NOTES & TIPS

Electrophoresis of Proteins and Protein–Protein Complexes in a Native Agarose Gel

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An important tool for the biochemist is the ability to analyze proteins in their native state. Electrophoresis of proteins and protein:protein complexes in native polyacrylamide gels using a horizontal gel apparatus has been previously described (1). While this method works well, we have developed an alternate procedure using a native agarose gel that is much easier to set up, takes considerably less time to run, and avoids the use of toxic components. This system has the additional advantage of allowing the detection of both positively and negatively charged proteins as well as protein: protein complexes in the same gel. A protein in native agarose can have either a positive or negative charge depending on its isoelectric point (p*I*)[2] and the pH of the buffer used to perform the electrophoresis. Proteins with a p*I* lower than the buffer pH carry a net negative charge and migrate toward the anode, whereas proteins with a p*I* higher than the buffer pH carry a positive charge and migrate toward the cathode. The gel is run in a submerged horizontal platform with the wells positioned in the center of the gel. This allows for negatively and positively charged proteins to migrate toward the anode and cathode, respectively. Proteins with different molecular weights and p*I* values were tested as well as proteins that form complexes, whether they be two pure proteins forming a complex or a complex formed after incubating a pure protein with a crude extract. Once a complex is identified, it can be cut from the gel and the components isolated using a gel nebulizer (2). This method does not replace isoelectric focusing gels since it does not determine the p*I* of a protein but it does facilitate the detection of protein:protein complexes.

Materials and methods. Agarose (low electroendosmosis) was purchased from Research Organics (Cleveland, OH). The following proteins were purified in our laboratory: single-chain monellin (SCM) (3), *Methanococcus jannaschii* small heat shock protein (Mj HSP16.5) (4) and Mj0577 protein (5), glutathione *S*-transferase, and *Pyrococcus horikoshii* 1704 (Ph1704). Bovine serum albumin was purchased from Sigma (St. Louis, MO). The *M. jannaschii* cell extract was prepared as described by Zarembinski *et al.* (5).

Native agarose electrophoresis was carried out using a submerged horizontal Gibco BRL Horizon 58 unit. The horizontal 0.8% agarose gel (8 cm × 5.5 cm × 3 mm) was prepared in Buffer A (25 mM Tris–HCl, pH 8.5, 19.2 mM glycine) and the comb placed in the center of the gel. The gel was submerged in a reservoir containing Buffer A and electrophoresis was performed at a constant voltage of 50 V for 1 h at room temperature. The samples (5 μg) were mixed 1:1 with sample buffer (20% glycerol, 0.2% Bromophenol blue, 0.12 M Tris base) prior to loading. Gels were stained in 0.12% Coomassie brilliant blue R, 45% methanol, 10% acetic acid for 20 min and destained in 45% methanol, 10% acetic acid and dried between two layers of cellophane membrane (Idea Scientific, Minneapolis, MN).

To isolate the components of a protein:protein complex, two identical samples were loaded on adjacent lanes. The position of the complex was determined by staining one of the two lanes containing the complex and using this information; the unstained gel fragment was cut out with a razor blade and placed into a gel nebulizer that was inserted into a Micropure-0.22 unit (Millipore Corp., Bedford, MA) (2). These two units were centrifuged at 12,000*g*, allowing the gel to go through the orifice of the nebulizer thus converting the gel to a fine slurry. The gel particles were then captured by the Micropure-0.22 and the soluble biomolecules were extruded and passed through the Micro-

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[2] Abbreviations used: p*I*, isoelectric point; SCM, single-chain monellin; Mj HSP16.5, *Methanococcus jannaschii* small heat shock protein; Mj 0577, *Methanococcus jannaschii* 0577 protein; Ph1704, *Pyrococcus horikoshii* 1704 protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

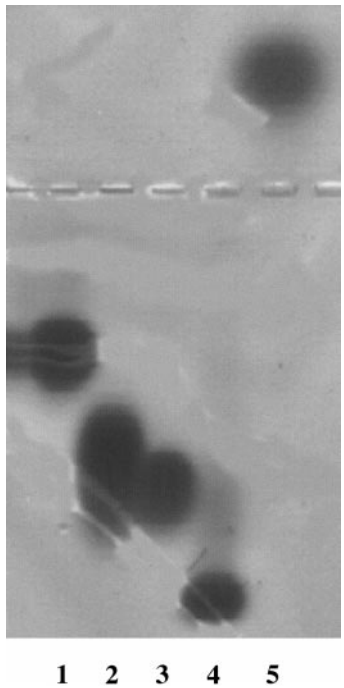

FIG. 1. Migration of negatively and positively charged proteins. Proteins were analyzed on a 0.8% native agarose gel as described under Materials and Methods. Proteins (5 μg) were loaded in the following order: Lane 1, Ph1704 (18.8 kDa, p*I* 6.1); lane 2, Glutathione *S*-transferase (25.5 kDa, p*I* 6.0); lane 3, bovine serum albumin (66 kDa, pH 4.9); lane 4, Mj HSP16.5 (16.5 kDa, p*I* 5.0); Lane 5, SCM (11.1 kDa, p*I* 8.7).

pure filter into a Microcon 10 concentrator (Millipore Corp., Bedford, MA) that allowed the sample to be concentrated prior to loading onto a sodium dodecyl sulfate–Laemmli polyacrylamide gel (15% SDS–PAGE) (6) for determination of the components of the protein:protein complex band.

Results and discussion. Figure 1 illustrates the migration of proteins of differing molecular weights and p*I*'s on a native 0.8% agarose gel. The migration of the proteins is dependent on their molecular weight, p*I*, and conformation. As an example, Mj HSP16.5, with a molecular weight of 16.5 kDa and a p*I* of 5.0, migrates toward the anode while SCM with a molecular weight of 11 kDa and a p*I* of 8.7 migrates toward the cathode. Mj HSP16.5 is a small heat shock protein that confers thermal protection of other proteins such as SCM at 80°C (4).

To investigate complex formation, Mj HSP16.5 (7.4 μg) and SCM (5 μg) were mixed at a 1:1 molar ratio in 25 mM Hepes, pH 7.5, 0.1 M NaCl at different temperatures for 20 min and spun down at 14,000*g* in a microcentrifuge and the supernatant was loaded on a 0.8% native agarose gel using Buffer A in the reservoir. Figure 2 shows that the apparent complex formation (Band C) increases dramatically at 70 and at 80°C. The

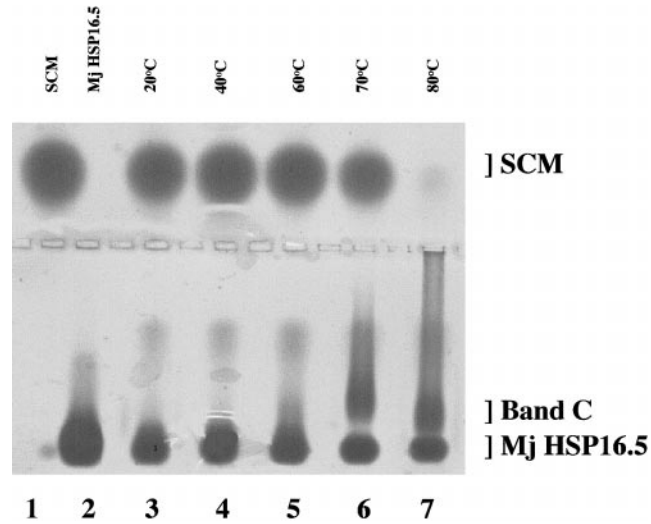

FIG. 2. Analysis of complex formation of Mj HSP16.5 and SCM. Mj HSP16.5 and SCM were incubated at a 1:1 molar ratio in 25 mM Hepes, pH 7.5, 0.1 M NaCl for 20 min at the indicated temperatures and centrifuged 5 min at 14,000*g* and the supernatants were loaded onto a 0.8% native agarose gel as described under Materials and Methods. Lane 1, SCM (5 μg); lane 2, Mj HSP16.5 (7.4 μg); Mj HSP16.5:SCM (1:1 molar ratio); lane 3, 20°C; lane 4, 40°C; lane 5, 60°C; lane 6, 70°C, lane 7, 80°C. Right margin indicates the complex (Band C) and the Mj HSP16.5 band.

SCM band is no longer present in an unbound form after 80°C incubation with Mj HSP16.5. SCM is resistant to precipitation up to 65°C (data not shown) suggesting that Mj HSP16.5 interacts with SCM when it is in a denatured state. Note that as the temperature of incubation is increased the amount of uncomplexed Mj HSP16.5 decreases as more Band C is formed.

To ascertain whether Band C is indeed a complex, we ran the 70°C heated sample of Mj HSP16.5 and SCM on two lanes of a gel, stained one lane, and used those

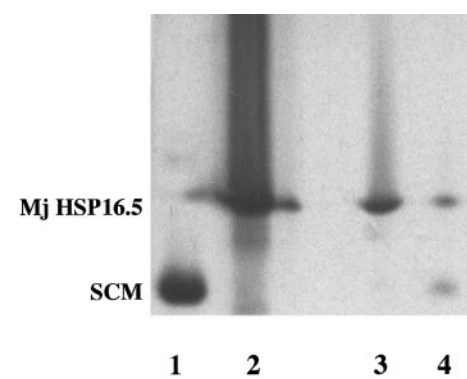

FIG. 3. Extraction of Band C from a native agarose gel. Isolated Band C and the Mj HSP16.5 band from a native agarose gel were extracted according to Materials and Methods and analyzed on a 15% SDS–PAGE gel. Lane 1, SCM (5 μg); lane 2, Mj HSP16.5 (5 μg); lane 3, extracted Mj HSP16.5 band; lane 4, extracted Band C.

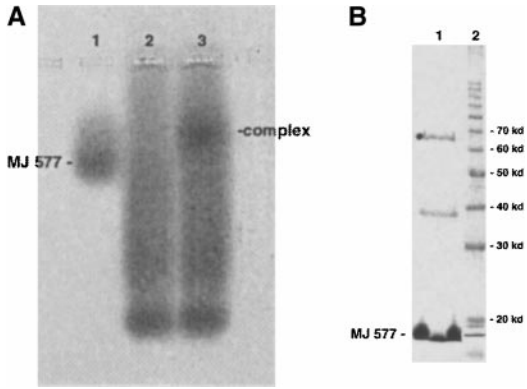

FIG. 4. Analysis of complex formation of Mj0577 and *M. jannaschii* crude extract. (A) Mj0577 (6 μg) and *M. jannaschii* crude extract (45 μg) were incubated together in the presence of 5 mM $MgCl_2$ and 2.5 mM $MnCl_2$ at 80°C for 30 min. The sample was centrifuged 5 min at 14,000*g* and the supernatant loaded onto a 0.8% native agarose gel as described under Materials and Methods. Lane 1, Mj0577 (6 μg) heated at 80°C; lane 2, *M. jannaschii* crude extract (45 μg), heated at 80°C; lane 3, Mj0577 (6 μg): *M. jannaschii* crude extract (45 μg), heated at 80°C and centrifuged and the supernatant loaded on the gel. Presence of Mj0577 and complex band are indicated. (B) Extraction of the complex band was performed as described under Materials and Methods and analyzed on a 15% SDS–PAGE gel. Lane 1, extracted complex band; lane 2, 10-kDa protein standard.

bands as markers to cut the corresponding Band C as well as the Mj HSP16.5 band from the unstained lane. The proteins were extracted from the gel as described under Materials and Methods and were analyzed on a 15% SDS–PAGE gel. Figure 3 shows that Band C (Lane 4) is composed of both Mj HSP16.5 and SCM while the band corresponding to unbound Mj HSP16.5 only contains the small heat shock protein (Lane 3). This same approach has been used to isolate proteins that bind to Mj0577, a hyperthermophilic protein whose structure has been determined in our laboratory as part of a structural genomics project (5). The structure contained a bound ATP and was shown to hydrolyze ATP in the presence of a *M. jannaschii* cell extract. Figure 4 shows the formation of a complex upon incubation of Mj0577 and the *M. jannaschii* cell extract at 80°C. When the complex band was isolated and run on a 15% SDS–PAGE gel, two proteins (38 and 65 kDa in size) in addition to Mj0577 could be identified. Further characterization of these two proteins is in progress.

Conclusion. This study has shown that a native agarose gel can be used to analyze both acidic and basic proteins and is especially useful for studies of protein: protein complexes. Since all components involved in complex formation can be visualized on a single lane, variables such as stoichiometry, buffers, and incubation conditions can be readily assessed. These complexes can be easily extracted from the agarose gel and then run on an SDS–PAGE gel for further analysis. The rapidity and ease of this method compared to other previously described ones may facilitate studies of protein:protein complex formation.

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Phosphohistidine Analysis Using Reversed-Phase Thin-Layer Chromatography[1]

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Proteins phosphorylated on histidine residues which are common to bacteria as part of the two-component signaling mechanism (1) are also becoming of increasing interest in eukaryotic signaling pathways (2). Many of these proteins are phosphorylated transiently on histidine, with the phosphate moiety subsequently

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