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Estimation of molecular weights of proteins by agarose gel filtration

At the present time gel filtration is widely used, as both an analytical and a preparative tool, for the fractionation, isolation and purification of proteins, enzymes, hormones, antibiotics, nucleic acids, etc. DETERMANN's[1] monograph has exhaustively reviewed the literature concerning this subject.

Gel filtration on agarose has recently been introduced[2,3]. The use of agarose gel beads for chromatographic molecular sieving of proteins of high molecular weights has proven to have advantages over the use of other gels. Furthermore, these gels make it possible to extend the working range up to limits which are far beyond those of dextran and polyacrylamide gels.

Among the principal features of filtration with agarose bead gels are the high flow rates which are obtained and the absence of the packing procedure.

The present paper describes a procedure for the evaluation of molecular weights of proteins and the splitting off of a "nucleoprotein-enzyme complex" by means of an agarose gel. Comparative studies with two commercial types of agarose gels are also reported.

Materials and methods

Proteins used in this study are listed in Table I. Aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase, EC 4.2.1.24) from soybean callus tissue system was

TABLE I

PROTEINS EMPLOYED IN THE STUDY

Protein	*Source*	*Mol. wt.* $\times 10^{-3}$	*Reference*	*Amounts used (mg)*
Thyroglobulin (bovine) type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	670	EDELHOCH[4]	8–15
Fibrinogen (bovine) fraction I citrated type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	330–340	SHULMAN[5]	10–30
Catalase (from ox liver) purified powder	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	230–250	SAMEJIMA AND YANG[6]	10–20
γ-Globulins (bovine) Cohn Fraction II	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	150–170	PHELPS AND PUTNAM[7]	10–20
Serum albumin dimer (present in bovine serum albumin)		134		(2 × mol. weight of serum albumin)
Serum albumin bovine, fraction V from bovine plasma	Armour Pharmaceutical Co. Ltd., Chicago, Ill., U.S.A.	67	PHELPS AND PUTNAM[7]	10–20
Ovoalbumin	Schering-Kahlbaum AG, Berlin	45	WARNER[8]	10–20
Myoglobin (sperm whale)	L. Light & Co. Ltd., Colnbrook, Bucks., England	17.8	EDMUNDSON AND HIRS[9]	5–10
Cytochrome c from horse heart, type I	Sigma Chemical Co., Saint Louis, Mo., U.S.A.	12.4	MARGOLIASH[10]	4–7

partially purified, and its activity was determined according to TIGIER *et al.*[11]. Blue Dextran 2000 (containing some material of very high molecular weight) and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio Gel-A 1.5 m was the generous gift of Bio Rad Laboratories. Richmond, Calif., U.S.A.

SR 25/45 columns from Pharmacia Fine Chemicals, Uppsala, Sweden, and vertical glass tubes (60 cm long; 2.5 cm I.D.) equipped with capillary outlets were used. In the latter a disc of polypropylene tissue was used as bed support.

Sepharose 4B and Bio Gel-A 1.5 m, the agarose gels used in this work, were obtained as a slurry in distilled water or Tris buffer containing 0.02% sodium azide as a bacteriostatic agent. Each column was filled with buffer, and the gel, previously deaerated under reduced pressure, was poured into the column by using an extension tube. The head of water was gradually increased until reaching a pressure of 30 to 45 cm of level difference. Operating flow rates were between 25 to 35 ml/h. The columns were ready for use after equilibrating with two bed volumes of the appropriate buffer. This was done either with 0.05 *M* Tris–HCl buffer (pH 7.4) or 0.1 *M* glycine–NaOH buffer (pH 9.0) unless otherwise stated.

All experiments were done at room temperature or at 4°; no significative differences in elution volumes were observed at either temperature. Samples were dissolved in the elution buffer and applied to the top of the column by layering under the solution already present. Sucrose was added, when necessary, in order to increase solution density.

Column eluates were collected with an automatic fraction collector (LKB, Stockholm, Sweden) equipped with a Uvicord I effluent monitor and chopper bar recorder, registering light absorption at 254 nm. When columns were run at 4°, a Colora low-temperature bath (KT 305, Lorch, Württenberg, Germany) was used.

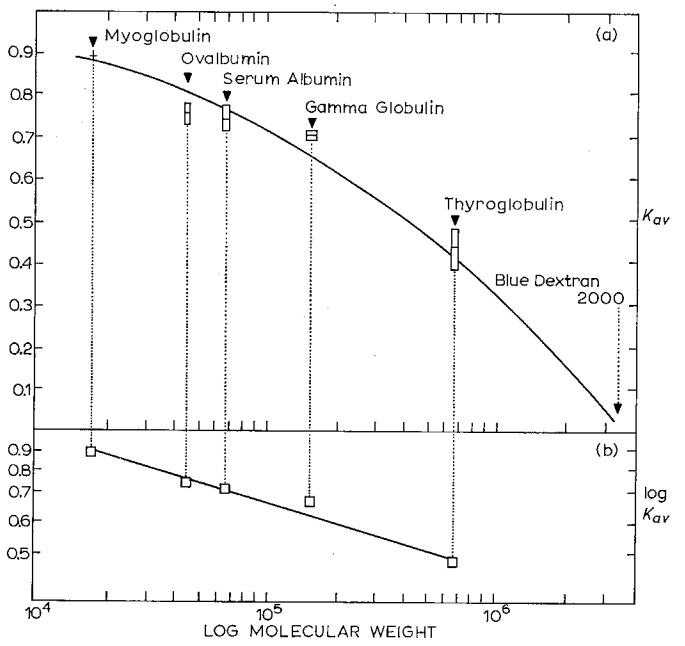

Fig. 1. (a) Plots of K_{av} against log(mol. wt.) and (b) plots of log K_{av} against log (mol. wt.) for proteins on Sepharose 4B.

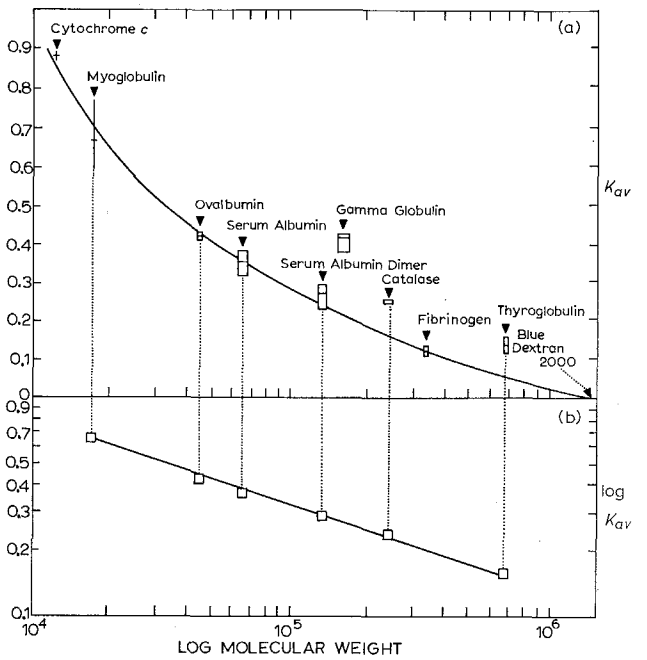

Fig. 2. (a) Plots of K_{av} against log(mol. wt.) and (b) plots of log K_{av} against log(mol. wt.) for proteins on Bio Gel-A 1.5 m.

Results and discussion

The elution behaviour of a substance in gel chromatography can be described by different variables which are derived from the elution volume. These relationships have been compiled by DETERMANN[12]; some of them are empirical and some were derived on the basis of theoretical considerations.

A magnitude independent of the geometry and packing density of the column and easily measured, defined by LAURENT AND KILLANDER[13] as K_{av}, was plotted versus the log of the molecular weight. Fig. 1a and 2a show plots of K_{av} values from Bio Gel-A 1.5 m and Sepharose 4B columns versus the log of the molecular weight for a set of standards.

Differences in the preparation of these agarose gels and/or raw materials might account for the different shapes of these curves. Furthermore, even though the non-linearity of these relationships is still an open question, a possible reason for this might be found in the intrinsic nature of agarose gels, which are known to possess no cross-linking, as dextran and polyacrylamide gels do, but rather hydrogen bonding between different portions of a chain or between different chains.

From Figs. 1a and 2a, it is clear that Sepharose 4B provided better resolution for proteins in the high molecular weight zone, whereas Bio Gel-A 1.5 m would be better for the lower zone, even though the latter provided a wide working range.

As has been mentioned, a non-linear dependence of the K_{av} (or elution volume) or the log of the molecular weight was observed for agarose gels; but we have found that a straight line is obtained if the log of K_{av} is plotted against the log of the molecular weight, as shown in Figs. 1b and 2b.

As it has already been pointed out for other gels, some proteins also exhibited abnormal behaviour on these agarose gels.

Carbohydrate content, differences in shape of native proteins and uncertainty regarding the state of aggregation[1,14–17] might account for this fact. In addition, some proteins deviated to a different extent on each agarose gel; for example, fibrinogen exhibited a normal behaviour on Bio Gel-A 1.5 m, whereas variable values of K_{av} were obtained on Sepharose 4B. On the other hand, γ-globulin and thyroglobulin are retarded on Bio Gel-A 1.5 m, particularly the former, which deviated to a lesser extent on Sepharose 4B; thyroglobulin, however, fitted the curve. Moreover, the observed values for catalase, especially on Sepharose 4B at different pH values (not shown in Fig. 1a) would suggest some variation in its state of aggregation[18].

Further purification of aminolaevulinate dehydratase

When aminolaevulinate dehydratase, obtained as mentioned above, was run on Sephadex G-200 or Bio Gel P-300 columns, it was systematically eluted with the void volume. Evidence was obtained which suggested that the enzyme was associated with a nucleoprotein; therefore, attempts were made to separate the components. Among them, protamin sulphate treatment was performed, but the resulting supernatant was absolutely devoid of activity and all efforts to split the "enzyme–nucleoprotein complex" were unsuccessful[19].

When preparations of such a complex were run on Sepharose 4B columns at 4°, with 0.1 *M* glycine–NaOH buffer (pH 9.0) as elution buffer, three peaks were obtained (Fig. 3); the first one was eluted with the void volume and contained the unwanted

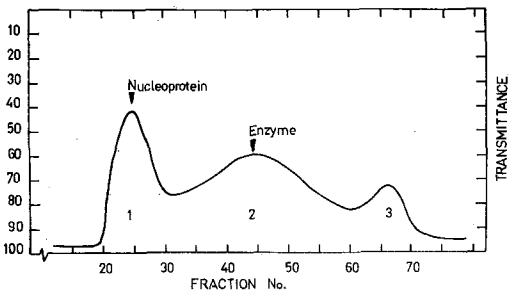

Fig. 3. Gel filtration on Sepharose 4B of a "nucleoprotein–aminolaevulinate complex" from a soybean callus tissue system. 8–10 ml of solution in 0.1 *M* glycine–NaOH buffer (pH 9.0) containing approx. 80 mg of protein were applied to a SR 25/45 Pharmacia column of Sepharose 4B equilibrated with the same buffer. Flow rate: 0.5 ml/min. Peak 1 was eluted with the void volume and contained the unwanted nucleoprotein; all enzymic activity was associated with the second protein peak.

nucleoproteins, while all the enzymic activity was associated with the second peak. Thus, this technique allowed a further purification of the enzyme, yielding a 40-fold over-all enrichment.

By running the highly purified enzyme through calibrated columns of Sepharose 4B, Sephadex G-200 and Bio Gel P-300, an estimated molecular weight of 280,000 was obtained.

Attempts to dissociate the complex into its nucleoprotein and enzyme com-

ponents on Bio Gel-A 1.5 m columns with different buffers containing 2 *M* NaCl[20] were unsuccessful. A single wide peak eluted with the void volume was always obtained.

Summing up, the use of column chromatography on agarose gels offers advantages over dextran and polyacrylamide gels, permitting the estimation of molecular weights of proteins within wider molecular weight ranges, the use of high flow rates and the absence of packing of the column. Moreover, gel filtration on Sepharose allows a good and rapid separation of nucleoproteins (or nucleic acids) and proteins from a complex of both, while with dextran or acrylamide gels it is difficult to dissociate those components.

Finally, the present methods of application have the advantages of simplicity, speed and fairly good reproducibility.

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