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Review

Electrophoresis of hydrophobic proteins

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Abstract

The electrophoresis of hydrophobic proteins underwent significant development during the last decade. In native gel electrophoresis, the induced charge shift, the involvement of mild detergents as well as a constant ionic strength in the electrophoresis, overcame the difficulty of the low solubility and aggregation of the hydrophobic proteins, and critically improved the results of their native PAGE [H. Schägger, G. von Jagow, Anal. Biochem. 166 (1987) 368]. In isoelectric focusing electrophoresis, the most difficult one for hydrophobic proteins, the including of mild detergents, the use of diluted samples with immobilized pH gradient, as well as a more hydrophobic media such as polyethylene glycol methacrylate–acrylamide copolymer improved the resolution and yield of these proteins [J. Schüpbach, R.W. Ammann, A.U. Freiburghaus. Anal. Biochem. 196 (1991) 337]. In SDS PAGE, contradicting the general belief that it is denaturing, a new development in this technique may permit the complete removal and exchange of SDS bound to proteins and restoration of the latters' activity [M. Dong, L.G. Bagget, P. Felson, M. LeMaire, F. Pennin, Anal. Biochem. 247 (1997) 333]. With the use of mild detergents and charge shift of the proteins, native electrophoresis resolving proteins while maintaining activities is now possible. Preparation of active membrane proteins with electrophoresis principles can thus be realized and improved by some new technique such as "Free-flow electrophoresis". Membrane protein preparation will be more and more sophisticated, contributing to the characterization, crystallization and structure determination of these biologically important macromolecules.

Keywords: Hydrophobic proteins; Charge shift; Detergents; Aggregates; Blue-Nature PAGE

1. Introduction

The electrophoresis of hydrophobic proteins, especially native electrophoresis, has been much more difficult to achieve than that of soluble proteins. The major difficulties come from their low solubility and aggregation in the electrophoretic field. Many attempts have been made to improve this technique, but no standard procedures have been accepted (methods to solubilize hydrophobic proteins for their purification can be used for the sample preparation in electrophoresis).

Here, we summarize the major work accomplished and the development of this technique during the past decades. We also speculate on its future development. In fact, the hydrophobic proteins are mostly membrane associated or integral membrane proteins. The present study is thus important for these proteins, which have critical biological functions, while their mechanism remains much less understood than the soluble proteins. Indeed, membrane proteins have

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been crystallized only since the 1980s, yielding few crystal structures, of which very few with high resolutions among hundreds of them until now [1–3]. The efficient analysis, especially the preparation of these proteins will provide important basis for their crystallization and structure determination, while the latter remains the most important means in the structural biology nowadays.

2. Native electrophoresis

The critical points to improve the native gel electrophoresis for hydrophobic proteins are: making use of the protein's own charge and especially inducing the charge shift on the protein; improving the solubilization and disaggregation of these proteins. In fact, the charge, solubilization and disaggregation of hydrophobic proteins are closely related. For example, the salting-in effect results in the solubility increase of proteins at low salt concentration (<0.5 M), while the salting out results in a decrease of protein solubility, which is governed by hydrophobic interactions at high salt concentration [4]. With high concentrations of salts (ammonium sulfate and phosphate) in the presence of detergents, the solubility of a few membrane protein–detergent complexes have been decreased, resulting in their crystallization [5].

2.1. Making use of the protein's own charge or inducing the charge shift

Attempts in this respect have been made by many protein scientists since 1970s. A representative work was provided by Newby and Chrambach [6], who found that a mixture of ionic and non-ionic detergents can provide charge shift with ionic detergent binding, disaggregation of adenylate cyclase as well as an increase in the recovery of the enzyme activity in the electrophoresis. In fact, the use of ionic but mild detergents to induce a charge shift on the detergent bound-proteins which migrate in the same direction had already been attempted before the above report. A Golgi membrane α-mannosidase from rat liver had been successfully electrophoresed only in the presence of both Triton X-100 and sodium deoxycholate (detergents incorporated both in the gel and the electrophoresis buffer, similar to the text below) [7].

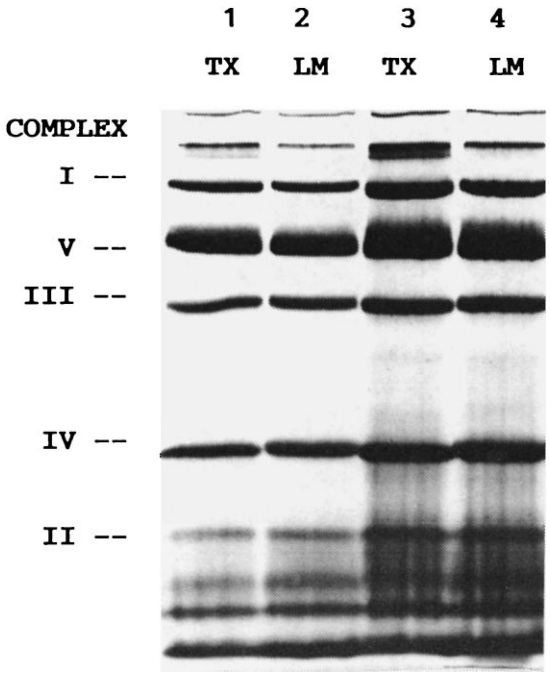

Fig. 1. Blue Native PAGE for resolution of membrane protein complexes from whole mitochondria, the first dimension Blue Native PAGE with the cathode buffer throughout the whole run was performed after solubilization of the proteins by laurylmaltoside (LM) or Triton X-100 (TX); 0.7 mm gels with 1% acrylamide steps from 6% T to 10% T were used. The protein load was 75 μg in lanes 1 and 2, 150 μg in lanes 3 and 4. Electrophoresis was started at 100 V (30 min) and continued at 500 V (2 h) at 4°C (from [11] with permission).

Deoxycholate, sarkosyl, or a mix of Triton X-100 and deoxycholate were the detergents mostly used for this purpose [8–10].

Later, Schägger and von Jagow [11] further developed the charge induction and a more general protocol for native electrophoresis of hydrophobic proteins by (1) using Coomassie blue G native gel electrophoresis for large membrane proteins (Fig. 1), and (2) using the mild taurodeoxycholate for lower molecular mass proteins. In both cases, charge shift was introduced in the presence of aminocaproic acid to assist the solubilization of membrane proteins. In contrast to detergents that dissociate from membrane proteins below the critical micelle concentration, Coomassie dye such as Serva blue G remains slightly bound to the protein. The method of ''Blue Native Gel'' reported by these authors has been proven efficient for many

mitochondria membrane proteins from different sources, and has also been successfully used for other membrane proteins such as the membrane anchor subunit of succinate dehydrogenase from *Saccharomyces cerevisiae* [12].

2.2. Detergents use

For the solubilization and disaggregation of hydrophobic proteins, the detergent incorporation into the electrophoretic system was an important step. In the 1970s, membrane protein samples were treated with detergents and 70–100% solubilization was achieved [8,13,14]. The same detergents were incorporated into the gel and (upper) electrode buffer to provide a constant concentration of the solubilizing agent and thus prevent the hydrophobic proteins from reaggregating. Triton X-100 and sodium deoxycholate (DOC) were apparently the most utilized in the native electrophoresis of hydrophobic proteins. Triton X-100 was successfully used for many membrane proteins (e.g., esterases, alkaline phosphatase, phosphodiesterase 1, β-glucuronidase, L-leucyl-β-naphthylamidase, etc.), with effective solubilization, maintenance of enzyme activity, and sharp resolution in electrophoresis. It has been demonstrated that the electrophoretic mobility of the hydrophilic proteins remained unaffected while that of the amphiphilic proteins was shifted in the presence of detergent mixtures, providing a sensitive method for distinguishing these two kinds of proteins [9]. Nevertheless, we might look for specific detergents for various hydrophobic proteins with different properties, a necessity similar to that of finding compatible detergents for their purification. Methods of systematic selection of detergents for hydrophobic proteins in electrophoresis have been provided by Hjemeland and Chrambach [15,16]. Photosystem I protein complex was purified through native gel electrophoresis of β-dodecyl maltoside (a non-ionic detergent) solubilized thylakoid membrane preparation from wild-type and T398 strains of Anabaena variabilis ATCC 29413. Finding a suitable detergent or detergent mixture which solubilizes the hydrophobic protein while maintaining its activity is essentially the same problem for these proteins' electrophoresis and for their purification. The classification, roles, and

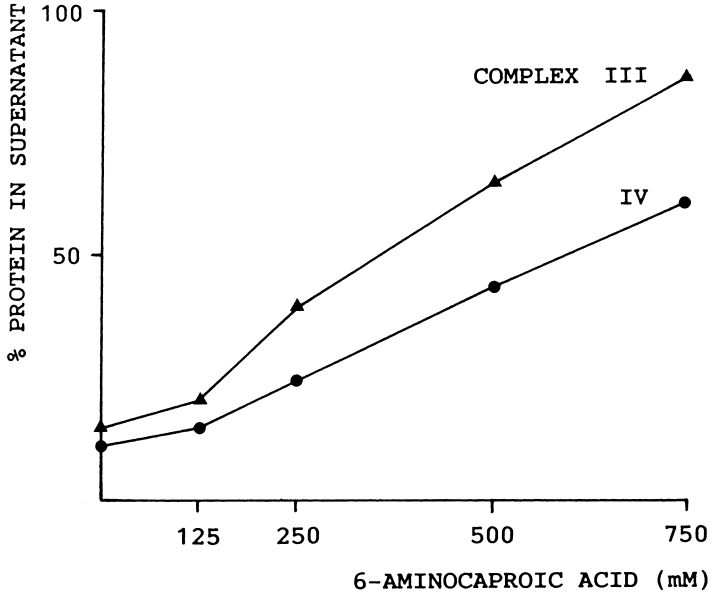

Fig. 2. Solubilization of membrane proteins by Triton X-100 and aminocaproic acid. Of mitochondrial membrane proteins, only the solubilization of complexes III and IV could easily be quantitated photometrically. Bovine heart mitochondria were treated with Triton X-100 (3%) in the presence of varying concentrations of aminocaproic acid. The protein concentration before centrifugation was 13 mg/ml. In the presence of 750 mM aminocaproic acid the same percentage of the membrane protein complexes III and IV was found to be solubilized as in the presence of 500 mM NaCl (from [11] with permission).

usage of detergents have been better understood and the search for new detergents greatly developed as a need for this knowledge appeared for the crystallization of membrane proteins since the 1980s. A systematic discussion of this aspect can be found in chapter of ''General and Practical aspects of membrane protein crystallization'' [17] and ''Detergent phenomena in membrane protein crystallization'' [18].

2.3. A suitable ionic strength, another point closely related to the detergent solubilization

The solubilization of membrane proteins in detergents is often difficult at low ionic strength (Fig. 2), salt such as NaCl was frequently used to increase the solubility. Since Cl^- has a much higher mobility than the proteins under electrophoresis, the salt at high concentration in the sample will be separated from the proteins during the electrophoresis, causing the concentration and aggregation of the protein. So Schägger and Jagow used 6-aminocaproic acid instead of NaCl. A concentration of 750 mM of aminocaproic acid had the same effect on the solubility of proteins from mitochondria to that of 500 mM NaCl. Contrary to NaCl, no migration of the compound occurs at a pH of 7–7.5 in an electric field, with relative p*K* values of 10.7 and 4.4 for the amino and carboxylic groups, respectively.

3. IEF (isoelectric focusing)

Schüpbach et al. [19], developed a ''Universal method for two-dimensional polyacrylamide gel electrophoresis of membrane proteins using isoelectric focusing on immobilized pH gradients in the first dimension'' with pig pancreatic zymogen granule membrane proteins. Optimized solubilization procedures for hydrophobic membrane proteins were presented and the use of diluted samples was shown to be essential to overcome the major problems in isoelectric focusing (IEF). The latter electrophoresis with diluted samples gave good results in 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate) without urea. The authors also indicated the importance of avoiding heat denaturation.

To overcome the important difficulties encountered in IEF, i.e., the tendency of proteins to precipitate more at their isoelectric point, and the fact that the chemical environment in IEF is incapable of counteracting the aggregation of the hydrophobic proteins, Harrington and Zewert have used a more hydrophobic media than polyacrylamide (i.e., polyethylene glycol methacrylate–acrylamide copolymer), combined with organic solvents and/or detergents. Using this method, improved resolution and yield of IEF separation for the hydrophobic proteins was achieved [20]. This approach appears promising for high resolution IEF electrophoresis.

4. SDS PAGE

In contradiction with the general belief that SDS PAGE is a denaturing electrophoresis, a new development in this technique is the complete removal and exchange of SDS bound to proteins (including hydrophobic proteins) and restoration of the latter's activity [21]. This involves loading the SDS–protein complexes onto the ceramic hydroxyapatite column, extensive washing of bound proteins with a mild detergent in a phosphate buffer, followed by an elution of the retained protein with a phosphate gradient. Complete exchange of SDS with non-ionic detergent such as dodecyl maltoside was achieved with 90–100% protein recovery. It was proposed that the efficiency of SDS removal from protein was due to the combined effect of phosphate ions and the hydrophobic tail of non-ionic detergent. In fact, the phosphate ion mimics the sulfate part of SDS and the mild detergent mimics the dodecyl carbon chain of SDS. SDS-treated membrane P-glycoprotein, and soluble glutamate dehydrogenase, bovine serum albumin and lysozyme fully recovered their enzymatic activities after the above chromatography. This was also the case for lysozyme electrophoresed with SDS PAGE. The authors suggest that the denaturation caused by SDS is incomplete and reversible, allowing the use of the high solubilization capacity of SDS without causing irreversible loss of enzymatic activity. Of course, electrophoresis with SDS for activity recovery should be different from the conventional SDS-PAGE: in this case the samples should not be heated nor incubated with high amounts of reducing compounds in order to avoid the dissociation of the protein subunits and the irreversible inactivation. Furthermore, the protein

should not be fixed nor stained with Coomassie blue before electroelution.

This finding may bring an important development in the electrophoresis technique as well as provide a new preparation of hydrophobic proteins. Of course, this new method needs to be applied to a number of hydrophobic proteins, for the establishment of a more general way for activity recovery. In the case of Ca^{2+}-ATPase, the SDS removal was complete but reactivation of the enzyme activity is still being attempted in the presence of lipids or high calcium concentrations [22]. Among the detergents used in this protocol, the authors indicated that dodecyl maltoside gave excellent results in SDS removal and activity recovery of the proteins. Other classes of detergents, such as $C_{12}E_8$ and Chaps, have also been successfully used. This indicates that most detergents are able to exchange SDS, when the protein solubility is ensured and the detergent does not precipitate with SDS by electrostatic interaction. Hydroxyapatite is widely used in protein preparation, especially for membrane proteins, as this matrix allows chromatography in the presence of most detergents [21,23–25]. These also demonstrate the general applicability of the present method.

Another interesting development in SDS PAGE in the late 1980s is the use of tricine as a trailing ion, permitting the resolution of small proteins at lower acrylamide concentration than in a glycine–SDS-PAGE system. High resolution of proteins in the range of 5–20 kDa was achieved without adding urea, while the SDS PAGE with glycine had been difficult for proteins in this range. The present method thus provides an excellent SDS PAGE system for proteins ranging from 1 to 100 Kda. It has also been proven efficient in the electrophoresis of membrane proteins [26].

5. Development of native gel electrophoresis for molecular mass determination and preparation of hydrophobic proteins

Determination of molecular mass has been reported with a series of gels at increasing polyacrylamide concentrations under nondenaturing conditions for many soluble proteins [27] and some membrane-associated proteins such as human estrogenic 17β-hydroxysteroid dehydrogenase [28]. With the above

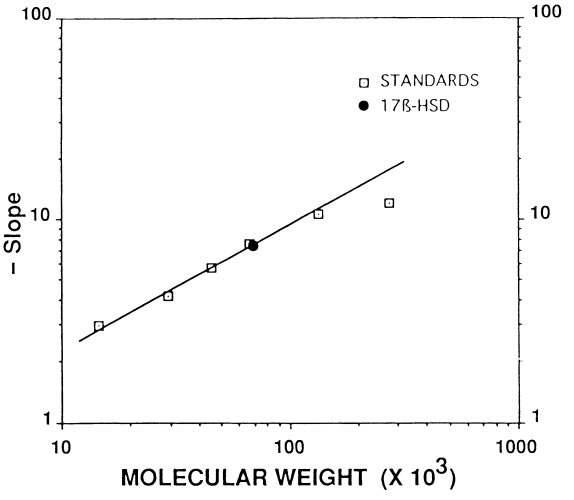

Fig. 3. Evaluation of 17β-Hydroxysteroid dehydrogenase molecular mass by pore gradient native gels. The standards are: α-lactalbumin (14.2 kDa), carbonic anhydrase (79 kDa), chicken egg albumin (45 kDa), bovine serum albumin (66 kDa) for monomer and 132 kDa for dimer), and urease (272 kDa for trimer) from Sigma (from [28] with permission).

method, slopes of R_f versus gel concentrations are obtained. From a standard curve of the slopes for the known molecular mass, the unknown protein molecular mass could be derived (Fig. 3). Differently, in Blue Native PAGE (with Coomassie dye) or native PAGE (with taurodeoxycholate) for high and low molecular mass hydrophobic proteins, respectively, charge shift are induced in both cases, yielding protein separation in principle according to their molecular mass on the same gel with a gradient of polyacrylamide concentration [11]. This provides the possibility of a further simplification and generalization of molecular mass determination under nondenaturing conditions, similar to the established SDS PAGE for subunit mass determination of all proteins. Of course, more systematic assay with various proteins is necessary for the establishment of such a general method.

As mentioned above, with the use of mild mixed detergents or Blue Native PAGE, native electrophoresis resolving proteins maintaining activities is now possible. Combined with the recent development of "Free-flow electrophoresis" [29], the preparation of active hydrophobic proteins making use of the resolution power of electrophoresis could be achieved. During free-flow electrophoresis, a thin layer of elec-

trolyte solution flows laminarly between two parallel cooling plates with a perpendicular electric field to the flow direction. This results in the differential deflection of charged solutes, the latter being separated according to their electrophoretic mobility or isoelectric point. The purification of membrane proteins will attract many biochemists in the study of protein structure-function, especially for the crystallization and structure determination of membrane proteins, which play important biological roles, while their structures remain largely unknown until now [17].

For the preparation of hydrophobic proteins, a large-scale electrophoresis method has also been reported exploiting isoelectricity [30]. With this new technique, the need for proteins of interest to pass the gel has been eliminated, only the contaminants traverse the gel. Thus the drawbacks of low loading and low recovery in routine preparative electrophoresis could be eliminated, only the contaminants traverse the gel. The authors demonstrated that this method provided high rate of sample processing (up to 1 g h^{-1}), efficient purification and high recovery. A large-scale membrane apparatus was built, with large Pt electrode disks to provide an even current. In this electrolyser, 10 g of recombinant Elgin C was purified to homogeneity in about 10 h.

6. A specific technique: capillary electrophoresis

A new methodology of gel electrophoresis for hydrophobic proteins was also reported for polymer-coated fused-silica capillary electrophoresis columns [31]. It is appropriate here to mention this emerging technique for rapid analysis of purity and homogeneity of the hydrophobic proteins. Two capillary electrophoresis separation techniques with either simultaneous solvent flow induced by hydrostatic pressure or followed by low pressurization with helium were developed for the analysis of extremely hydrophobic proteins [32]. For these two related procedures, buffer solutions containing up to 70% of 2-propanol were used for the capillary electrophoresis separation. This highly concentrated organic cosolvent which was used to solubilize the protein can reduce the electrophoresis flow in aminopropyltrimethoxysilane-treated fused-silica capillaries. With this combination of pressurization and capillary electrophoresis, it is not necessary to use any detergent or involatile buffer additions, which are usually needed to solubilize extremely hydrophobic lipoproteins. It is therefore applicable to on-line coupling with electrospray mass spectrometry for the direct structural characterization of hydrophobic proteins.

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