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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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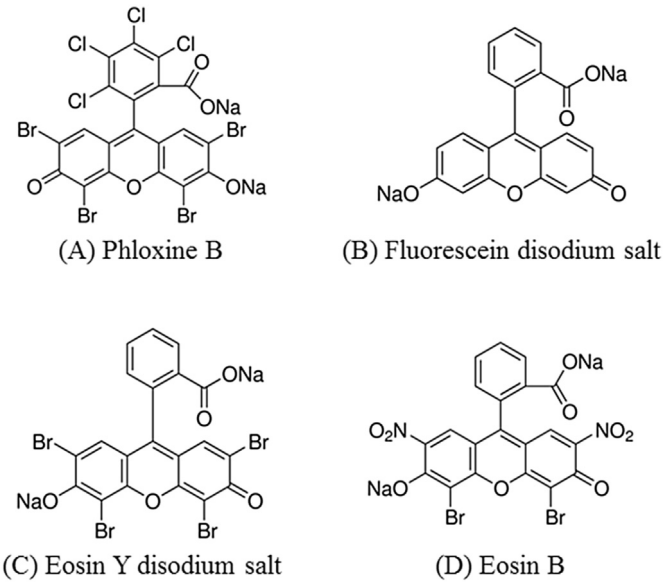

Fig. 1. Structures of PB and fluorescein derivatives.

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].

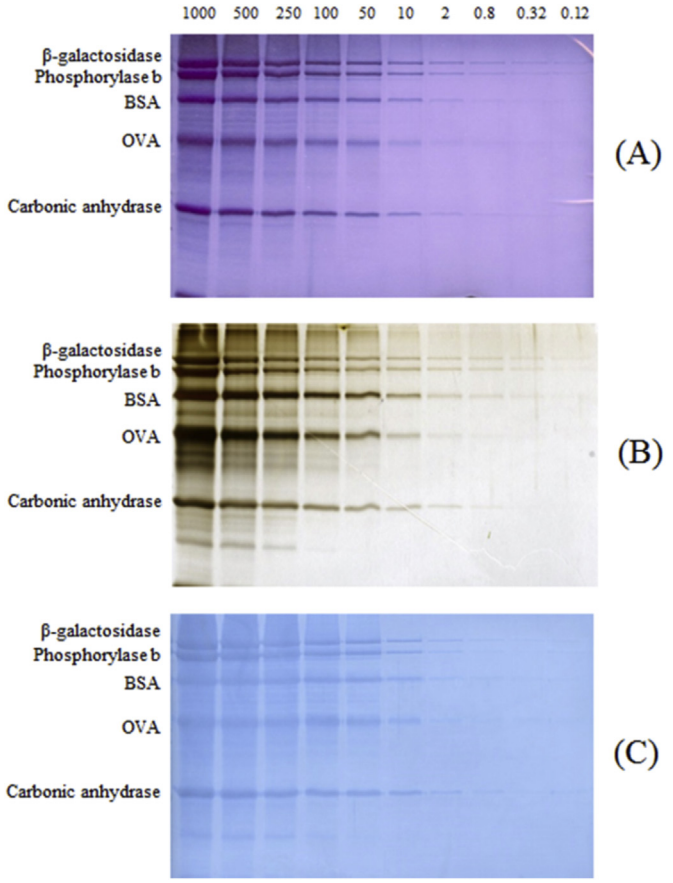

Fig. 2. Comparisons of the sensitivity of PB stain with commercial silver and IZ stain in 1-D SDS-PAGE. The amounts of each standard marker protein (β-galactosidase, phosphorylase b, BSA, OVA, carbonic anhydrase) are as follows: lane (1) 1000; (2) 500; (3) 250; (4) 100; (5) 50; (6) 10; (7) 2; (8) 0.8;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

Table 1
Comparison of the characteristics of PB stain with IZ, EY and silver stain.

	PB staining	IZ staining	Silver staining
Fix	20 min	–	30 min
Enhance	–	–	30 min
Wash	5 min × 2	30 s	5 min × 3
Incubate	15 min	15 min	20 min
Stain	–	–	–
Wash	–	–	1 min × 2
Develop	~2 min	30 s	~15 min (to stop)
Total Steps	5 steps	3 steps	10 steps
Total Times	47 min	16 min	110 min
Sensitivity	0.1–0.4 ng	0.8–2 ng	0.1–0.8 ng
Retention Time	Several hours	1–2 min	Several hours

for 10 min to stop development.

IZ stain

IZ staining was essentially according to Castellanos-Serra et al. [2]. After electrophoresis, a gel was rinsed in DW for 30 s and then incubated in 50 mL of 0.2 M imidazole, 0.1% (w/v) SDS solution for 15 min. Once completed, the solutions were discarded and the gel was incubated in 50 mL of 0.2 M zinc sulfate solution until the protein bands become transparent on a deep white background (~30 s). Finally, the incubation was stopped by rinsing the gel with abundant DW.

Image analysis

The stained gels with negative staining methods were shown up as transparent bands or spots against an opaque background. When the gel is placed on a black board, however, it will be appeared with a positive gel image, which was converted into dark-colored bands or spots against a purple (PB stain) or light blue (IZ stain) background. For PB and IZ image acquisition, therefore, each gel was placed on the scanner (V700, Epson Co., Seiko, Japan) and covered with a black board. All the images were scanned at resolutions of 200 dpi and were adjusted for optimal contrast. On the other hand, the silver-stained gel images were obtained under a white board at 200 dpi resolution with the scanner.

Results

Determination of optimal staining condition

A number of factors such as the concentration of dyes, a kind of solvent, pH, and consumed time may affect the sensitivity and contrast of staining. Considering these factors, the staining process was developed as following 4 steps: fixing, washing, incubation, and development. In this study, several experiments have been conducted to determine the optimal staining condition, and we compared sensitivity and contrast from the results obtained when gels were under different conditions. The effects with changing of staining condition were shown in supporting information figures. For better results, protein-separated gels were fixed and washed to remove substances (such as buffer or SDS) that interfere with staining. Furthermore, fixing is also necessary to prevent proteins from diffusing out of the gel. Therefore, an electrophoresed gel is typically fixed in aqueous solution containing organic solvents such as MeOH, EtOH and HAc. In this study, the gels were fixed in 50% (v/v) EtOH and 10% (v/v) HAc solution for 20 min and washed with 50% (v/v) MeOH for 5 min twice. Comparing the results obtained with or without fixing and washing step, the processes are necessary to increase the sensitivity and contrast (supporting information Fig. 1). After fixing and washing, the gels were incubated with 0.3% (w/v) PB in 50% (v/v) MeOH. According to the result, the proteins were detected by pink color at a concentration less than 0.1% (w/v) PB, which is shown similar sensitivity to that of CBBR stain. In contrast, when the concentration of PB is increased to 0.3% (w/v) or higher, the proteins were changed into transparent in development step (supporting information Fig. 2). In order to maximize the staining performance, PB was dissolved in 50% (v/v) MeOH solution to allow effective infiltration of the dye into the gel matrix (supporting information Fig. 3). The developing step was conducted by employing HAc in varying concentrations from 0.01% (v/v) to 1.6% (v/v) (supporting information Fig. 4). As shown in supporting information Fig. 5A, the gel was all orange-red color before development because PB is bound with the proteins and gel background. According to the result, the HAc in the developing solution is the important ingredient, which allows protein bands to clear. There was distinct improvement in the transparency of proteins by increasing the concentration of HAc. However, increasing more than 0.8% (v/v) HAc resulted in a slight decrease in the sensitivity, then, the stained gel was developed with 0.4% (v/v) aqueous HAc solution. When the gel was placed on scanner and covered with a board, in addition, there was a big difference between white and black board (supporting information Fig. 5B and C). Consequently, it enables the detection for proteins within 50 min without long time destaining. The optimized protocol of PB

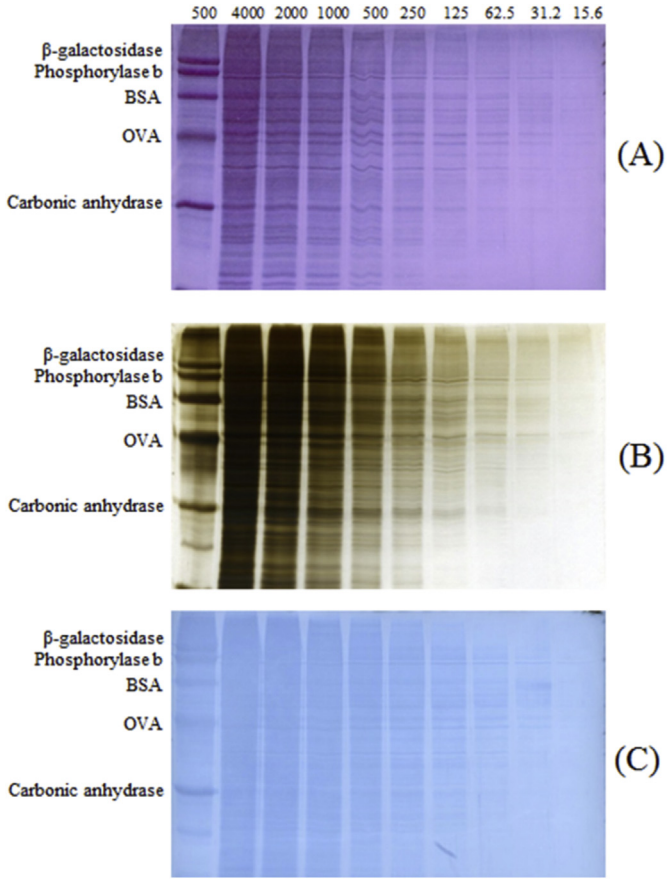

Fig. 3. Comparisons of the sensitivity of PB stain with commercial silver and IZ stain in 1-D SDS-PAGE with total cell proteins of Bosc23. The amounts of Bosc23 are as follows: lane (1) 500 ng of standard marker protein; (2) 4000; (3) 2000; (4) 1000; (5) 500; (6) 250; (7) 125; (8) 62; (9) 31; (10) 15 ng/lane. (A) PB stain; (B) silver stain; (C) IZ stain.

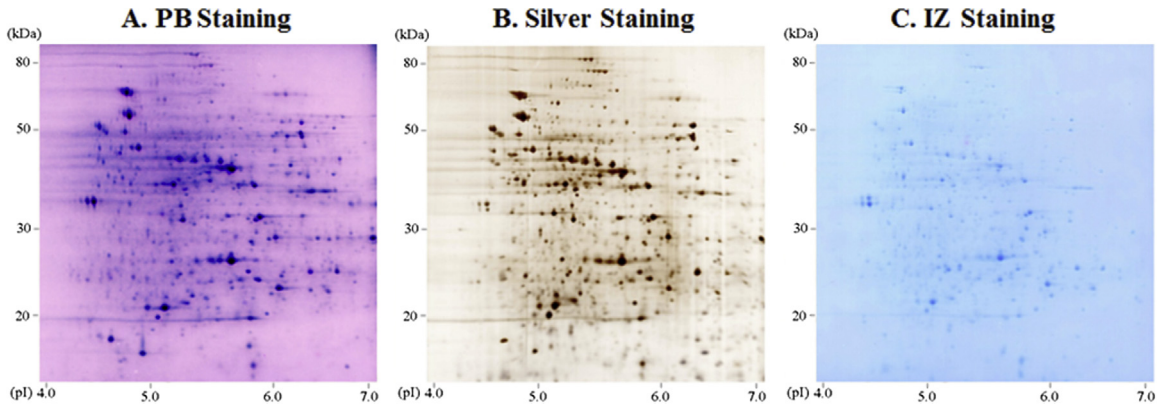

Fig. 4. Comparisons of the sensitivity of PB stain with commercial silver and IZ stain in 2-D SDS-PAGE with total cell proteins of Bosc23. 50 μg of total cell proteins were resolved in 4–7 linear pH gradient (IPG strips; 130 mm) and 12.5% SDS-PAGE (1500 × 1500 × 1 mm) (A) PB stain; (B) silver stain; (C) IZ stain.

stain is shown in Table 1.

Protein detection in SDS-PAGE

To compare the sensitivity with other stains, the mixture of five different protein marker (β-galactosidase, phosphorylase b, BSA, OVA, and carbonic anhydrase) was loaded on SDS-PAGE mini-gels ranging from 0.12 ng to 1000 ng, separated by 1-D and stained with PB, commercial silver and IZ staining, respectively. As can be seen in Fig. 2, the sensitivity of PB staining was 0.1 ng of β-galactosidase and phosphorylase b, 0.4 ng of BSA, OVA, and carbonic anhydrase, respectively. Comparing with other two staining techniques, PB stain offers the sensitivity of down to 0.1–0.4 ng of proteins similar to that obtained by the ultrasensitive silver stain. Furthermore, it is 5–10-fold more sensitive than IZ stain that detect 1–2 ng of the same proteins. The contrast between protein bands and the opaque background was clearly observed by PB staining, while IZ staining was shown a relatively hazy resolution.

In addition, to confirm sensitivities and compare protein-staining patterns, total proteins from Bosc23 were separated by 1-DE and stained by different methods, respectively. In Fig. 3, we may further conclude that PB provides a better sensitivity, resolution and color contrast than silver stain and IZ stain. 2-DE of this new method was further demonstrated (Fig. 4), and the results are consistent with that of 1-DE.

Reproducibility

The reproducibility of PB and silver stains were performed by consecutive staining for five pieces of differently separated 1-DE. The band intensities of five proteins (at 500 ng and 100 ng per band) were estimated. According to the result, the consistent staining intensities from gel to gel were obtained both by PB and silver stains. The values of the RSD varied from 0.88% to 3.33% for PB staining compared with 0.70%–3.29% obtained by silver staining, which indicate an acceptable degree of reproducibility (Table 2).

Table 2
The reproducibility of each protein stained by PB and commercial silver.

No. protein ID		RSD (%)			
		500 ng (n = 5)		100 ng (n = 5)	
		PB	Commercial silver	PB	Commercial silver
1	β-Galactosidase	1.29	3.29	1.55	3.09
2	Phosphorylase b	0.88	1.25	1.54	2.79
3	BSA	0.94	1.01	2.22	1.55
4	OVA	1.42	2.11	3.33	1.37
5	Carbonic anhydrase	1.12	2.13	1.55	0.70

Discussion

Xanthene dyes exist as a variety of neutral and ionic forms in solution. The properties of each form are highly dependent on the local environment of the dye such as temperature, pH, solvent, concentration and other factors [10–14].

PB differs from fluorescein, eosin Y and eosin B by the presence four bromine atoms at positions 2, 4, 5 and 7 of the xanthene ring and four chlorine atoms in the carboxyphenyl ring (Fig. 1). Due to these differences attached to the xanthene skeleton, the color chemical properties of PB dye differ significantly from eosin Y and eosin B. Indeed, PB is used an acid/base indicator that undergoes a color change based on its pKa. PB has a pKa of around pH 2.9, thereby, PB is colorless (neutral form) below about pH 2, while, red (ionic form) above about pH 3.3.

In this study, ionic PB can form a stable water–soluble complex with the proteins, and then be converted into its neutral form under acidic condition. PB coupled with the proteins was shown transparent bands/spots, while PB in the gel matrix was water-insoluble form and precipitated with an opaque background. Therefore, proteins contacting with PB in gels were detectable via a color change from red to transparent at a pH below about 2. Taking into account the property of this dye, PB was successfully introduced in negative stain method for protein detection, and achieved remarkable effect on color contrast between proteins and background.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.07.015.

Conflicts of interest

The authors have declared no conflict of interest.

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