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THIN-LAYER GEL CHROMATOGRAPHY OF PROTEINS IN MILD AND DENATURING DETERGENTS

JAMES F. COLLAWN, Jr., DAVID J. COX, LARRY M. HAMLIN and WAYNE W. FISH
Department of Biochemistry, Medical University of South Carolina, Charleston, S.C. 29403 (U.S.A.)
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SUMMARY

Thin-layer gel chromatography of proteins in the presence of the mild detergents, sodium deoxycholate and Triton X-100, or in the presence of a denaturing detergent, sodium dodecyl sulfate, is described in terms of application to (glyco)-proteins of membrane origin. In these detergent systems, a nearly linear relationship was observed between the value of the distribution coefficient of a protein and the protein's equivalent hydrodynamic radius. The fractionation properties of the gel employed in this investigation appeared to be different in Triton X-100 than in the other two detergent systems. The fixation and replica-staining of glycoproteins separated in the presence of detergent is also discussed.

INTRODUCTION

Thin-layer gel chromatography (TLG)[1,2] has found ready application for the rapid, inexpensive separation and/or size estimation of microgram quantities of water-soluble proteins[3–5]. Recently, this method was adapted for the molecular weight estimations of polypeptide chains in random coil-producing solvents[6].

The study of membrane proteins and glycoproteins is complicated by the fact that most integral membrane proteins are insoluble in dilute, aqueous buffers and must be solubilized by the presence of a detergent in the aqueous solvent. Additionally, microgram quantities of material are routinely encountered in membrane protein research, and these protein quantities are eminently suited to TLG applications. Before its possible application to any membrane protein systems, TLG of some common hydrophilic proteins in mild or denaturing detergents was examined in our laboratory to document the specific method and to determine the hydrodynamic sizes to which the method might be applicable. The results of this investigation are reported herein.

EXPERIMENTAL

Materials

Sephadex G-200 with a particle size of 10–40 μm was obtained from Sigma (St. Louis, Mo., U.S.A.).

α_1-acid glycoprotein (human glycoprotein fraction VI), human globulin fraction II (IgG), and ovalbumin were obtained from Miles Labs (Elkhart, Ind., U.S.A.). Chymotrypsinogen-A and ovomucoid were obtained from Worthington Biochemicals (Freehold, N.J., U.S.A.). Dinitrophenyl-bovine serum albumin (DNP-BSA) was prepared by the method of Little and Eisen[7]. The nominal DNP-content of the preparation as estimated spectrophotometrically was 16.5 moles/mole of BSA. Myosin was prepared by the method of Perry[8].

Sodium dodecyl sulfate (SDS), specially pure, was obtained from BDH (Poole, Great Britain); all other chemicals were reagent grade or the best commercially available.

Methods

Three solvent systems were employed; each contained a detergent with properties rather distinct from the other two. These solvent systems represented a denaturing detergent solvent system[9], SDS, a non-ionic, mild detergent solvent system[10], Triton X-100, and a mild anionic detergent solvent system[11], sodium deoxycholate (DOC). The compositions of the three solvent systems employed are given in Table I.

TABLE I

DETERGENT SOLVENT SYSTEMS

Detergent	*Running buffer*	*Sample preparation buffers*
SDS	0.04 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.1% SDS	0.04 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 10.0% SDS 0.13 *M* β-Mercaptoethanol
Triton X-100	0.025 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.05% Triton X-100	0.025 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.1% Triton X-100
Deoxycholate	0.025 *M* Carbonate, pH 9.7 (I = 0.1) 0.02% NaN_3 0.01 *M* DOC	0.025 *M* Sodium carbonate, pH 9.7 0.02% NaN_3 0.02 *M* Sodium deoxycholate

TLG was performed in a gel bed 0.8 mm thick which was spread on a 20 × 30 cm heavy glass plate. The gel filtration slurry was prepared by the addition of 6 g of Sephadex G-200 to 150 ml of the appropriate detergent solvent system. This was sufficient Sephadex slurry to prepare two plates. Upon addition of the Sephadex to the solvent, the flask was gently swirled or vortexed for a length of time sufficient to insure complete mixing. The Sephadex gel was then allowed to hydrate for at least 24 h before pouring the plates. A swelling time of 72 h, as recommended by the manufacturer, produced no improvement in the protein zone resolution. One side of a glass plate was marked with a line which was 3.5 cm from a 20-cm edge and parallel to it; this line served as a guide for sample application. Routinely, seven or eight samples were applied at equally spaced intervals along the line.

Immediately before pouring a plate, the Sephadex slurry was gently vortexed and deaerated to remove or bring to the top of the slurry any recalcitrant air bubbles;

these bubbles were then removed by gentle aspiration. Once all bubbles were removed, the slurry was gently swirled to produce an even suspension, and about 60 ml of the slurry were poured across the same end of the plate as the inscribed line but on the opposite side. The gel bed was spread to a thickness of 0.8 mm with a commercial TLG-spreader; in our hands, the TLG-spreader purchased from Pharmacia (Piscataway, N.J., U.S.A.) yielded the best results. The spreader was placed parallel to the narrow edge of the plate and moved slowly towards the opposite end, pushing a ridge of Sephadex before it. A second line of Sephadex could be added to the plate if more were needed. However, if the spreader was stopped and restarted, a slight discontinuity in the gel was apparent at that point. Bubbles which were occasionally present and which adhered to the trailing edge of the spreader produced thinning or channels in the gel bed. This situation resulted in a discontinuity of solvent flow rates across the width of the gel bed.

Experiments were performed in a Plexi-glass chamber (40 × 28 × 18 cm) similar to that described by Morris[3]. Whatman No. 3MM chromatography paper (22 × 7 cm) soaked in the appropriate solvent served as the wicks between the gel bed and the buffer reservoirs. The eluent was allowed to percolate through the gel overnight to equilibrate the plate prior to sample application. A plate incline of 15 degrees was routinely employed and produced an eluent flow-rate sufficient to move the void volume marker about three-fourths the length of the plate in 5 h.

Protein sample solutions contained about 10 mg/ml of each protein species in the detergent system which was under investigation. When the SDS solvent system was employed, the SDS–protein complexes were formed by heating the proteins in the sample preparation buffer at 100° for 10 min[12]. Myosin was employed as the void volume marker and dinitrophenyl glycine (DNP-Gly) as the internal volume marker in the SDS system. DNP-BSA or BSA could equally serve as void volume markers in SDS because each possessed the same TLG mobility as myosin. DNP-BSA and DNP-Gly served as void volume and internal volume markers, respectively, in the DOC and Triton X-100 systems. Although the hydrodynamic radius of native BSA is sufficiently small to be partially included in the G-200 pore volume, DNP-BSA behaved hydrodynamically as though it were much larger (see the Results and discussion section for an explanation) and migrated at the same rate in G-200 by TLG as myosin.

A sample volume of up to 5 μl could be applied to the gel bed without noticeable zone spreading. We routinely applied 2.5 μl loads (25 μg of each protein species in the sample), as this volume yielded the best resolution in our hands. The experimental run was stopped when the void volume marker was approximately 3 cm from the distal end of the plate.

Proteins and/or glycoproteins were detected by using the technique of replica-staining[5] on Whatman No. 3MM chromatography paper. The plate was removed from the chromatography chamber and the Whatman No. 3MM paper, cut to the dimensions of the gel bed, was carefully layered (rolled) onto the surface of the gel bed and equilibrated for 5 min. The paper was carefully removed from the gel surface and dried for 30 min at 80°. At this point, the DNP-Gly and DNP-BSA zones were visible and were circled in pencil for reference. Staining for protein was performed in Coomassie Brilliant Blue (0.25% in methanol–acetic acid, 9:1, v/v) for 5 min, the replica was rinsed in distilled water and destained in methanol–acetic acid–water

(5:1:5, v/v) for 15 min. The paper was dried and the protein migration distances measured.

Fixation of glycoproteins prior to glycoprotein-specific staining was accomplished by a slightly different fixation procedure. The Whatman paper was carefully removed from the gel surface and was washed with three successive changes of phosphotungstic acid (10%, w/v in 2 *N* hydrochloric acid). This treatment not only fixed glycoproteins some of which are soluble in trichloroacetic acid[13], but also removed the Sephadex beads and detergent from the chromatogram which significantly reduced its background color after glycoprotein staining. The chromatogram was continuously agitated in the third phosphotungstic acid wash for 5 min or until the Sephadex beads no longer adhered to the paper and the wash solution lost its "soapy" appearance. The phosphotungstic acid fixative was removed from the replica by agitation in three successive washes, the first two of which were distilled/deionized water, and the last 95% ethanol. The chromatogram was then air dried. Periodate oxidation–fuschin Schiff base staining, a glycoprotein-specific stain, was performed according to the procedure of McGuckin and McKenzie[14]. Steps 1, 3 and 7 from that procedure were performed in the dark.

Analyses of the data

TLG migration distances of the proteins were expressed either (a) relative to the migration distance of one of the proteins[3,5], (b) relative to the migration distance of the void volume marker[6], (c) as the distribution coefficient, K_t, defined by Klaus *et al.*[6], or (d) as the TLG distribution coefficient, K_d, defined by Ackers[15].

The molecular weights and equivalent hydrodynamic radii (R_e) of the reduced polypeptides complexed with SDS and chromatographed in the presence of SDS were taken from refs. 12 and 16. The R_e values for most of the native proteins in each of the two mild detergent systems were calculated from literature values for the intrinsic viscosity or the sedimentation coefficient of the native protein in dilute buffer[12,16].

The equivalent hydrodynamic radii of BSA and IgG in each of the mild detergents were experimentally estimated from sedimentation velocity measurements. BSA exhibited an $s^0_{20,w}$ of 4.3 S in each of the mild detergent solvent systems as well as in dilute buffer. In Triton X-100, however, the sedimentation coefficient exhibited a slight positive dependence on BSA concentration; this suggests a rapidly associating–dissociating system. IgG exhibited an $s^0_{20,w}$ of 6.9 S in the DOC solvent system and $s^0_{20,w}$ of 7.2 S $\pm$ 0.1 S in the Triton X-100 solvent system. These sedimentation coefficients yielded values for R_e of 50 Å and 48 Å, respectively.

RESULTS AND DISCUSSION

The fractionation properties of the chromatographic support medium, G-200, was compared among the three solvent systems. This was made possible by expressing the sizes of the proteins and polypeptides utilized in the investigation in terms of an equivalent hydrodynamic radius[9]. The equivalent hydrodynamic radius, R_e, takes into account the intrinsic native conformational and the detergent-induced conformational differences among the proteins and thus permits a comparison among solvents[9]. The equivalent hydrodynamic radius of a protein (polypeptide) is plotted

versus the error function compliment of the protein's K_d value; this is known as the Ackers equation[17]. That is,

$$R_e = A + B \operatorname{erf}^{-1}(1 - K_d),$$

where A and B are constants for a specific gel chromatography support medium in a given solvent system. Differences in the properties of a support medium between two different solvent systems are manifested by a change in the slope and/or intercept of the data plotted according to the Ackers equation[9]. Protein migration rates in the three detergent solvent systems, plotted according to the aforementioned equation, are presented in Fig. 1.

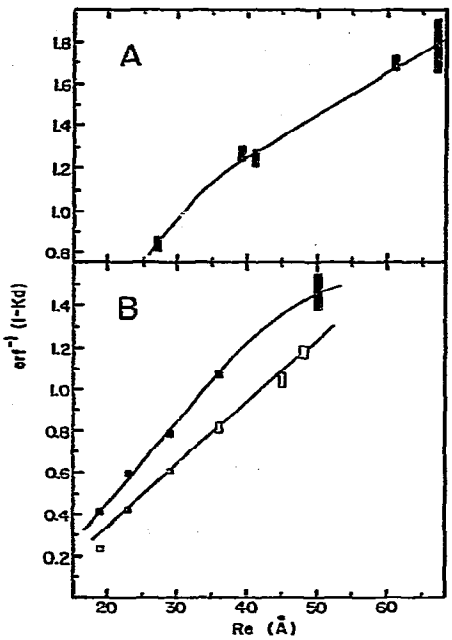

Fig. 1. Thin-layer gel chromatography in the presence of detergents. Data are presented according to the relationship given by Ackers[17]. A, Reduced polypeptides in the presence of SDS. The polypeptides and their respective hydrodynamic radii are: IgG heavy chain, 67 Å; ovalbumin, 61 Å; chymotrypsinigen A, 41 Å; IgG light chain, 39 Å and lysozyme, 27 Å. B, Proteins in the presence of Triton X-100 (□) or in the presence of sodium deoxycholate (■). The proteins and their respective hydrodynamic radii are: IgG, 50 Å in DOC and 48 Å in Triton X-100; aldolase, 45 Å; BSA, 36 Å; ovalbumin, 29 Å; chymotrypsinogen A, 23 Å and ribonuclease A, 19 Å.

A comparison of the data among the three detergent systems strongly suggests that the size discriminatory properties of G-200 do not remain constant among the systems. The data for polypeptides in the SDS and DOC systems would appear to all lie on the same biphasic curve; the solutes of $R_e < \approx 30$ Å form a linear segment of slope greater than that linear segment formed by solutes of $R_e > 30$ Å. The Triton X-100 system exhibits fractionation properties which are distinctly different from other

two detergent systems. A solute of given R_e will be retarded to a greater extent by the G-200 in the Triton X-100 system than in the other two detergent systems which we examined. For example, K_d for a solute of $R_e = 40$ Å is 0.18 in Triton X-100 and 0.08 in DOC or SDS. These results are somewhat puzzling in view of the fact that column gel chromatography has suggested that agarose gels possess fractionation properties which remain identical in dilute buffers, 6 *M* guanidinium chloride, and a number of detergent systems[9,16,18]. It was, however, recently inferred that the fractionation characteristics of gel chromatographic support media may be affected by the presence of some detergents[18]. Thus, it is to be expected that K_d for a given polypeptide on a given gel chromatographic support medium may vary from one detergent system to the next. This occurs because the detergent affects either the protein's conformation or the chromatographic support medium's fractionation properties, or both.

The data of Fig. 1 also demonstrate the fractionation range of the G-200 in the detergent systems. As can be seen, the upper limit of the fractionation range is an R_e of about 70 Å; this corresponds to a polypeptide of molecular weight 50,000 complexed with SDS in the usual fashion or a globular protein of about 500,000 molecular weight*. This limit may be compared to an upper limit for R_e of about 85 Å inferred from the data for Sephadex G-200 in 6 *M* Gdm·Cl[6]. Resolution of components in each of the three detergent solvent systems was comparable to the resolution reported for TLG in dilute buffers[3,5]; components with equivalent hydrodynamic radii of 65, 40, 30, 23 and 19 Å could be resolved from one another when initially applied to the gel as a mixture.

As expected from previous observations by column gel chromatography of reduced polypeptides in SDS[9,19], plots of the logarithm of polypeptide molecular weight *versus* any one of a number of functions of TLG migration distance (see Experimental) were relatively linear. Thus, though perhaps not quite as precise, TLG in SDS may substitute for column gel chromatography in SDS as an empirical molecular weight estimation method when only microgram quantities of protein are available. The apparent molecular weight given by TLG in SDS for a polypeptide of unknown molecular weight was within two percent of the value estimated by column gel chromatography in SDS and within ten percent of the molecular weight estimated by sedimentation equilibrium.

In our hands, the procedural aspects, the sensitivity, and the resolution of TLG in the presence of detergent was quite similar to that in dilute buffers. With the replica-staining technique which we employed for zone detection on TLG, about 10 μg of protein in the 2.5 μl sample application volume were required for a well-defined spot. About 25 μg appeared to be the optimal quantity of protein, and it became increasingly more difficult to measure precisely migration distances with sample quantities in excess of this amount. For the detection of glycoprotein-associated carbohydrate, about 12 μg of carbohydrate represented the lower limit of detection. Additionally, as much as 100 μg of a carbohydrate-free protein gave no color

* Because of the asymmetric nature of many proteins and of the propensity of integral membrane proteins to bind large amounts of detergent, it is actually meaningless to talk about molecular weights in this context. The values referred to above with reference to globular proteins are intended to present the reader with a more common reference to visualize the hydrodynamic radii which are mentioned.

development by the staining procedure we employed. Thus, for the routine sample loads of 25 to 50 μg of material, a glycoprotein with a typical oligosaccharide structure would have to possess at least 25% carbohydrate to be visualized by the detection procedure employed. In practice, the carbohydrate detection limits depend on the nature of the oligosaccharide side chains attached to the polypeptide since the chemical nature of the sugar constituents and the degree of chain branching will ultimately determine the color yield with the Schiff base reagent[20]. Obviously, the sensitivity of detection for either proteins or glycoproteins can be increased by employing radioactive labeling, either *in vivo* or *in vitro*[20,21].

It is possible to utilize DNP-BSA as a TLG void volume marker in the presence of the mild detergents only because the derivatized protein apparently undergoes a partial unfolding and exhibits a rapid association–dissociation in dilute buffer or in the mild detergent solvent systems. In the analytical ultracentrifuge, DNP-BSA appears as a polydisperse system with species of sedimentation coefficients from 3 S to 8 S; the weight-average sedimentation coefficient in the DOC solvent system is 7.6 S. Native BSA in DOC exhibites a single sedimenting boundary of 4.3 S. Since both unfolding and aggregation increase the hydrodynamic radius, a combination of the two effects is apparently sufficient to produce DNP-BSA in a molecular state whose average hydrodynamic dimensions precluded its entrance into any of the pore volume of G-200, *i.e.*, $R_e > \approx 70$ Å. Of course, in the typical SDS complex, the reduced polypeptide chain of DNP-BSA (or BSA) of $R_e = 85$ Å is also excluded from G-200. As a void volume marker for TLG, DNP-BSA offers advantages over Blue Dextran or large, non-chromophoric proteins. Like Blue Dextran, it is visible at all times during the run, yet, like other large proteins, it migrates as a discrete zone on TLG rather than exhibiting irregular zone spreading as does Blue Dextran[6].

It should be mentioned at this point that we have intentionally not emphasized the use of TLG in SDS as an empirical means for the estimation of polypeptide chain molecular weights or the use of TLG in mild detergents to estimate empirically equivalent hydrodynamic radii. Obviously, TLG may serve these purposes, but its application in such endeavors should be done only with the realization of the many potential pitfalls associated with these types of empirical measurements[12,18]. In these detergent systems, the internal volume marker, DNP-Gly, is slightly retarded in its migration by weak interactions with the gel matrix. This slight retardation of the internal volume marker yields absolute K_d values which are slightly smaller than if a totally inert volume marker, such as NaCl, had been employed. Though the absolute K_d values are slightly affected, there is no effect on the relation between R_e and $\text{erf}^{-1}(1 - K_d)$.

In conclusion, TLG in the presence of detergents is a simple, inexpensive method for separating microgram quantities of (glyco)proteins which are inherently insoluble in dilute buffers. This, of course, suggests that its greatest utility may lie with the separation, according to size, of the small quantities of detergent soluble proteins frequently attending membrane research.

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