AGAROSE GEL FILTRATION OF FLUORESCENT LABELED PROTEIN-SODIUM DODECYL SULFATE COMPLEXES

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Received February 26,1974

SUMMARY

A procedure has been developed for estimation of the molecular weight of fluorescent labeled protein-SDS complexes by gel filtration of 4% agarose. The fluorescent label allows high sensitivity while the nature of the technique readily lends itself also to preparative separations. Evidence is presented for the linear dependence of log molecular weight of the fluorescent complexes on the relative elution volume. A representative preparative run is also shown illustrating a separation of the chains of gamma globulin.

INTRODUCTION

The use of sodium dodecyl sulfate-protein complex formation as an aid in the determination of molecular weights has been widely exploited since the initial description of the technique by Shapiro et al (1). The separation of such complexes in acrylamide gel electrophoresis and the correlation between their electrophoritic mobilities and molecular weights has been extensively studied (2),(3),(4). Reynolds and Tanford (5) have shown in gel filtration studies that these protein-detergent complexes take the form of semi-rigid rods, with a length directly proportional to the size of the polypeptide chain.

In the course of a study of thyroid protein struc-

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ture, we have developed a procedure for gel-filtration in the presence of sodium dodecyl sulfate which is not only suitable for molecular weight determinations on very small quantities of protein ($\sim$ 2.0 µg) after labeling with a fluorescent dye, but may also be easily stepped up to large sample sizes for preparative experiments. A typical standard curve is presented here along with an example of a preparative separation of the two chains of γ-globulin. The materials recovered after such preparative experiments may be readily freed of detergent and are useful for chemical studies on the individual separated components.

MATERIALS AND METHODS

Samples of from 0.1 to 25 mg were dissolved in 0.75 ml of 1.45 M Tris-HCl buffer, pH 8.3. After the addition of 0.075 ml of 5% ethylenediamine tetraacetate, 0.902 gms of solid urea was dissolved in the solution. When the urea was fully dissolved, 0.025 ml of β-mercaptoethanol was added and the total sample volume was brought to 1.9 ml with water and then to 3.0 ml with 8 M urea containing 0.2% EDTA. The tube was then flushed with nitrogen, sealed and allowed to stand for 4 hours at room temperature. At the conclusion of the reduction period, the tube was opened and 75.5 mg of sodium iodoacetate dissolved in 0.25 ml of water was added with rapid mixing. Alkylation was allowed to proceed in the dark for 20 minutes at room temperature. The reduced and alkylated samples were dialyzed in the dark against distilled water (12 hours) and then against 0.1 M ammonium bicarbonate (24 hours). After dialysis

against the ammonium bicarbonate buffer, dimethylamino-naphthalene sulfonyl chloride in acetone was added (molar ratio of dye to protein, 5:1) followed by sufficient acetone to make the solution 10% (vol:vol) in acetone. The dansylation was allowed to proceed at 4° with periodic shaking for 4 hours and was followed by dialysis against 0.05 M glycine, pH 9.5, containing 0.5% sodium dodecyl sulfate in order to remove excess dye and equilibrate the sample with eluting buffer.

Samples of the reduced and alkylated proteins were applied to 1 ml of eluting buffer to a column of Biogel A-15M, 100-200 mesh. The column dimensions were 0.9 cm x 88 cm and the eluting buffer was 0.05 M glycine, pH 9.5, containing 0.5% sodium dodecyl sulfate. Fractions of 1.5 ml were collected and relative fluorescence was determined on either an Aminco Bowman Spectrofluorometer with excitation at 340 nm and emission set at 495 nm or on a right angle polarization of fluorescence instrument of our own design (6). The volume of each fraction was carefully measured to within 5% as each tube was assayed for fluorescence. The exclusion volume of such a column as determined by Blue Dextran was 28.0 ml. Samples of reduced and alkylated porcine gamma globulin chains were run in SDS-acrylamide gel electrophoresis before and after being resolved by gel filtration in order to evaluate the completeness of the separation.

RESULTS

The operational range of this procedure was from 5,000 up to 200,000 in molecular weight. Material of significantly larger or smaller molecular weight was

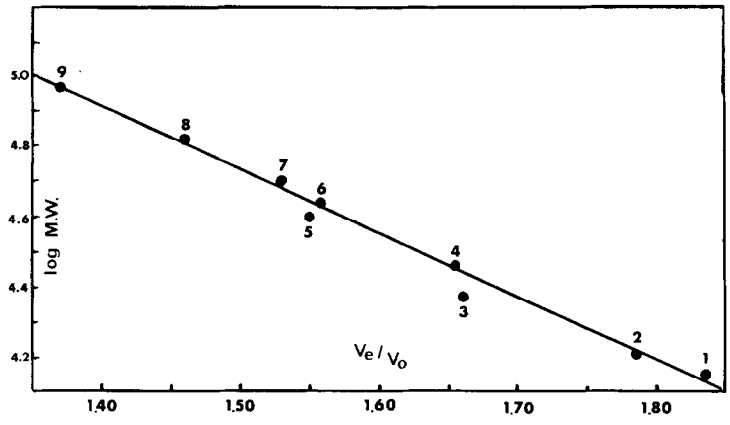

Figure 1. A plot of log molecular weight versus the elution volume divided by the exclusion volume. The proteins of known molecular weight are indicated as follows: (1) Egg White Lysozyme (14,300); (2) Hemoglobin subunit (16,000); (3) Gamma Globulin light chain (23,000); (4) Carbonic Anhydrase (49,000); (5) Rabbit Aldolase (40,000); (6) Ovalbumin (43,500); (7) Gamma Globulin heavy chain (50,000); (8) Bovine Serum Albumin (66,000); (9) Phosphorylase subunit (94,000). Each point is the average of at least duplicate runs.

found either at the exclusion volume or at the column volume. Figure 1 illustrates the linearity of a plot of log molecular weight versus $Ve/_{Vo}$, where Ve is the elution volume of the material in question and Vo is the exclusion volume of the column.

While the sensitivity of this procedure will be a function of the fluorometer employed to determine the relative fluorescence and the amount of background fluorescence (due to solvent and buffer components), we have been able to obtain clearly defined elution peaks with samples containing as little as 2 μg of protein and believe further increases in sensitivity are possible with appropriate fluorometric instrumentation and careful buffer preparation.

An example of a typical separation of the chains in

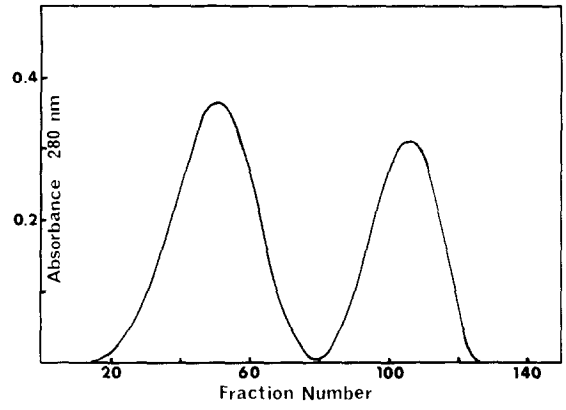

Figure 2. A separation of the chains of Porcine Gamma Globulin as SDS complexes after reduction and alkylation. This profile was obtained after two passes through a column of 4% Agarose (2.5 x 110 cm) equilibrated with the SDS-glycine buffer system given in the text. The column was run upward flow in this case to facilitate recycling. The molecular weights of the two peaks were determined by SDS-acrylamide gel electrophoresis as ∿ 23,000 and ∿ 50,000, thus verifying the separation of the gamma globulin chains.

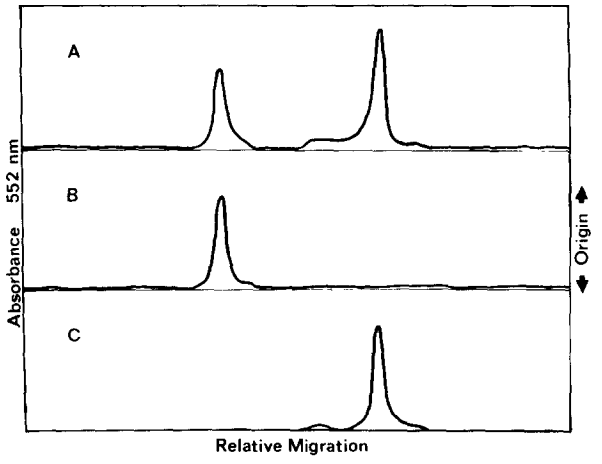

Figure 3. Densitometer tracings of gamma globulin chains separated by SDS-gel electrophoresis. The electrophoresis was carried out according to Weber and Osborn (2) and stained with Coomassie Brilliant Blue. (A) reduced and alkylated porcine gamma globulin, (B) isolated light chain, (C) isolated heavy chain

a multi-chain protein is shown in Figure 2, where the chains of reduced and alkylated porcine gamma globulins have been resolved. Such a separation illustrates the potential for preparative as well as analytical applica-

tions of gel filtration of protein-detergent complexes under the conditions described here. The completeness of the chain separation is illustrated in Figure 3 where densitometer tracings of SDS gel electrophoretic runs are shown after staining with Coomassie Blue (1). Figure 3 shows a mixture of heavy and light chains, isolated heavy and isolated light chain respectively. While the resolution in the gel filtration procedure is somewhat reduced when compared to gel electrophoresis, the ability to carry out recycling chromatography offers at least partial compensation. In preparative experiments the detergent may be removed by either acetone precipitation (2) or ion-exchange chromatography (7). This procedure's use of fluorescence as a means of detection allows the sensitivity required for small analytical runs, while the nature of the gel filtration process allows recovery of samples for further analyses. The latter capability is of importance particularly in the case of proteins where sodium dodecyl sulfate is a more effective dissociating agent than urea or guanidine hydrochloride and the chemical nature of separated components is of interest.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Mrs. S. Wilczynski for excellent technical assistance. This work was supported by NIH Grant AM14553.

References

1. Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr., Biophys. Res. Commun., 28, 815 (1967).
2. Weber, Klaus and Osborn, Mary, J. Biol. Chem. 244 4406 (1969)

3. Dunker, A. K. and Rueckert, Roland R., J. Biol. Chem. 244 5074 (1969).
4. Tung, Jwu-Sheng and Knight, C. A., Analyt. Biochem. 48 153 (1972).
5. Reynolds, Jacqueline A. and Tanford, Charles, J. Biol. Chem. 245, 5161 (1970).
6. Rawitch, Allen B., (Unpublished).
7. Weber, Klaus and Kuter, David J., J. Biol. Chem. 246 4505 (1971).