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MOLECULAR WEIGHT MEASUREMENT OF HUMAN SATIETIN

J. NAGY*, L. VÁRADY, H. KALÁSZ and J. KNOLL
Department of Pharmacology, Semmelweis University of Medicine, Nagyvárad tér 4, 1089 Budapest (Hungary)

SUMMARY

Attempts were made to estimate the actual molecular weight of satietin, an endogenous food intake inhibitor, by means of classical and high-performance gel filtration and gel electrophoresis. Satietin, which proved to be a glycoprotein, was isolated from human plasma. Sephacryl S-300 gel and TSK-GEL G 3000 SW columns were used as media for gel filtration and gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), respectively. A relatively good correlation was established between the molecular weight measurements carried out by different methods, although satietin as a carbohydrate-rich glycoprotein gave an underestimated value by gel filtration when the calibration graphs were constructed with simple proteins. The molecular weight of satietin was determined to be about 43,000 daltons by SDS-PAGE and 29,000 daltons by gel filtration when using a buffer containing 1% of sodium dodecyl sulphate.

INTRODUCTION

Knoll[1] discovered a highly potent and selective anorexogenic substance in human plasma and named it satietin. Further analysis of the highly purified preparations supported the hypothesis that this substance may be responsible for the food intake regulation of the mammalian organism[2,3]. Later, we achieved the isolation and preliminary characterization of this endogenous material. According to our previous findings, this substance has unexpectedly high carbohydrate (60–65%) and low peptide (22–24%) contents[4,5]. Some of the other properties of this material have also been described, including our first attempts at molecular weight measurement.

Several analytical methods for determining the actual molecular weight of satietin gave different results, as it is a carbohydrate-rich glycoprotein and natural glycoproteins are known to present anomalies in size compared with simple proteins in different systems[6,7]. Several workers[8–10] have reported this deviant behaviour when the molecular weight values of natural glycoproteins were compared with those of standard serum proteins by SDS-PAGE and gel filtration, which have been the most widely used methods in the last 15 years for the determination of the molecular weight distributions of proteins. Although the use of Sephadex G-100 and Bio-Gel P-100 type gels is generally accepted for studying the molecular behaviour of proteins

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in the commonly used denaturing buffers, *e.g.*, solutions of 6 *M* guanidine hydrochloride or 8 *M* urea[11,12], we have found that these molecular sieving media showed very slow solvent velocities as well as an increased viscosity.

This paper demonstrates the usefulness of 1% SDS solution in 0.1 *M* Tris buffer, adjusted to pH 7.2, for gel filtration. This solvent also ensures the complete denaturation of proteins investigated and the absence of any adsorptive interaction between the proteins and the gel matrix. These are the most important criteria of the validity of molecular weight determinations by molecular sieving. In addition, Sephacryl S-300 was a much more convenient gel regarding its hydrodynamic behaviour than the Sephadex G or Bio-Gel P type of gels, as it can endure high flow-rates without collapse of the gel and provides a good resolving power[13].

Several pilot experiments with SDS-PAGE under various conditions for the molecular weight determination of human satietin gave values in the wide range 48,000–67,000 daltons[4,5]. This paper reports our efforts and presents a comparison of data obtained by different techniques: gel filtration on Sephacryl S-300 and on TSK-GEL G 3000 SW columns and gel electrophoresis in the presence of sodium dodecyl sulphate.

EXPERIMENTAL

Materials

Satietin-D was isolated from human plasma by a series of separation techniques, including a proteolytic digestion step, as previously described[14]. Sephacryl S-300 gel and Blue Dextran 2000 were obtained from Pharmacia (Uppsala, Sweden). A low-molecular-weight standard protein calibration kit and the reagents for SDS gel electrophoretic studies were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Purified proteins used as standards for gel filtration were obtained from standard sources: bovine serum albumin, ovalbumin, alcohol dehydrogenase, carboxypeptidase A, chymotrypsinogen, trypsin, trypsin inhibitor, myoglobin and cytochrome c. All other reagents were of analytical-reagent grade and supplied by Reanal (Budapest, Hungary).

Methods

SDS gel electrophoresis. This was performed on polyacrylamide rods in a twelve-tube gel electrophoresis cell (Reanal) according to the method described by Laemmli[15].

SDS–Tris PAGE. The total acrylamide concentration was varied over a range 7.5–15%; 2.5-μg loads of standard proteins and 50–100-μg loads of satietin were solubilized in 2% (w/v) of SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.001% bromphenol blue, 0.0625 *M* Tris–HCl (pH 6.8). Samples were incubated at 80°C for 10 min.

Electrophoresis. Electrophoresis was performed at room temperature at a constant current of 1 mA per gel rod for 20 min, and 3 mA per gel rod was maintained until the dye front approached the lower end of the gel tube. The position of the tracking dye was measured, and gels were fixed and stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 in acetic acid–methanol–water (1:5:5) for 20–60 min, depending on how recently the dye mixture used had been prepared. The gels were destained by diffusion in 10% (v/v) acetic acid.

Gel filtration. Gel filtration on Sephacryl S-300 gel was carried out on a Pharmacia K 26/100 glass column (84 × 2.5 cm I.D.). A Pharmacia P-3 peristaltic pump delivered the solvent and the separation was followed with an LKB Uvicord II instrument at 280 nm. The freshly packed column was equilibrated for 48 h with 0.1 *M* Tris-HCl buffer (pH 7.2) containing 1% of sodium dodecyl sulphate and 0.05% of sodium azide. During this period, more and more gel was added until the column length no longer changed and the bed height was 84 cm. The system was operated at room temperature and the solvent flow-rate was set at 40 ml/h. Highly purified and homogeneous satietin preparations (15–20 mg) and the standard proteins were dissolved in the same buffer, incubated at 37°C for 15 min and loaded on to the column by means of a peristaltic pump. The sample volume was 8 ml in each instance. Artificial mixture of Blue Dextran 2000, standard proteins and acetone were dissolved like the samples to be investigated and they were treated in a similar manner. Fractions were collected with a laboratory-built fraction collector. The fraction size was 10 ml. An analysis could be completed within 11 h.

Gel filtration on TSK-GEL G 3000 SW was kindly performed by Professor N. Ui (Department of Physical Biochemistry, Gunma University, Maebashi, Japan).

RESULTS

Gel filtration

Gel filtration on Sephacryl S-300 column was applied to determine the actual molecular weight of satietin by making comparisons with simple proteins and to examine its chromatographic behaviour. Generally, the distribution coefficients (K_d) of standard proteins of known molecular weight can be employed for the determination of the size of similarly shaped proteins. In this work, K_d, plotted against the molecular weight (M), as described by Fish *et al.*[12] and Ui[16], gave a linear relationship between $K_d^{1/3}$ and $M^{0.555}$.

The elution volumes (V_e) of standard proteins and our glycoprotein were measured as the distances (not the elution volumes) of the substance from the sample injection. The void volume (V_0) was detected by the elution of Blue Dextran 2000 and the total permeation volume of the gel column (V_∞) was detected with acetone. Acetone does not interfere with the gel matrix, and elution can be fairly easily fol-

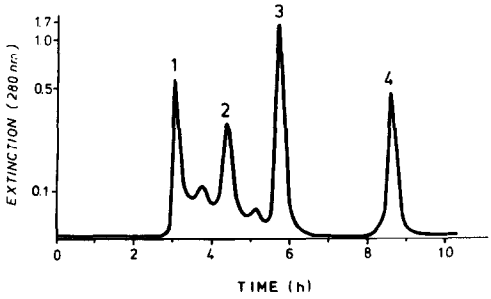

Fig. 1. Gel filtration of an artificial mixture of Blue Dextran 2000, bovine serum albumin, cytochrome c and acetone. The column (84 × 2.5 cm I.D.) was equilibrated with 0.1 *M* Tris, containing 1% SDS and 0.05% NaN_3 buffer (pH 7.2).

TABLE I

MOLECULAR WEIGHT (M), DISTRIBUTION COEFFICIENT (K_d) AND $M^{0.555}$ AND $K_d^{1/3}$ VALUES OF SIMPLE PROTEINS, DETERMINED BY GEL FILTRATION ON A SEPHACRYL S-300 COLUMN AND USED FOR CONSTRUCTING THE CALIBRATION GRAPH

Protein	M	$M^{0.555}$	K_d	$K_d^{1/3}$
Bovine serum albumin	67,000	476.9	0.233	0.615
Ovalbumin	43,000	372.8	0.304	0.672
Alcohol dehydrogenase (liver)	41,000	363.1	0.348	0.703
Carboxypeptidase A	34,600	335.5	0.310	0.677
Chymotrypsinogen	25,700	280.2	0.331	0.718
Trypsin	23,300	265.3	0.439	0.760
Trypsin inhibitor (soybean)	20,100	244.5	0.410	0.742
Myoglobin (horse)	17,200	224.5	0.401	0.737
Cytochrome *c*	11,700	181.0	0.475	0.780
Satietin-D (calculated)	28,200	295.0	0.368	0.717

lowed at 254 or 280 nm[17]. The distribution coefficient was calculated according to the equation

$$K_d = \frac{V_e - V_0}{V_\infty - V_0} = \frac{V_e - V_{\text{Blue Dextran}}}{V_{\text{acetone}} - V_{\text{Blue Dextran}}} \quad (1)$$

A typical elution profile is illustrated in Fig. 1, obtained with a Sephacryl S-300 column by using an artificially mixed sample of Blue Dextran 2000, bovine serum albumin, cytochrome c and acetone. The gel-chromatographic behaviour and molecular-weight data of the standard proteins used are summarized in Table I.

Elution diagrams of human satietin samples, which were loaded on to the same column, are illustrated in Fig. 2a and b. Fig. 2a shows the chromatogram of a highly purified satietin-D preparation, and Fig. 2b represents the elution of the homogeneous product (peak No. 2). This column proved to be capable of handling 15–20 mg of satietin preparation per loading, and as it provided sufficient purity, it also served for preparative purposes. The molecular weights and K_d values of standard proteins and peptides (Table I) allowed us to estimate the apparent molecular size

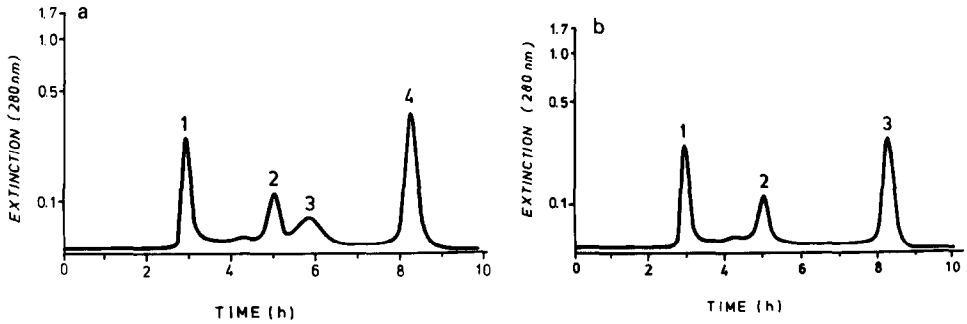

Fig. 2. Elution pattern of highly purified satietin-D (a) and homogeneous satietin (b) on a Sephacryl S-300 column (84 × 2.5 cm I.D.). Eluent as in Fig. 1.

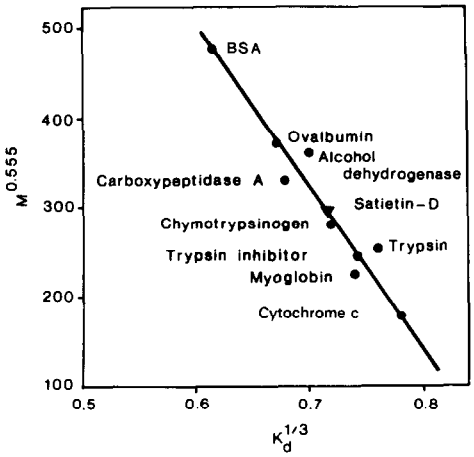

Fig. 3. $M^{0.555}$ *vs.* $K_d^{1/3}$ calibration graph for various proteins established by gel filtration on a Sephacryl S-300 column with the same buffer as in Fig. 1.

of satietin. On the basis of these measurements, a calibration graph was constructed of $K_d^{1/3}$ versus $M^{0.555}$. When the appropriate $K_d^{1/3}$ value of our endogenous sample was located on the calibration graph, the actual molecular size of satietin-D was found to be 28,000–29,000 (Fig. 3).

This experiment was confirmed by high-performance gel filtration on a TSK-GEL G 3000 SW column with 6 *M* guanidine hydrochloride solution in 0.01 *M* phosphate buffer (pH 6.5) as the eluent. The elution profile for this experiment is presented in Fig. 4. The molecular weight of satietin-D was calculated from its K_d values as above and found to be 29,000.

Gel electrophoresis

Some preliminary experiments were performed in order to measure the molecular weight of satietin by simple polyacrylamide gel electrophoresis (PAGE). Then SDS-PAGE with a definite acrylamide concentration was applied, which resulted in

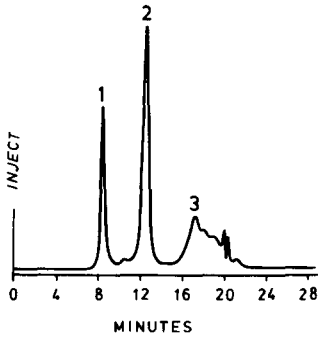

Fig. 4. Gel filtration of pure satietin-D on a TSK G 3000 SW column (30 cm × 7.8 mm I.D.) with 6 *M* guanidine hydrochloride–10 m*M* phosphate–1 m*M* EDTA buffer (pH 6.5) as eluent. 1 = Marker protein for measuring the void volume; 2 = satietin-D; 3 = low-molecular-weight substances for measuring the total permeation volume.

a molecular weight range of around 67,000 daltons[5]. Later, more experiments were performed to verify the validity of these data. When a 12.5% acrylamide concentration of SDS–Tris PAGE was used and the electrophoretic mobilities were calculated by the method of Weber and Osborn[18], a value of around 49,000 daltons was obtained[14]. As we are dealing with a carbohydrate-rich glycoprotein, it seemed to be necessary to use the Ferguson plots besides the usual SDS–Tris PAGE. This type of treatment presumably divides the relative electrophoretic mobility of a solute into two different functions, namely its free electrophoretic mobility and the sieving effect of the gel matrix[19]. The free electrophoretic mobility (M_0) and retardation coefficient (K_R) depend solely on the molecular size when SDS-PAGE is used for determining the migration rates in gels of different acrylamide concentrations. We used the equation first proposed by Ferguson[20]:

$$\log (R_F) = \log (M_0) - K_R T \tag{2}$$

where R_F is the electrophoretic mobility in a gel of a given acrylamide concentration (T). Plots of log R_F *versus* T (Ferguson plots) are linear with a slope of K_R and intercept log M_0 (at $T = 0$). As demonstrated by the data in Fig. 5, the five common polypeptides and satietin-D behaved similarly.

Fig. 6 shows the plot of K_R *versus* molecular weight for five common polypeptides and satietin-D. As measurement of the retardation coefficient was used to es-

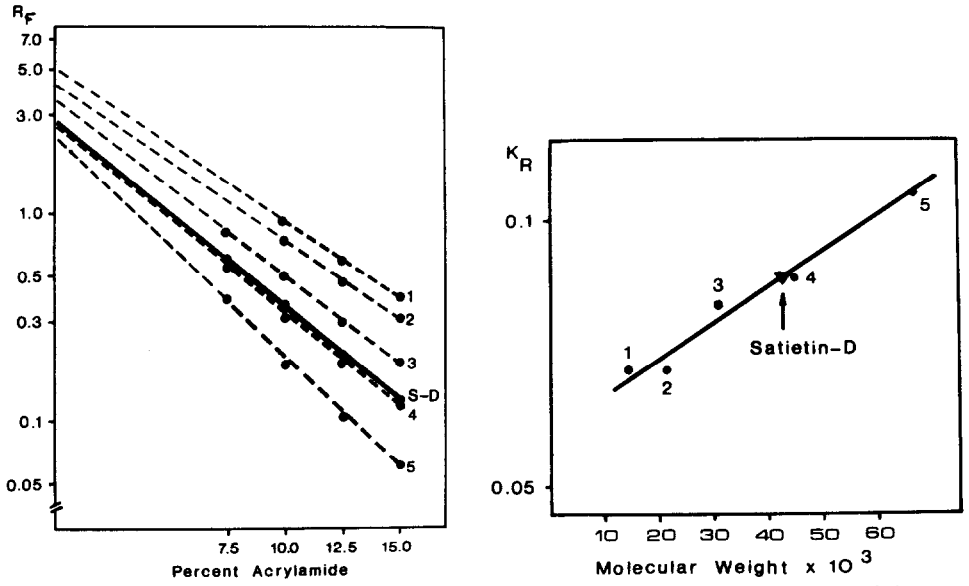

Fig. 5. Ferguson plots of protein standards and satietin-D (S-D). Each point is the mean of three R_F measurements. 1 = Lysozyme; 2 = soybean trypsin inhibitor; 3 = carbonic anhydrase; 4 = ovalbumin; 5 = bovine serum albumin.

Fig. 6. Relationship between molecular weight and retardation coefficient (K_R) for standard proteins and satietin-D. Values of K_R were calculated from eqn. 2; the molecular weight of satietin-D was found to be 43,000. The correlation coefficient was 0.99 (mean of three independent experiments). Symbols as in Fig. 5.

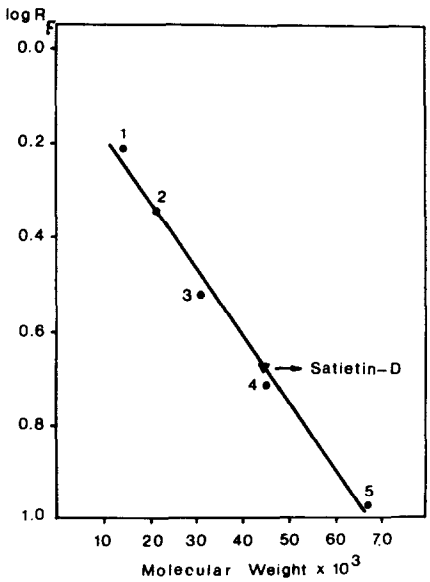

Fig. 7. Relationship between molecular weight and the logarithm of mobility in SDS-PAGE. Mobility is expressed relative to bromophenol blue. Gels contained 12.5% (w/v) of acrylamide. Each value is the mean of three measurements. The correlation coefficient was 0.996. Symbols as in Fig. 5.

timate the molecular size of soluble proteins, it might be suggested that this value can be used to estimate molecular weights of anomalous proteins in SDS-PAGE. The application of this value essentially corrects any unusual free mobility and might be useful when this is believed to account for unusual migration rates[6].

DISCUSSION

Comparison of data obtained by classical gel filtration, high-performance gel filtration and SDS-PAGE for the measurement of the molecular weight of satietin will be discussed.

Classical gel filtration on a Sephacryl S-300 column in a solvent buffer of 0.1 *M* Tris containing 1% of sodium dodecyl sulphate proved to be an appropriate method for measuring the molecular size of satietin. All of the standard proteins used produced a linear calibration graph when the SDS-containing buffer was used as the denaturing solvent instead of 6 *M* guanidine hydrochloride or 8 *M* urea, which are generally applied for estimating molecular weights of proteins. All of these denaturing solvents prevent gel–solute interactions and eliminate or at least strongly decrease aggregation. Moreover, this SDS-containing buffer was found to dissolve satietin better than the others. As the SDS-containing buffer offers a fair solubility for satietin and prevents aggregation, we think that this method gives closer agreement between the two commonly used methods for molecular weight estimation, classical gel filtration and SDS-PAGE.

The presence or absence of 2-mercaptoethanol made no difference in the molecular weight distribution in SDS-PAGE. Therefore we did not use the reduced form

of satietin in the gel chromatographic studies; this is probably due to the high carbohydrate content of satietin and the fact that it could not be precipitated by trichloroacetic acid treatment.

Gel filtration on both Sephacryl gel and TSK-GEL columns gave similar results. The resolution was naturally better with SDS-PAGE than that obtained by gel chromatography. The difference between the data obtained by the different methods was found to be about 30%. We obtained consistently higher values for the molecular weight by SDS-PAGE than by the chromatographic methods. When we used a method adapted from Neville[19], the found value was 45,000 and the calculated correlation coefficient was 0.996 (Fig. 7). Calculating the molecular weight according to Ferguson gave a slightly lower value, namely 43,000 (Fig. 6), also with a satisfactory correlation coefficient of 0.990.

The higher molecular weight obtained with the SDS-PAGE technique may be explained by the anomalous behaviour generally known for glycoproteins. As our material contained an extremely large amount of sugars, one can expect a similar behaviour. It may give different retardations in simple gel filtration and in SDS-PAGE.

Finally, it can be concluded that both types of method are useful for the determination of the molecular weight or size of these kinds of substances and also for obtaining more information about the properties of the glycoprotein molecule.

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