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THE SOLUBILIZATION AND GEL ELECTROPHORESIS OF MEMBRANE ENZYMES BY USE OF DETERGENTS

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SUMMARY

The solubilization and electrophoresis of membrane proteins with phenol and acetic acid or urea usually have deleterious effects on enzyme activities. A method is reported of solubilizing rat-liver plasma membrane proteins, by the use of the detergents sodium dodecyl sulfate, sodium deoxycholate, and Triton X-100, for subsequent electrophoresis of the enzymes on 7 % polyacrylamide gel. An important feature of the method is the incorporation of detergent into the gel and the electrophoresis buffer. By use of this procedure, non-specific esterases, an alkaline phosphatase, and alkaline phosphodiesterase are detectable on the gels. The method is compared with other techniques currently being used, and it would appear that this method will have general usefulness in the study of membrane enzymes.

INTRODUCTION

Electrophoresis on polyacrylamide gel results in excellent resolution of soluble proteins. However, large lipoprotein molecules will not enter gels composed of 5–10 % acrylamide, which is the best compromise between firmness and large pore size, or, if they enter, will not separate well from one another. Plasma lipoproteins have been electrophoresed in 3.75 % polyacrylamide gel[1] and in gel containing an even higher concentration of acrylamide[2], but resolution has not been so good as that obtained with smaller proteins. Consequently, membranes have usually been treated according to the method of TAKAYAMA *et al.*[3] or modifications thereof[4,5], which require solubilization with phenol–acetic acid–water and subsequent electrophoresis on 7.5 % gel containing 5 M urea and 35 % acetic acid. This method cannot be used if the investigator desires to retain enzyme activities.

We wish to report a method for the solubilization and electrophoresis on polyacrylamide gel of a major part of rat-liver plasma membrane protein and the identification on the gels of certain enzyme activities.

METHODS

The standard 7 % polyacrylamide gel formulation was used according to instructions provided by Canal Industrial Corporation (Canalco)[6] or by DAVIS[7]. A Canalco Model 6 analytical disc electrophoresis unit was employed, with gel tubes 2.5 inch

in length. Sample gel was not used. Stacking (spacer) gel and separating gel were polymerized after addition to the gel solutions of sufficient detergent (sodium dodecyl sulfate, sodium deoxycholate, or Triton X-100, added as a 10 % (w/v) solution) to give a final concentration of 0.1 %. Detergent was added to the upper buffer so that the final concentration was 0.1 %, and 50–100 μl of a 0.05 % solution of bromphenol blue was added to the buffer as a tracking dye. After insertion of gel columns into the silicone column adapters, addition of lower buffer, and assembling of the apparatus, upper buffer was poured in and samples were added (see the following paragraph). Samples were electrophoresed toward the anode at 2–4 mA per column for 1–2 h. Gels were removed from the columns by squirting a fine stream of water from a hypodermic needle between the gel and the glass wall.

Membrane solutions of a concentration of 1–2 mg/ml were obtained by adding detergent to a final concentration of 1 % (w/v), after the suspension had been made 10 % (w/v) in sucrose and 0.1 M with respect to Tris–HCl buffer (pH 8.5). Membranes were centrifuged at $6000000 \times g \cdot$min after addition of detergent if the solutions appeared opalescent; the amount of protein solubilized was found to vary between 70 and 100 % in different preparations. About 100 μg of protein in 50–100 μl of the membrane solution were added to the small well remaining above each gel column in its adapter.

In order to demonstrate phosphodiesterase, the gel was placed into a solution of 5 mM *p*-nitrophenyl 5′-thymidylate–0.02 M Tris–HCl (pH 9) on ice for 15–30 min. A yellow band of liberated *p*-nitrophenol localized the activity.

Phosphatase was demonstrated by a modification of the method of BARKA[8]. Solutions of 4 % (w/v) $NaNO_2$ in water and 4 % pararosaniline–HCl in 2 M HCl were prepared and cooled in ice. After about 5 min, 1 ml of the clear portion of the pararosaniline solution was mixed with 1 ml of $NaNO_2$ solution and allowed to stand for 5 min. A second solution was prepared consisting of 1 ml of 0.1 M Tris–HCl (pH 7.4), 6 ml of water, and 5 mg α-naphthyl phosphate (sodium salt), to which was added sufficient 1 M NaOH, about 1.9 ml, to neutralize the HCl in the pararosaniline–diazonium salt solution. The basic solution was immediately poured into the diazonium solution and mixed, and the gel was inserted into the solution and incubated at 4° for 15–30 min or until phosphatase activity was evinced by appearance of a pink band of diazotized α-naphthol. If the solution clouded, the gel was transferred to fresh incubation medium.

Esterases were visualized as follows: to a solution of 1 ml of 1.0 M sodium phosphate buffer (pH 7.4) and 9 ml of water, about 10 mg of fast blue RR salt were added, and the mixture was rapidly and thoroughly mixed and filtered. About 100 μl of a solution of 10 mg of α-naphthyl acetate in 1 ml of methanol were then added dropwise to the diazonium salt solution with vigorous mixing to prevent precipitation. The gel was placed in the solution and incubated in ice for 15–30 min until brown or purplish bands of α-naphthol diazotate appeared. The gel was transferred to a fresh solution if cloudiness occurred.

Proteins were stained by immersing gels for 15 min in a fresh solution of acetic acid (7 %, v/v) containing 0.1 % (w/v) coomassie blue and then washing out unbound dye with 7 % acetic acid with gentle agitation over a period of 1–2 days.

HIO_4-oxidizable material capable of reacting with Schiff reagent was visualized by incubating gels in a 1 % (w/v) solution of HIO_4 in 7 % acetic acid for 1 h with

gentle rocking. Diffusible Schiff-reacting materials were then removed by rinsing the gels in several changes of large volumes (100 ml) of 7 % acetic acid for a total of 12–18 h. Gels were placed for 2 min in Schiff reagent[9] and were finally transferred to water. Immediate note was taken of the position of pink bands before pink or reddish background color developed.

Plasma membranes were prepared by a method developed by H. HENDRICKSON in this laboratory (to be submitted) and involves (a) flotation of cell particulates (except nuclei) in 2.1 M sucrose followed by (b) separation of the plasma membrane fraction in a discontinuous sucrose gradient and (c) washing in a sucrose–KCl solution to remove adsorbed protein. The membrane fraction floats at a density of about 1.11–1.13 and is characterized by electron microscopy as consisting largely of closed vesicles with no desmosomes. It is highly enriched (10–20-fold) in 5′-nucleotidase, the most widely accepted plasma membrane marker, and is low in enzyme markers of other subcellular fractions.

MATERIALS

Reagents were obtained from the following sources: fast blue RR salt (diazotized product of 4-benzoylamino-2,5-dimethoxyaniline-$ZnCl_2$, C.I. 37155) from Matheson, Coleman and Bell; α-naphthyl phosphate (sodium salt) from Sigma Chemical Co.; α-naphthyl acetate from Nutritional Biochemicals Corp.; *p*-nitrophenyl 5′-thymidylate from Calbiochem; pararosaniline–HCl and basic fuchsin (both C.I. 42500) from Fisher Scientific Co.; coomassie brilliant blue R250 from Colab Laboratories; sodium dodecyl sulfate and Triton X-100 from Mann Research Laboratories; and sodium deoxycholate from General Biochemicals. All chemicals for preparing gels were purchased from Canalco except that sucrose was a Fisher product; Tris was purchased from Eastman or Mann and glycine from Sigma. Acrylamide and bisacrylamide were recrystallized according to LOENING[10] from chloroform and acetone, respectively.

RESULTS

After solubilization of rat-liver plasma membranes with 1 % Triton X-100 and electrophoresis in polyacrylamide gel containing Triton, staining of the gels resulted in the bands indicated in Fig. 1.

Solubilization with sodium deoxycholate and electrophoresis in the presence of this detergent also permitted the visualization of several protein bands and of various enzymes on the gel (Fig. 2). Unlike the result with Triton X-100, part of the phosphodiesterase is seen to bind to the point of application of the sample while part smears along the first part of the separating gel. The Schiff stain gave negative results after use of sodium deoxycholate.

Sodium dodecyl sulfate, like sodium deoxycholate an anionic detergent, solubilized membranes and permitted the electrophoretic separation of their proteins apparently as effectively as sodium deoxycholate (Fig. 3). It should be noted that sodium dodecyl sulfate results in the disappearance of esterase activity.

Certain technical difficulties have been encountered while working with the systems described here. For example, sodium deoxycholate precipitates in the gel in the presence of 7 % acetic acid and other fixatives such as sulfosalicylic acid, resulting

in a diffuse background against which it may be difficult to discern fine bands of protein after the coomassie blue stain. Resolution of finer protein bands may be improved by increasing sample size to 150–200 μg of protein, although this may

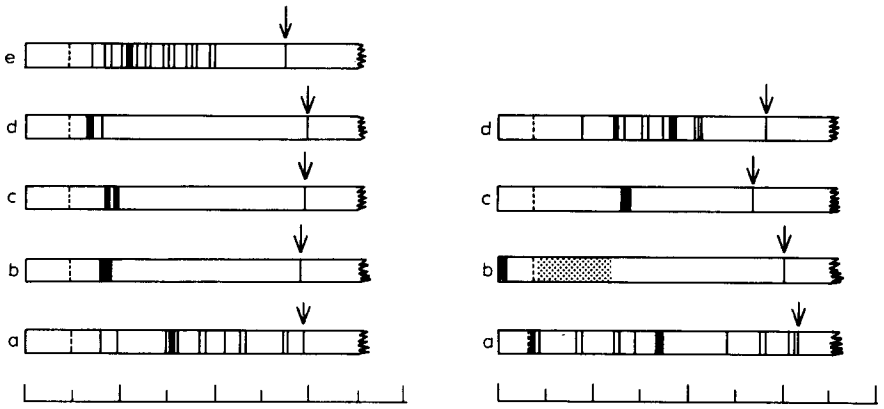

Fig. 1. Plasma membranes were solubilized with Triton X-100 and electrophoresed and stained as described in METHODS. (a) Protein; (b) phosphodiesterase; (c) alkaline phosphatase; (d) Schiff-positive material; (e) esterases. The dashed line marks the junction between stacking gel and separating gel; the arrows indicate the position of tracking dye. The scale is calibrated in half-inch segments.

Fig. 2. Plasma membranes were solubilized with sodium deoxycholate and treated as described in METHODS. (a) Protein; (b) phosphodiestesase; (c) an alkaline phosphatase; (d) esterases.

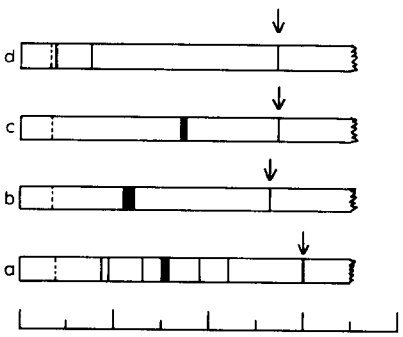

Fig. 3. Plasma membranes were solubilized with sodium dodecyl sulfate and treated as described in METHODS. (a) Protein; (b) phosphodiesterase; (c) an alkaline phosphatase; (d) Schiff-positive material.

result in distortion of some bands representing proteins present in higher concentration in the membranes. The presence of impurities necessitates keeping the solutions of stabilized diazonium salts cold and filtering them immediately before use. Such salts may also only couple under quite restricted conditions of pH and ionic strength. Furthermore, coupling techniques which may be excellent for demonstrating enzymes in tissue sections and in starch gels may not be applicable to polyacrylamide gels, perhaps due to the chemical composition of the gel.

The phosphodiesterase activity may be ascribed to the enzyme demonstrated by LANSING *et al.*[11] in liver plasma membrane. The reason why this enzyme fails to

migrate into sodium deoxycholate gels is unknown. It is not known what enzyme is represented by the α-naphthyl phosphatase activity on these gels. Both 5′-nucleotidase (5′-AMP as substrate) and alkaline phosphatase (β-glycerophosphate as substrate) are known to be present in the liver plasma membrane[12], and either might act on the substrate used here. Whereas bands of esterase activity were seen after treatment with sodium deoxycholate and Triton X-100, none was detected when sodium dodecyl sulfate was used. It is known, however, that some liver esterases are inhibited by sodium dodecyl sulfate[13], and this may be the reason for negative results in the presence of this detergent. The specific nature of the Schiff-positive bands is not certain[14].

Membranes other than liver plasma membranes, including microsomes, lysosomal membranes and mitochondrial membranes, have also been solubilized and electrophoresed by the use of this technique, to yield gels showing the presence of various enzyme activities.

DISCUSSION

A variety of treatments (urea, acetic acid) have been employed to solubilize and electrophorese non-enzymatically active membrane proteins on gels[3,15–17]. However, not all enzyme activities need be lost after urea treatment, for example, alkaline phosphatase from red blood cell stroma[18] and NADH oxidase and ATPase of *Escherichia coli* B cell "envelope membranes"[19]. Some aspects of the technique used in the present work have been incorporated in recent studies; *i.e.* COUTINHO *et al.*[20] have reported adding Triton X-100 to homogenates of the mid-gut of *Rhynchoschiara angelae* before electrophoresis of the extract, and FITZPATRICK *et al.*[21] have solubilized about 70 % of rat-kidney plasma membrane protein, retaining $(Na^+ - K^+)$-dependent ATPase activity, and have electrophoresed this material.

It is clear that the use of detergents, particularly Triton X-100, to solubilize proteins is preferable to reagents, such as urea and acid, with a strong tendency to cause denaturation, and that incorporation of detergent into both buffer and gels surrounds the membrane proteins with a constant environment of the solubilizing agent. It is possible that the solubilizing action of the detergent is the result of the dispersal of lipid from the immediate environment of membrane proteins. SALTON AND SCHMITT[22], using ^{32}P-labeled *Micrococcus luteus* (*M. lysodeikticus*) membrane dissolved in sodium deoxycholate, have indicated that almost all of the label migrates as a single peak ahead of the proteins. Also, SHAPIRO *et al.*[23] electrophoresed proteins in gels containing sodium dodecyl sulfate, found that migration rates were inversely related to the log of the molecular weight, and suggested that sodium dodecyl sulfate minimizes charge differences and that "all proteins migrate as anions as the result of complex formation with sodium dodecyl sulfate". It is not known whether in the present system such a dissociation of lipid from membrane protein occurs. What is clear is that at least some of the enzyme activities are indeed retained by liver plasma membrane proteins after solubilization by, and electrophoresis in the presence of, detergent. It is hoped that this technique will greatly extend the usefulness of polyacrylamide-disc electrophoresis in the study of membrane enzymes.

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REFERENCES

1 S. RAYMOND, J. L. MILES AND J. C. J. LEE, *Science*, 151 (1966) 346.
2 K. A. NARAYAN, H. L. CREININ AND F. A. KUMMAROW, *J. Lipid Res.*, 7 (1966) 150.
3 K. TAKAYAMA, D. H. MACLENNAN, A. TZAGOLOFF AND C. D. STONER, *Arch. Biochem. Biophys.*, 114 (1964) 223.
4 D. M. NEVILLE, JR., *Biochim. Biophys. Acta*, 133 (1967) 168.
5 W. L. ZAHLER, A. SAITO AND S. FLEISCHER, *Biochem. Biophys. Res. Commun.*, 32 (1968) 512.
6 Canalco, *Chemical Formulation for Disc Electrophoresis* (April 1965) and *The Model 6 System Instructions*.
7 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
8 T. BARKA, *Nature*, 187 (1960) 248.
9 L. G. LUNA, *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, McGraw-Hill, The Blakiston Div., New York, 2nd Ed., 1960, p. 159.
10 U. E. LOENING, *Biochem. J.*, 102 (1967) 251.
11 A. I. LANSING, M. L. BELKHODE, W. E. LYNCH AND I. LIEBERMAN, *J. Biol. Chem.*, 242 (1967) 1772.
12 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND P. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
13 G. GOMORI, *J. Histochem. Cytochem.*, 3 (1955) 479.
14 A. G. E. PEARSE, *Histochemistry*, Little, Brown and Co., Boston, 2nd Ed., 1960, p. 234.
15 E. D. KIEHN AND J. J. HOLLAND, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1370.
16 J. H. STRAUSS, JR., B. W. BURGE, E. R. PFEFFERKORN AND J. E. DARNELL, JR., *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 533.
17 J. SUSZ AND W. R. ADEY, *Federation Proc.*, 28 (1969) 830.
18 L. J. SCHNEIDERMAN, *Biochem. Biophys. Res. Commun.*, 20 (1965) 763.
19 G. WEINBAUM AND R. MARKMAN, *Biochim. Biophys. Acta*, 124 (1966) 207.
20 H. B. COUTINHO, E. KATCHBURIAN AND A. G. E. PEARSE, *J. Clin. Pathol.*, 19 (1966) 617.
21 D. F. FITZPATRICK, G. R. DAVENPORT, L. FORTE AND E. J. LANDON, *J. Biol. Chem.*, 244 (1969) 3561.
22 M. R. J. SALTON AND M. D. SCHMITT, *Biochem. Biophys. Res. Commun.*, 27 (1967) 529.
23 A. L. SHAPIRO, E. VIÑUELA AND J. V. MAIZEL, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.