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Advanced negative detection method comparable to silver stain for SDS-PAGE separated proteins detection

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ABSTRACT

In order to achieve an easy, rapid and sensitive protocol to detect proteins in polyacrylamide gel, an advanced negative detection method comparable to silver stain is described. When a gel was incubated with Phloxine B and followed by the development in acidic solution, the zones where forming protein-dye complex were selectively transparent, unlike opaque gel background. Within 50 min after electrophoresis, down to 0.1–0.4 ng of gel-separated proteins (similar with silver stain) could be observed, without labor-intensive and time-consuming procedure. Comparing with the most common negative stain method, Imidazole-zinc stain, Phloxine B stain has been shown higher sensitivity and distinct contrast between the transparent protein bands/spots and opaque background than those; furthermore, it is no longer necessary to concern about retention time of observation. This technique may provide a sensitive and practical choice for proteomics researches.

Introduction

Gel-based proteomics is an easy and useful method for protein separation, which provide the efficiency of large scale protein expression screening, and identification and quantification of thousands proteins in a sample. For decades, SDS-PAGE has been paired with chromogenic dye-based detection methods such as coomassie brilliant blue (CBB) or silver staining. However, the time-consuming and labor-intensive staining cannot keep up with the rapid growth of proteomics. As a choice of the proper visualization method for gel-separated proteins, a proteomics compatible negative stain was developed [1,2]. Imidazole-Zinc (IZ) staining has been one of the most common negative detection methods, which is forming precipitates on the surface of gels except protein bands binding with a metallic salt. However, it is not easy to differentiate the color contrast between the transparent protein bands/spots and the opaque background, which relies on the subtle color shift. This method has a short retention time of observation and difficulty in storage, although it can detect proteins on SDS-PAGE within a short time [3]. Negative staining has improved following researches using eosin Y or eosin B [4,5], which provides a higher sensitivity and contrast as compared to the prior detection methods.

In this study, we have taken a great step forward through using a new xanthene derivative reagent, phloxine B (PB), as a rational substitute. PB is used as an additive for foods, drugs and cosmetics [6], and for Gram staining [7]. PB, which is one of xanthene dyes similar to eosin Y and eosin B, has been proved to be a useful class of luminescent and triplet forming dyes [8]. However, it differs from fluorescein, eosin Y and eosin B by the presence four bromine atoms in the xanthene ring and four chlorine atoms in the carboxyphenyl ring (Fig. 1). Due to these characteristics of PB different from eosin Y and eosin B, PB staining is achieved remarkable effect on sensitivity and color contrast between protein bands and background.

The negative staining method may advance considerably through this novel PB stain, which allows high sensitivity and resolution of protein bands on the gel as those of silver staining. As indicated in Fig. 2, within 50 min after electrophoresis, down to 0.1–0.4 ng of proteins could be observed similar to the ultrasensitive silver stain without labor-intensive and time-consuming procedure (Table 1). Furthermore, a sensitivity of PB stain is about 5–10-fold higher than that of the representative negative staining method, IZ stain, and about 60–320-fold higher than that of CBBR stain.

Abbreviations used: DW, deionized water; EtOH, ethanol; HAc, acetic acid; IZ, imidazole/zinc; MeOH, methanol; PB, phloxine B.

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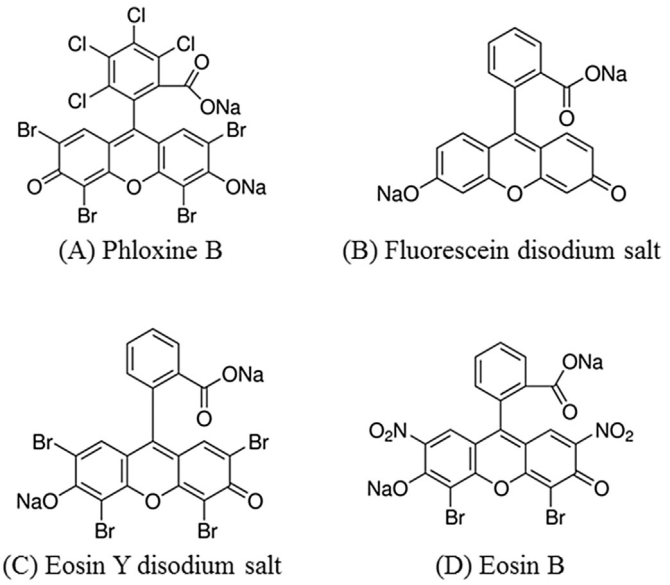

Fig. 1. Structures of PB and fluorescein derivatives.

Materials and methods

Materials

Acrylamide, Bis, APS, TEMED, Tris base, glycine, SDS, glycerol, CHAPS, bromophenol blue, β-galactosidase (E.coli), phosphorylase b (rabbit muscle), BSA (bovine), OVA (egg), carbonic anhydrase (bovine erythrocytes), PB (Cat. #P4030), imidazole, and zinc sulfate were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO, USA). CHAPS, DTT, urea, IPG strip, cover oil, IPG buffer and Silver staining kit for protein was purchased from GE Healthcare™ (Uppsala, Sweden). All other reagents and chemicals used were of analytical grade and were obtained from various commercial sources.

Preparation and separation of protein samples in SDS-PAGE

Each protein standard marker was dissolved in 1 × sample buffer containing 60 mM Tris (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, and 2% (v/v) β-mercaptoethanol, and then heated at 100 °C for 5 min in a boiling water bath. For 2D electrophoresis, practical protein samples, Bosc23 cells were harvested by centrifugation at 3000 rpm for 10 min, consecutively washed thrice with ice-cold PBS and lysed on ice in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, and 0.4 mM PMSF. The cells were then sonicated 5 times for 1 min each, and were centrifuged at 15000 rpm for 20 min at 4 °C. The protein amount in the supernatant was determined by Bradford's method using the Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, CA, USA).

1-D Electrophoresis was carried out on polyacrylamide gels (60 × 80 × 0.75 mm) using the discontinuous buffer system [9]. For electrophoresis, protein mixture was serially diluted with sample buffer containing 0.1% (w/v) bromophenol blue, and loaded into the gel lanes composed of a 4% stacking gel and an 11% separating gel with an acrylamide: Bis ratio of 30: 0.8. The gel was electrophoresed at a constant current of 22 mA per slab gel in 1 × running buffer (0.025 M Tris, 0.2 M glycine, and 0.1% SDS) using a Miniprotein III dual slab cell (Bio-Rad Lab, Hercules, CA, USA) and a Power PAC 300 (Bio-Rad). 2-D electrophoresis was performed according to Cong et al. [4].

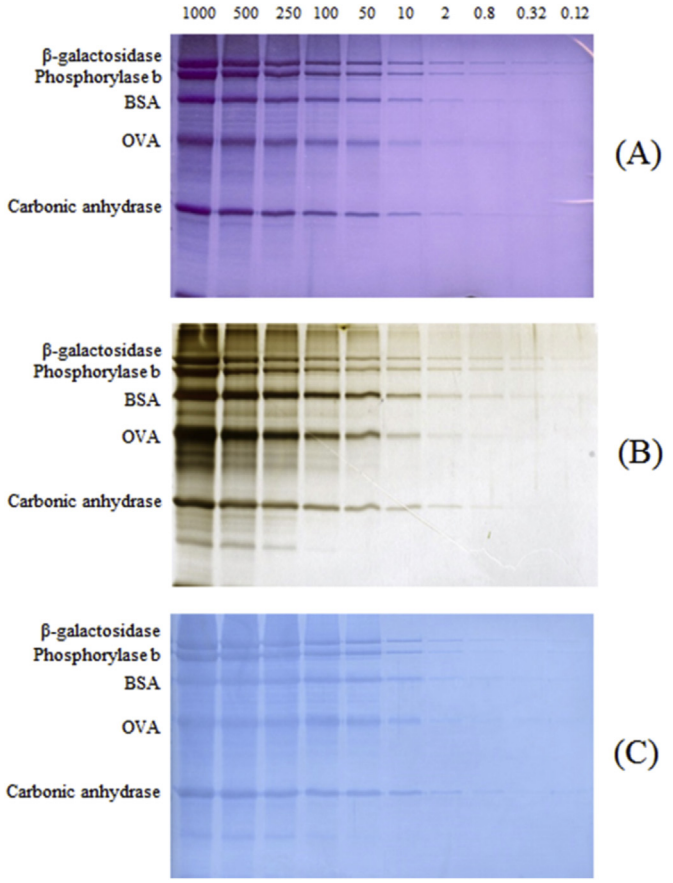

Fig. 2. Comparisons of the sensitivity of PB stain with commercial silver and IZ stain in 1-D SDS-PAGE. The amounts of each standard marker protein (β-galactosidase, phosphorylase b, BSA, OVA, carbonic anhydrase) are as follows: lane (1) 1000; (2) 500; (3) 250; (4) 100; (5) 50; (6) 10; (7) 2; (8) 0.8; (9) 0.3; (10) 0.1 ng/band. (A) PB stain; (B) silver stain; (C) IZ stain.

Protein staining

PB stain

After electrophoresis, a gel was fixed in 100 mL of 50% (v/v) ethanol (EtOH) with 10% (v/v) acetic acid (HAc) for 20 min. Then, the gel was washed with 100 mL of 50% (v/v) methanol (MeOH) for 5 min twice, followed by 15 min incubation in 50 mL of PB staining solution. Finally, the gel was developed in 100 mL of 0.4% (v/v) HAc to visualize the transparent protein bands/spots on an opaque background. PB staining solution was prepared at a concentration of 0.3% (w/v) PB in 50% (v/v) MeOH as a working solution. In each case, the solutions of this study were prepared immediately before using.

Silver stain

The staining method using glutaraldehyde as a sensitizer was performed according to the manufacturer's instructions (GE Healthcare™). Briefly, after electrophoresis, a gel was fixed in 125 mL of 40% (v/v) EtOH, 10% (v/v) HAc solution for 30 min, and reacted in 125 mL of 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulfate and glutaraldehyde solution for 30 min, and then washed in 125 mL DW for 5 min thrice. The gel was then impregnated in 125 mL of 0.25% (w/v) silver nitrate solution for 20 min, washed in 125 mL DW for 1 min twice, and immersed in 125 mL of 3% (w/v) sodium carbonate with formaldehyde solution. After silver ion reduction, the gel was immersed in 125 mL of 1.5% (w/v) EDTA