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Fast Protein Staining in Sodium Dodecyl Sulfate Polyacrylamide Gel using Counter ion-Dyes, Coomassie Brilliant Blue R-250 and Neutral Red

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A fast and sensitive protein staining method in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using both an acidic dye, Coomassie Brilliant Blue R-250 (CBBR) and a basic dye, Neutral Red (NR) is described. It is based on a counter ion-dye staining technique that employs oppositely charged two dyes to form an ion-pair complex. The selective binding of the free dye molecules to proteins in an acidic solution enhances the staining effect of CBBR on protein bands, and also reduces gel background. It is a rapid staining procedure, involving fixing and staining steps with short destaining that are completed in about 1 h. As the result, it showed two to fourfold increase in sensitivity comparing with CBBR staining. The stained protein bands can be visualized at the same time of staining.

Key words: Protein stain, Polyacrylamide gel, Electrophoresis, Counter ion-dye stain, Coomassie Brilliant Blue R, Neutral Red

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is a widely used technique for the separation and characterization of proteins. The detection method of proteins in PAGE depends upon both the amount and the type of proteins separated in gels. Various methods using organic dyes, silver and fluorescence stain have been developed for the detection of proteins in polyacrylamide gels (Patton W. F., 2002; Wirth P. J., and Romano A, 1995). Among the organic dye stains available, Coomassie brilliant blue R-250 (CBBR, C.I. 42660) is the most popularly used organic dye stain (Merril, 1990). However, the staining/destaining process is time-consuming. To overcome the disadvantages of CBBR stain, colloidal Coomassie brilliant blue G (CBBG) staining has been developed improving the sensitivity up to the range of 8-10 ng (Neuhoff *et al.*, 1985; Neuhoff *et al.*, 1990). However it still takes a long staining time and the relatively complicated ingredients for the formation of colloidal particles makes its use inconvenient. Here we developed a visible dye staining method improved in sensitivity and speed. We have previously reported that a counter ion-dye staining technique reduces an undesirable background stain, which results in high sensitivity as well as fast staining/destaining (Jung *et al.*, 1998; Jung *et al.*, 1998).

In this study, the counter ion-dye staining method using CBBR/NR has been developed to detect proteins in polyacrylamide gels using low concentrations of dye. It has the advantages over the conventional CBBR stain in speed and sensitivity. This method can detect as little as 10-20 ng of protein within about 1 h.

MATERIALS AND METHODS

Chemicals

Acrylamide, *N, N'*-methylenebisacrylamide (Bis), TEMED, ammonium persulfate, Tris base, glycine, sodium dodecyl sulfate (SDS), CBBR, molecular weight marker proteins including myosin heavy chain, galactosidase, phosphorylase b, bovine serum albumin (BSA), ovalbumin (OVA), carbonic anhydrase (CA), tripsin inhibitor (TrpI) and other electrophoresis grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Neutral Red

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(NR) was from Aldrich Chemical Co. (St. Louis, MO, USA). Bradford reagent for a protein assay was purchased from Bio-Rad (Hercules, CA, USA). All other chemicals used were of analytical grade from commercial sources.

Apparatus

Electrophoretic separation was carried out using a Mini-protein II dual slab cell (Bio-Rad Lab., Hercules, CA, USA). Power was supplied by Power PAC 300 or Power PAC 1000 (Bio-Rad Lab., Hercules, CA, USA). For gel staining, gel was shaken by an adjustable tilt rocker (National Labnet Co., Woodbridge, NJ, USA). Gel was dried using a model 583 gel dryer (Bio-Rad Lab., Hercules, CA, USA).

Electrophoresis

Electrophoresis was carried out on polyacrylamide slab gels (60 × 80 × 0.75 mm) containing 0.1% SDS using the discontinuous buffer system of Laemmli (Laemmli, 1970). Protein concentrations were determined by Bradford's method using Bio-Rad protein assay kit with standard I (bovine plasma -globulin) as a standard (Bradford, 1976). Protein samples were dissolved into buffer containing 62 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.005% bromophenol blue. It was heated at 100 for 1 min and then cooled to room temperature before electrophoresis. The running buffer was 0.025 M Tris, 0.2 M glycine, 0.1% SDS, pH 8.3. Stacking gels 4.5%) of 1.5 cm in length was overlaid on separating gel of 10% polyacrylamide with an acrylamide: bisacrylamide ratio of 29.2: 0.8. Gels were run in a Mini-Protein II dual slab cell (Bio-Rad Lab., Hercules, CA, USA). Electrophoresis was performed at constant current of 20 mA per slab gel for 50 min.

Staining of proteins

Coomassie blue R staining

CBBR staining was performed by washing a gel in 50 ml of 40% methanol (MeOH)/7% acetic acid (HAc) for 5 min and staining in 30 ml of 0.25% CBBR in 40% MeOH/7% HAc for 60 min. The gels were destained by two consecutive changes of 40% MeOH/7% HAc solution (50 ml) for 2 to 4 h. Stained gels were dried on a filter paper at 60 for 40 min using a gel dryer.

Counter ion-dye staining

CBBR/NR staining was performed by washing a gel in 50 ml of 40% MeOH/7% HAc for 30 min and staining in 30 ml of 0.005% CBBR/0.0005% NR in 30% MeOH/7% HAc for 30 min. The solution for counter ion-dye stain was prepared by diluting the stock solution of 0.5% CBBR (in 40% MeOH/7% HAc), and 0.1% NR (in H_2O). The concentrations of CBBR and NR were calculated considering their dye content. This working solution should be prepared freshly just before use, because negatively charged CBBR and positively charged NR may have a tendency to precipitate each other. Stained gels were washed to remove excess dye on gel surface with 30% MeOH/7% HAc solution for 5 min and dried on a filter paper at 60 for 40 min using a gel dryer.

Densitometric analysis

Quantitation of the protein bands was performed with a Jet Scan Plus 8-bit scanner (Hewlett-Packard, Corvallis, OR, USA) interfaced to an IBM computer and image analysis was performed using a TINA 2.09 software program (Raytest Co., Straubenhardt, Germany).

RESULTS

Screening of counter ion-dyes

CBBR was selected as an acidic dye containing sulfonic (SO_3^-) groups. As a counter ion-dye of CBBR, the basic dyes containing positively charged amine groups were screened to form an ion pair complex. For a clear background gel matrix, we used relatively low concentrations of dye. Among the counter ion-dyes screened (NR, Rhodamine B, Pyronine Y, Ethyl violet, Methyl violet etc.) NR was selected to be the most competent for the counter ion-dye of CBBR in considering its staining effect, concentration of dye used, and staining time required.

Determination of optimal dye concentrations of CBBR and NR

Molecular weight marker proteins were electrophoresed in 0.75 mm thick 10% polyacrylamide gel. Before staining,

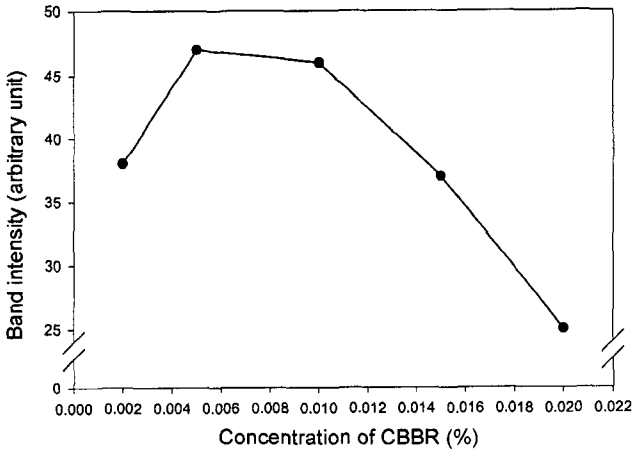

Fig. 1. Optimal CBBR concentration in staining solution. BSA separated on gels was stained with different CBBR concentrations having the range of 0.002-0.02 (w/v) %. Staining time was 30 min. Electrophoresis was carried out on a 0.75 mm thick, 10% SDS-polyacrylamide gel. Stained protein bands were quantified by using a TINA 2.09 software program.

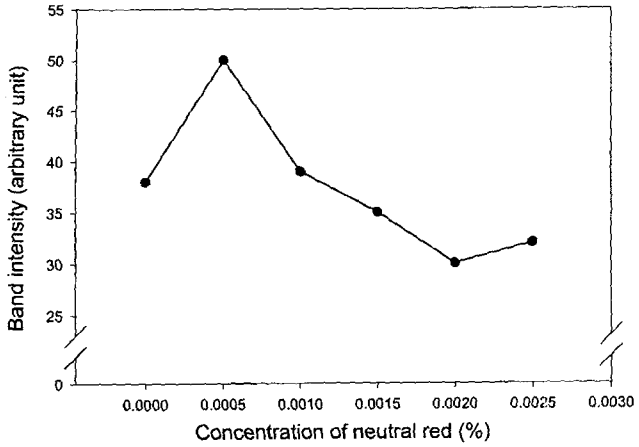

Fig. 2. Optimal NR concentration in staining solution for counter-ion dye staining. BSA separated on gels was stained for 30 min with 0.005 % CBBR solution containing different concentrations of NR. The final NR concentrations were from 0 to 0.0025 (w/v) %. Staining time was 30 min. Electrophoresis was carried out on a 0.75 mm thick, 10% SDS-polyacrylamide gel. Stained protein bands were quantified by using a TINA 2.09 software program.

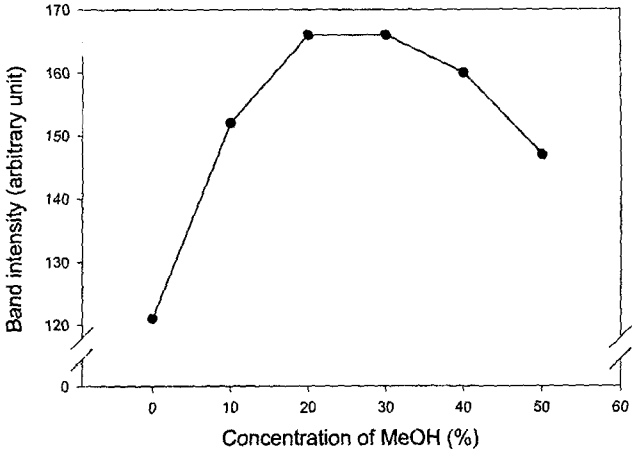

Fig. 3. Optimal MeOH composition in staining solution. BSA separated on gels was stained in optimal CBBR-NR staining solution determined for 30 min with different compositions of MeOH ranged from 0 to 50 (v/v) % with 7 % of HAc. Stained protein bands were quantified by using a TINA 2.09 software program.

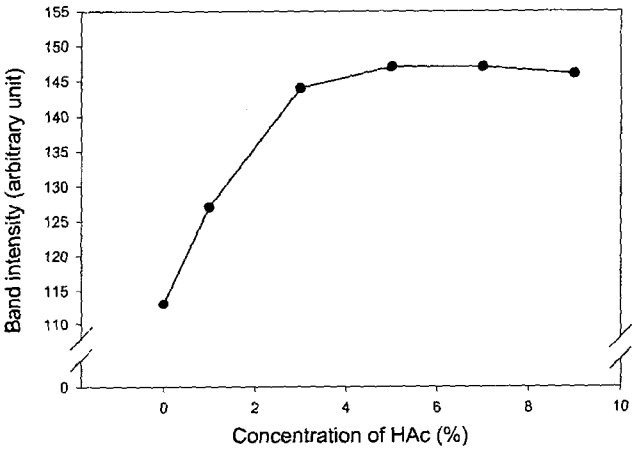

Fig. 4. Optimal HAc composition in staining solution. BSA separated on gels was stained in optimal CBBR-NR staining solution determined for 30 min with different compositions of HAc ranged from 0 to 9 (v/v) % with 30 % of MeOH. Stained protein bands were quantified by using a TINA 2.09 software program.

gels were washed/fixed in 40% MeOH/7% HAc for 30 min. To determine the optimal CBBR concentration, gels were stained with different concentrations of CBBR. Fig. 1 shows a staining effect according to the concentration of CBBR and the concentration of 0.005% was chosen as the optimal concentration considering a sensitivity and a low background. At the higher concentration of CBBR, the background stain was increased. To determine the optimum dye concentration of NR as the counter ion-dye of CBBR, the optimal ratio of CBBR and NR for staining solution was studied. NR stock solution was variably added to the fixed concentration of CBBR (0.005%). Fig. 2 indicates that the optimal concentration of NR in the counter ion-dye staining solution was 0.0005%. At the higher concentration of NR, the staining of protein band was less effective because negatively charged CBBR and positively charged NR had a tendency to precipitate each other. It may indicate that higher concentration of NR inhibits the binding of CBBR to protein bands as well as background gel matrix. At the concentration of NR, 0.0005%, gel background was clear, so it could help to shorten a destaining time.

Determination of optimal solvent composition of staining solution

To determine the compositions of MeOH and HAc in staining solution, gels were stained with the different solvent compositions of staining solution for 30 min. Fig. 3 and 4 illustrate the effects of MeOH and HAc on protein staining. According to the results, intense staining occurred at 30% MeOH and 7% HAc. Therefore, the optimal solvent composition of staining solution for CBBR/NR staining was chosen as 30% MeOH/7% HAc.

Determination of staining time

The total required staining time using CBBR-NR as a counter ion-dye pair was 30 to 60 min with less than 5 min destaining. Microgram ranges of BSA band became visible within several minutes and most of protein band became visible within 15 to 60 min.

Washing effect of gels in counter ion-dye staining

Before the counter ion-dye staining, gels should be washed to remove detergent, SDS, and buffer components which cause a high background of gel matrix (Nivinskas

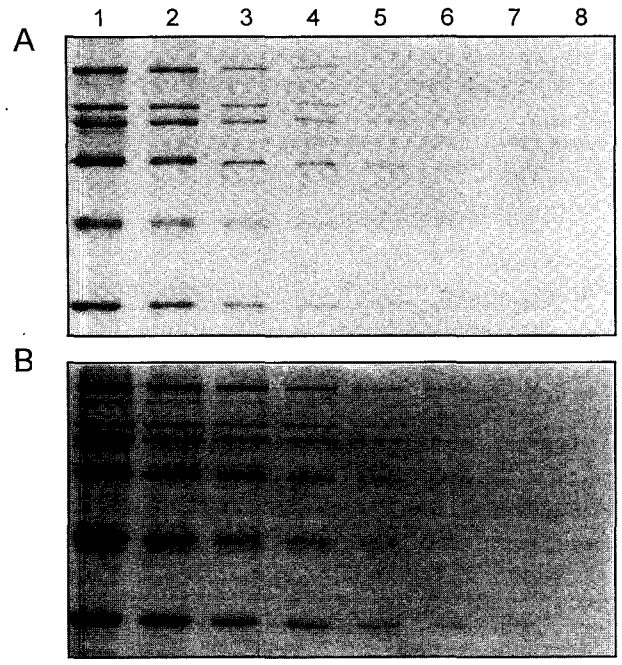

Fig. 5. Comparison of CBBR-NR stain with CBBR stain in protein band pattern and sensitivity with standard molecular mass markers of protein. Each of staining procedures (A) CBBR stain and (B) CBBR-NR stain was performed as described in Materials and methods. The protein mixture containing approximately equal amounts of six protein molecular mass standards were Myosin (205 kDa), -Galactosidase (116 kDa) Phosphorylase b (97.4 kDa), BSA (66 kDa), OVA (45 kDa) and CA (29 kDa) by turns from high molecular weight. Twofold serial dilutions of protein loaded onto the gel (from left to right) were: lane (1) 833; (2) 417; (3) 208; (4) 104; (5) 52; (6) 26; (7) 13, and (8) 7 ng per band respectively.

and Cole, 1996). Therefore gels were washed before staining. The best results were obtained by washing the gel with 50 ml of 40% MeOH/7% HAc for 30 min.

Comparison of CBBR/NR counter ion-dye stain with CBBR stain

The speed and sensitivity of counter ion-dye staining method compared with those of CBBR stain are illustrated in Fig. 5. The counter ion-dye staining method required 30 min for staining and optionally 5 min for washing to remove excess dye. While the staining time of CBBR (0.25%) was 60 min and destaining time was 2-4 hours. The sensitivity of CBBR/NR staining was increased up to 2~4-folds comparing with CBBR staining (Fig. 5). It shows that the protein band pattern of counter ion-dye stain was identical to that of CBBR staining method.

DISCUSSION

The conventional CBBR staining has been used most popularly throughout the years, but long destaining step resulting from strong background stain makes it inconvenient. The process of staining of protein in polyacrylamide gels with CBBR involves: (1) fixation and staining of protein bands (2) removal of the excess dye from gel background using destaining solution. The main drawback of this method is the long staining time necessary for stoichiometric binding of dye to proteins due to the slow diffusion of dye into gel. Similarly, the removal of excess dye from gel matrix is also a slow process (Sreeramulu and Singh, 1995). CBBR binding to proteins is mainly by electrostatic interaction between sulfonic (SO_3^-) group of dye and protonated NH_3^+ groups of proteins at acidic pH. Additionally, van der Waals' forces and hydrogen bonding contribute to the binding interaction between dye and protein. The number of CBBR ligands bound to each protein is approximately proportional to the number of positive charges on protein, about 1.5-3 dye molecules/charge (Tal *et al.*, 1985). Nonspecific binding of CBBR to gel matrix and protein is resulted from the structural similarity between protein and polyacrylamide gel matrix. It makes the selective protein staining on polyacrylamide gel matrix difficult. To improve the staining of protein on polyacrylamide gel in CBBR stain, selective dye binding to protein is necessary. In previous studies (Jung *et al.*, 1998; Jung *et al.*, 1998), it was proven that counter ion-dye staining technique reduces both background coloration and staining/destaining times through the formation of the ion-pair complex between an acidic dye and a basic dye in staining solution. During the counter ion-dye staining, the equilibrium exists between ion-pair complex and free forms of dyes in staining solution. The majority of dyes form an ion-pair complex while very small amount of free dye enters into gel matrix and stains preferentially protein. In addition, the selectivity of dye binding to protein is much improved by using relatively low dye concentrations comparing with other dye stains. In the previous results published (Jung *et al.*, 1998; Jung *et al.*, 1998), the amounts of dye used were much higher than those of CBBR/NR stain. As a result, destaining step was necessary even though the time was 20-30 min short because of the counter ion dye effect. Therefore in CBBR/NR stain, low dye concentrations used made destaining step unnecessary improving the sensitivity. It has been published that colloidal property of CBBG shows a sensitive and selective protein stain for polyacrylamide gels (Neuhoff *et al.*, 1985). Colloidal CBBG staining produces selective protein stain on a clear gel background. During the staining, equilibrium exists between colloidal and molecular dispersed forms of CBBG. The dispersed form of dye molecule enters gel matrix and stains proteins preferentially while colloidal form is excluded, avoiding background stain. However it still takes a long staining

time (at least for 3 h). Neutral red (*N, N*, 3-trimethyl-2,8-phenazinediamine) has been used for histological stain as 0.1% aqueous solution. It has been used for photometric determination of SCN^-, ClO_4^-, and Hg (Tsubouchi, 1971). It is a cationic basic dye that has a quaternary ammonium group and easily forms an ion-pair complex with CBBR. In CBBR/NR staining solution, CBBR and NR may form a colloidal state as in CBBG through an ion pair complex between two dyes. Therefore selective staining of proteins without background staining of gel may be possible improving the detection limit of proteins.

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