUSSION

Eleven reduced–alkylated glycopolypeptides were subjected to high-speed gel filtration and each elution volume was determined at least three times. A typical elution profile obtained with an artificial mixture of glycopolypeptides is shown in Fig. 1. Table I gives the distribution coefficients (K_d values) and standard deviations. Excellent reproducibility of the results is demonstrted by the small standard deviations. Also included in Table I are the molecular weights and carbohydrate contents of these glycopolypeptides.

In Fig. 2, the relationship between $K_d^{1/3}$ and $M^{0.555}$ for glycopolypeptides is illustrated and compared with a linear calibration graph constructed with standard polypeptides not containing carbohydrate. The apparent molecular weight of each glycoprotein was calculated on the basis of the calibration graph in Fig. 1 and the deviation from the actual molecular weight was calculated. These values are listed in Table I.

As can be seen from Table I, the gel filtration behaviour of most of the branched-chain glycopolypeptides studied did not deviate appreciably from that of the single-chain polypeptides obtained from simple proteins. Especially for glycoproteins with a carbohydrate content of less than 10%, the apparent molecular weight calculated from the K_d values coincided with the actual molecular weight, within the limits of experimental error and the uncertainty of the estimated molecular weight. How-

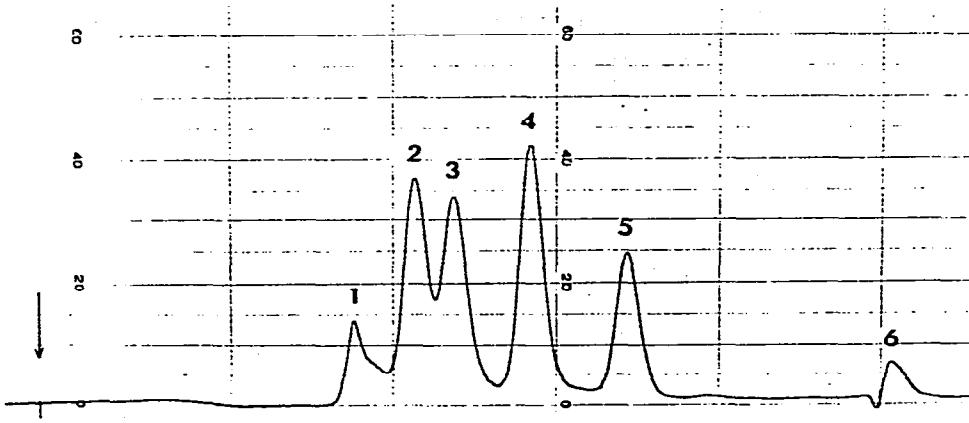

Fig. 1. Typical high-speed gel filtration profile obtained with a glycopolypeptide mixture in the presence of 6 *M* guanidine hydrochloride. The arrow on the left represents the position of sample application. 1 = Blue Dextran; 2 = transferin; 3 = Taka-amylase A; 4 = Japanese quail ovoinhibitor; 5 = ribonuclease B; 6 = 2,4-dinitrophenylalanine.

ever, two carbohydrate-rich glycopolypeptides, α_1-acid glycoprotein and acid carboxypeptidase, with 41.3% and 21.6% carbohydrate contents, respectively, gave definitely underestimated molecular weights. It should also be noted that the behaviour of fetuin and ovomucoid, the carbohydrate contents of which are nearly the same as that of acid carboxypeptidase, was not apparently anomalous, although the calculated molecular weights were only slightly lower than the actual values.

TABLE I

DISTRIBUTION COEFFICIENT (K_d) AND MOLECULAR WEIGHT (M) OF GLYCOPOLYPEPTIDES

Each K_d value listed is followed by the standard deviation of the mean. The molecular weight of each glycopolypeptide was calculated from the calibration graph prepared with simple polypeptides as shown in Fig. 1 (M from K_d) and compared with the actual value (M). The percentage difference of the former from the latter value is shown in the last column.

Protein	*M*	*CHO (%)*	K_d	*M from* K_d	*Difference in M values (%)*
Transferin	76,000	6	0.107 ± 0.003	77,700	+2.2
Acid carboxypeptidase (*A. niger*)	64,000	21.6	0.170 ± 0.002	57,600	−10.0
γ-Globulin H chain	51,500	4.9	0.195 ± 0.002	51,400	−0.2
Taka-amylase A	51,000	2.7	0.192 ± 0.003	52,100	+2.2
Ovoinhibitor (chicken)	48,300	12	0.197 ± 0.004	51,000	+5.6
Ovoinhibitor (Japanese quail)	48,300	12	0.202 ± 0.003	50,000	+3.5
Fetuin	48,000	22	0.220 ± 0.005	46,300	−3.5
Ovalbumin	44,300	3.6	0.238 ± 0.005	42,800	−3.4
α_1-Acid glycoprotein	36,500	41.3	0.312 ± 0.004	31,500	−13.7
Ovomucoid	28,000	23	0.350 ± 0.005	26,900	−3.9
Ribonuclease B	14,900	8.1	0.487 ± 0.006	14,700	−1.3

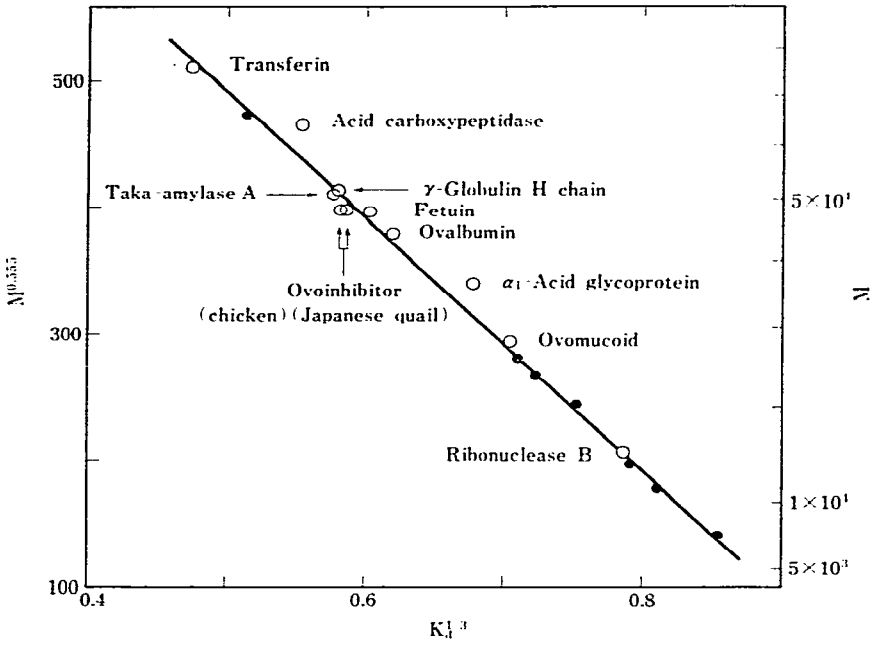

Fig. 2. Relationship between $M^{0.555}$ and $K_d^{1/3}$ obtained by high-speed gel filtration in 6 M guanidine hydrochloride. The straight line was drawn using the data for simple polypeptides (●) and compared with the data for glycopolypeptides (○).

It has been shown both theoretically and experimentally that a branched-chain homopolymer has a radius of gyration smaller than that of a linear polymer of the same species and total molecular weight[12]; thus, the K_d value of the polymer will increase with an increase in the extent of branching. The findings with α_1-acid glycoprotein and acid carboxypeptidase agreed qualitatively with this prediction. However, the shift in K_d observed with α_1-acid glycoprotein having five polysaccharide branches[13] was much smaller than that expected from the theoretical equation of Casassa and Berry[14,15], which accounts for the branching effect on the radius of gyration of comb-branched homopolymers. Further, the gel filtration behaviour of glycopolypeptides other than the two carbohydrate-rich glycoproteins mentioned above was not significantly different from that of simple polypeptides with the same molecular weights (Table I). Presumably, the unexpectedly weak influence of branching is attributable to the fact that glycopolypeptides are not branched homopolymers but branched heteropolymers. Because the nature of oligosaccharide chains would be different from that of polypeptide chains with respect to segment length, diameter, solvent–polymer interactions, etc., it is not unreasonable to assume that the effect of branching in causing a decrease in the radius of gyration is counteracted by the tendency for oligosaccharide branches to produce expansion of the polymer. The absence of a correlation between the extent of deviation and carbohydrate content (for example, compare the behaviour of acid carboxypeptidase, fetuin and ovomucoid having a similar carbohydrate content) may indicate that the number, location, length and structure of oligosaccharide branches have different influences in deter-

mining the overall hydrodynamic dimension of glycopolypeptides in 6 *M* guanidine hydrochloride. However, no quantitative explanation for the observed behaviour of glycopolypeptides is possible at present because of a lack of information concerning the hydrodynamic role of oligosaccharide branches.

During the preparation of this report, papers by Leach and co-workers[16,17] on the hydrodynamic behaviour of glycopolypeptides came to our notice. Although their gel filtration experiments in 6 *M* guanidine hydrochloride were performed on agarose gel at a low speed, they found, as we also did in the present investigation, that the disparity between the effective hydrodynamic volume of a glycopolypeptide and that of a linear polypeptide of the same mass was very small for most of the glycopolypeptides examined[17]. In contrast, they showed that the behaviour of SDS–glycopolypeptide complexes in gel electrophoresis and gel filtration was anomalous and no uniform correction could be found[16].

The overall conclusion from this and other investigations is that high-speed gel filtration is useful for the rapid empirical estimation of the molecular weights not only of simple polypeptides but also of glycopolypeptides. It must be remembered that occasionally some carbohydrate-rich glycopolypeptides may give an underestimated molecular weight. However, the present method is more insensitive to the presence of oligosaccharide branches than other empirical methods such as SDS–polyacrylamide gel electrophoresis[16]. In addition, the time required for a single gel filtration run (50 min) is even shorter than that required for SDS–polyacrylamide gel electrophoresis, and the reproducibility of the results is excellent. Therefore, high-speed gel filtration in 6 *M* guanidine hydrochloride appears to be the best method for estimating the molecular weights of glycopolypeptides rapidly and conveniently.

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