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Analytical Biochemistry 352 (2006) 296–298
ANALYTICAL BIOCHEMISTRY
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Notes & Tips

Detecting disulfide crosslinks of high-molecular weight complexes in mouse SVS proteins by diagonal electrophoresis

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Received 9 November 2005
Available online 20 March 2006

Since Brown and Hartley first developed two-dimensional electrophoresis on a paper sheet to examine the interpolypeptide disulfide linkage of chymotrypsinogen A in 1966 [1], diagonal electrophoresis has become one of the most useful methods for the protein study in this regard. In 1974, Sommer and Traut extended the concept to diagonal polyacrylamide–dodecyl sulfate electrophoresis for identification of ribosomal proteins crosslinked with methyl-4-mercaptobutyrimidate [2], with their protocol being generally followed since that time. Briefly, one strip of gel containing the protein sample is cut from the gel slab of first-dimensional electrophoresis being conducted under a nonreducing condition. After treatment with a disulfide-scissoring reagent, the gel is embedded into another polyacrylamide gel slab for the second-dimensional electrophoresis under a reducing condition. The resultant protein samples containing no interpolypeptide disulfide bond(s) will lie in a diagonal line on the second-dimensional gel slab, whereas a reduced sample will be resolved into smaller protein components. Given the various forms of high-molecular protein complexes (HMPCs)[1] in mouse seminal vesicle secretion (SVS) [3], we attempted to apply this conventional method to study the detail of interpolypeptide disulfide crosslinks of each HMPC. We discovered, however, that clear identification of HMPC from the protein spots on the gel slab of second-dimensional electrophoresis is somewhat problematic. In their study of rat SVS proteins in 1987, Wagner and Kistler reported similarly [4]. In this article, we present a modified method that improves the effectiveness of the established version.

Mouse SVS was collected from outbred ICR mice according to the established procedure [5–7]. The method of Laemmli was adopted to prepare the protein samples for SDS–PAGE [8]. Approximately 20 μg of SVS was dissolved in a sample buffer containing 2% SDS, 10% glycerol, 0.005% bromophenol blue, and 100 mM dithiothreitol (DTT) in 50 mM Tris–HCl at pH 6.8. The sample solution was heated at 95 °C for 5 min before loading for SDS–PAGE. According to the previous study [7], the major protein components in mouse SVS were designated SVS I (92 kDa), SVS II (38 kDa), SVS III (34 kDa), SVS IV (15 kDa), and SVS V (14 kDa), as well as a minor (and then unreported) protein component SVSP55 (55 kDa), based on their mobility of SDS–PAGE on a 14% gel slab under a reducing condition (Fig. 1A, lane 2). Meanwhile, SDS–PAGE was performed for the protein sample prepared in the sample buffer devoid of DTT on a 6% polyacrylamide slab under nonreducing conditions to improve resolution of the HMPC variants in mouse SVS. We found that distortion of the protein bands on the soft gel often occurred, in particular bending around their horizontal edge. This resulted in two bothersome disadvantages. First, further resolution of such distorted bands by second-dimensional electrophoresis produced tailing spots on the gel slab. This was problematic in terms of the assignment of HMPC components. Second, there were technical difficulties not only in cutting the protein-containing strip from the first-dimensional soft gel slab but also in inserting such a soft strip into the chamber for second-dimensional electrophoresis.

The above shortcomings can be rectified when the first-dimensional electrophoresis is performed on a gel slab prepared in an apparatus constructed using two plain glass plates, one of which is attached to a Gel Bond PAG film (Cambrex Bio Science, Rockland, ME, USA), which serves as a gel supporter. It was found that gels prepared from acrylamide concentrations as low as 4% still strongly adhere

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[1] *Abbreviations used:* HMPC, high-molecular protein complex; SVS, seminal vesicle secretion; DTT, dithiothreitol.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.03.005

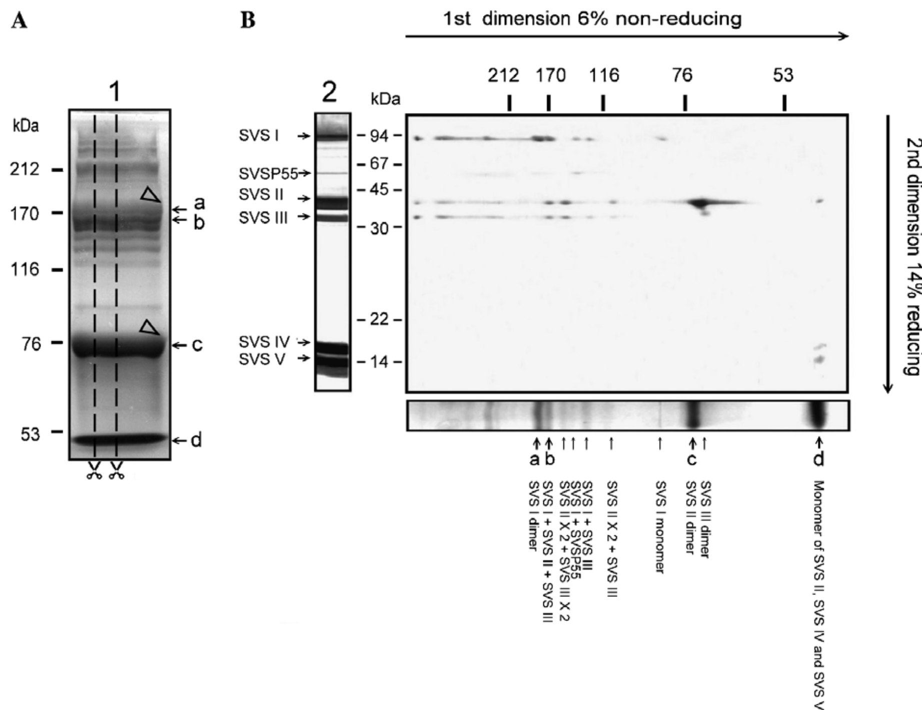

Fig. 1. Easy manipulation and high-resolution diagonal electrophoresis. (A) The protein components of mouse SVS (20 μg) were resolved by SDS–PAGE on a 14% gel slab under reducing conditions (lane 2). Alternatively, the electrophoresis was conducted on a Gel Bond film-supporting 6% gel slab under nonreducing conditions (lane 1). The film-supporting gel was stained and dried as described in the text. The arced edge and tailing of a protein band are indicated by open arrowheads. A strip of gel containing the distortion-free protein bands was scissored from the film-supporting gel slab (dashed lines of lane 1) for the second-dimensional electrophoresis, which was performed on 14% SDS–PAGE (8 × 10 cm) under reducing conditions (B). The major HMPC bands on the first dimension are denoted a–d, and the composition of each nonreducing protein band, denoted on the bottom of the first-dimensional gel, is revealed from the silver-stained protein spots on the two-dimensional gel pattern.

to the film. The electrophoresis of the SVS proteins was conducted on a film-supporting 6% polyacrylamide slab without disulfide reduction at 15 V/cm for 60 min. Without prefixation of the protein bands and after electrophoresis, the film adherent gel was directly washed twice with distilled water for 10 min, followed by staining with GelCode Blue reagent (Pierce, IL, USA) to reveal the protein band. As shown in lane 1 of Fig. 1A, the various forms of HMPC were well resolved. Four major bands emerged from the first-dimensional electrophoresis: a (184 kDa), b (164 kDa), c (76 kDa), and d (<53 kDa, appearing around the button of the gel slab). Minor bands bearing M_r values greater than 76 kDa also emerged. The appearance of various nonreduced protein bands that have M_r values greater than any of the reduced protein components manifests the complication of intermolecular disulfide bridges among the SVS protein components. Before the second-dimensional electrophoresis, the gel was dried overnight on the film at 37 °C after soaking in 2% glycerol for 1 h. This provides three technical advantages over the conventional second-dimensional method. First, the dried gel can be stored for more than 1 month without distortion of the protein bands. Second, the deformed portion around the horizontal edges of the stained protein bands could be scissored out easily before the second-dimensional electrophoresis (see lane 1 of Fig. 1A). Third, rehydration of the dried gel and the reduction reaction for the protein bands could proceed simultaneously. For the disulfide reduction, the dried gel was soaked in a reaction buffer containing 6 M urea, 20% glycerol, 2% SDS, 0.005% bromophenol blue, and 100 mM DTT in 50 mM Tris–HCl (pH 6.8) for 1 h at room temperature. After the reduction, the gel was incubated in the reaction buffer, in which DTT was replaced by 135 mM iodoacetamide to block the thio group. This treatment does not cause detachment of the polyacrylamide gel from the supporting film, facilitating its insertion into the chamber for second-dimensional electrophoresis.

The protein bands on the film-supporting gel were reduced as outlined above, and their components were resolved by second-dimensional electrophoresis on a 14% polyacrylamide slab under reducing conditions. After the gel slab was stained with silver nitrate, the protein spots on the two-dimensional gel pattern were clearly revealed (Fig. 1B). The unequivocal assignment of molecular size for the protein spots enabled confident elucidation of the interpolypeptide disulfide crosslinks in each HMPC, as shown at the bottom of Fig. 1B. There are no cysteine residues in SVS IV and SVS V, there is one cysteine residue in SVS II, there are three cysteine residues in SVS III, and there are six cysteine residues in SVS I. Band d contains mainly SVS II, SVS IV, and SVS V, suggesting no disulfide crosslinking among these three proteins. The interpolypeptide disulfide crosslinking among SVS I, SVS II, and SVS III may produce various forms of protein complexes. This is demonstrated by the presence of the homo-polypeptide dimers of SVS I (band a), SVS II (band c),

and SVS III; the hetero-polypeptide complexes formed by two SVS II plus one SVS III, one SVS I plus one SVS III, two SVS II plus two SVS III, one SVS I plus one SVS II plus SVS III (band b), one SVS I plus one SVSP55; and the even larger complexes with M_r values greater than 212 kDa formed by unknown stoichiometric ratios of SVS I, SVS II, and SVS III. These data provide a sound basis for future study to identify the roles of polypeptide complexes in semen coagulation that are important for the reproduction of a substantiated number of mammalian species [3].

In summary, the protocol reported here facilitates physical manipulation of a single soft gel prepared from a low concentration of acrylamide, providing good-quality identification of the high-molecular weight complexes formed by disulfide bridges. Our work may help to improve the reliability and applicability of diagonal electrophoresis.

Acknowledgments

This work was supported in part by Grants NSC94-2311-B-002-004 and NSC94-2311-B-001-016 from the National Sciences Council (Taiwan).

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