An improved Coomassie Brilliant Blue (CBB R-250) staining to proteins in gels

ZHOU Zhi-Dong[1,2], LIU Wang-Yi[2], LI Min-Qian[1]

([1] *Shanghai Institute of Nuclear Research, the Chinese Academy of Sciences, Shanghai 201800;*
[2] *Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, the Chinese Academy of Sciences, Shanghai 200031*)

Abstract An improved CBB staining with higher sensitivity than that of the typical CBB staining was reported. The main improvement was using a fixing step of 25% trichloroacetic acid (TCA) before CBB staining. For most proteins studied, the sensitivity of the improved CBB staining was about twice as high as that of the typical method. For basic and low molecular weight proteins such as ribosomal proteins, the sensitivity of this improved staining method was about 3.5–28 times that of the typical method. It was speculated that the improved procedure would be suitable for exact quantitative analysis of proteins fractionated by SDS-PAGE, especially for basic and low molecular weight proteins. On the other hand, this new modified method might be also applied to multidisciplinary studies, such as biological researches and nuclear sciences.

Keywords Basic and low molecular weight proteins, Coomasie Brilliant Blue (CBB) R-250, Ribosomal proteins, SDS-PAGE, 2-D electrophoresis

CLC numbers Q503, Q51

1 INTRODUCTION

The triphenylmethane organic dye, CBB R-250, currently the most commonly used protein stain, was introduced by Groth and was used by Maizel and Meyerin in polyacrylamide gel electrophoresis later.[1–3] The sensitivity of CBB staining was about 100 times less than that of silver staining, and so far improvements in CBB staining procedures were less than those in silver staining procedures. The most efficient staining recipe of CBB staining is 0.25% (*W/V*) CBB R-250 in 45% (*V/V*) methanol and 10% (*V/V*) acetic acid. Since the dye was introduced to bioscience researches, several alternative procedures have been developed. Chrambach reported that the CBB R-250 dissolved in 12.5% TCA could be directly used in protein staining.[4] This procedure could visualize protein bands more rapidly with a stained background of gels, which prevented detection of smaller bands immediately after staining. Removal of the background and intensification of protein bands could be obtained by storing the gel in 10% TCA for several

Corresponding author: LIU Wang-Yi, LI Min-Qian: mqli@srcap.stc.sh.cn
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hours. CBB G-250, a dimethyl substitute of the R-250 dye, could also be used because of its increased insolubility in TCA solution.[5,6] The colloidal suspension of dye in TCA solution could cause selective binding of dye to proteins and forming dye-protein complexes, whereas the dye would not penetrate into the gel matrix and avoid background staining. A procedure using formaldehyde to fix gels before carrying out CBB staining was also introduced and this procedure was suitable for staining low molecular weight proteins and polypepetides that could not be satisfactorily fixed by mixtures of acetic acid and methanol.[7,8] In summary, the sensitivity of most alternative procedures could not preponderate over that of the typical CBB R-250 staining.

In this work, a new CBB staining procedure was developed. Compared with the typical CBB staining the sensitivity of the new method was at least two times that for most proteins and could be much higher for basic and low molecular weight proteins. It was speculated that this new method would be suitable for more exact quantitative analysis of proteins separated by polyacrylamide gels.

2 MATERIALS AND METHODS

2.1 Materials

CBB R-250, 2-[N-Morpholine] ethane-sulfonic acid, bis-Tris and urea were from Amersco. Acrylamide was from Sigma. N, N'-Methylenebisacrylamide was from Fluka. Acetic acid, methanol, acetone, tricholoacetic acid and Fuchsin Basic were homemade. The six marker proteins used in the experiments were from Shanghai Li-Zhu-Dong-Feng Biotech. They were rabbit phosphorylase b (97,000), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,000) and hen egg white lysozyme (14,400). The two-dimensional gel electrophoresis apparatus was made in the experimental workshop according to Madjar with a little alteration.[9] The ImageMaster 2D system 3.01 version, GDS8000 system and GELWORK 3.01 version were from Gene Company Limited. Buffers and apparatus used for ribosomal extraction were all RNase free. Solutions for the preparation of buffers and polyacrylamide gels had to be prepared with double distilled water. The staining solutions for both the typical and the improved CBB staining were prepared freshly. 25% TCA solutions were diluted from 100% TCA storing solution before use. The concentration of TCA storing solution was 100%. They were stored in brown bottles and kept in refrigerator to avoid direct irradiation of sunlight, and should be used up within two weeks.

2.2 One-dimensional SDS polyacrylamide gel electrophoresis

One-dimensional SDS gel electrophoresis was carried out in vertical SDS polyacrylamide gels (10 cm× 8 cm×0.14 cm) with 1.5 cm long 4% stacking gel on top of 6.5 cm long 12.5% separation gel. Electrophoresis was carried out toward anode at 60 V in stocking

gel and 120 V in separation gel till the tracking dye touches the bottom of glass plate. Six marker proteins were dissolved in sample buffer (0.04 mol/L bis-Tris-acetic acid, pH 6.0; 6 mol/L urea; 1% SDS), heated at 95°C for 3 min and subjected to electrophoresis. After electrophoresis, gels were stained with different staining procedures.

2.3 Two-dimensional electrophoresis of rat liver ribosomal proteins

Rat liver ribosome was isolated according to Ogata.[10] Proteins from 80 S ribosomes are extracted by the acetic acid procedure already described, and dialyzed against 1 M acetic acid overnight and lyophilized.[11] 2-D electrophoresis of ribosomal protein was performed according to the method of Madjar with a little alteration.[12,13] Briefly, for first-dimensional electrophoresis, 100 mm high 1-D separation gel was prepared in glass tubes of 120 mm height and 2 mm inside diameter. Electrophoresis was carried out at 20°C toward the cathode at 150 V (constant voltage) for 6.5–7 h. After electrophoresis, the first-dimensional gel was taken out and equilibrated with dialyzing buffer (0.04 M bis-Tris-acetic acid, pH 6.0; 6 mol/L urea, 1% *W/V* SDS) at 20°C for about 15 min. For second-dimensional electrophoresis, 2.5 cm long 4% stacking gel were prepared on top of 10 cm high separation gel. The first dimensional gels were put horizontally on top of the second-dimensional gels. Electrophoresis was carried out at 20°C toward the anode using 3 W/per gel for 6–7 h. After electrophoresis, proteins were stained with different staining procedures respectively.

2.4 The typical CBB staining and the improved CBB staining procedures

The improved CBB staining was performed as follows. After electrophoresis, gels were fixed with 25% TCA solution for 0.5 h with gentle shaking, washed with 45% methanol and 10% acetic acid for about 1 min and then stained overnight at room temperature with the solution containing 45% methanol, 10% acetic acid, and 0.25% CBB (filtrated). Destaining was started with 45% methanol and 10% acetic acid without dye, and completed with 30% methanol and 10% acetic acid. Gels were usually destained overnight to accomplish satisfactory destaining. After washed with 40% ethanol for 20 min, gels were dried and analyzed. The typical CBB staining procedure was similar to that of the improved method, except that the preceding 25% TCA fixation step was devoid.

2.5 Quantitative and statistic analysis of protein contents in gels

The quantitative analysis of six marker protein bands in gels, either stained with the typical or the improved CBB staining procedures, was accomplished with GDS 8000 system and software of GELWORK 3.01 version. The relative color depth for each band was calculated by computer and displayed as the volume of each band of each protein. Spots of ribosomal proteins were scanned and analyzed by ImageMaster 2D system 3.01

version. The color depth of each spot was displayed as the volume of each spot for each protein. To ensure the conditions of analysis comparable, the dried gels were measured under the same scanning conditions on the same day. All experiments were repeated at least four times and quantitative data were statically analyzed with t-test and constructed with SigmaPlot 4.0.

3 RESULTS AND DISCUSSION

One of the important aims of gel fixation after electrophoresis was to rapidly precipitate the separated proteins in gel matrix and convert the diffusible species into insoluble precipitates. Mixtures of methanol, acetic acid and water were widely used as fixative reagents in the typical CBB staining. We had performed most of the published alternative CBB staining procedures and found that sensitivities of these procedures were poorer than or at best similar to that of the typical CBB staining. An exception was that the low molecular weight proteins could be stained better by the procedure using formaldehyde as fixative than by the typical CBB staining (data not shown). TCA was a preferable fixative that was usually found to be most efficient at the concentration of 20%–25%. Sulfosalicylic acid (10%–20%) or mixtures of TCA and sulfonsalicylic acids (10% *W/V* of each) were recommended sometimes, but it was found that their fixation effects were similar to that of using TCA alone.[14] Sivaraman had studied the mechanism of TCA induced protein precipitation and found that the mechanism of rapid fixation of proteins by TCA had close relationship with its protein-precipitation ability and the TCA induced protein precipitation was specific. The presence of three

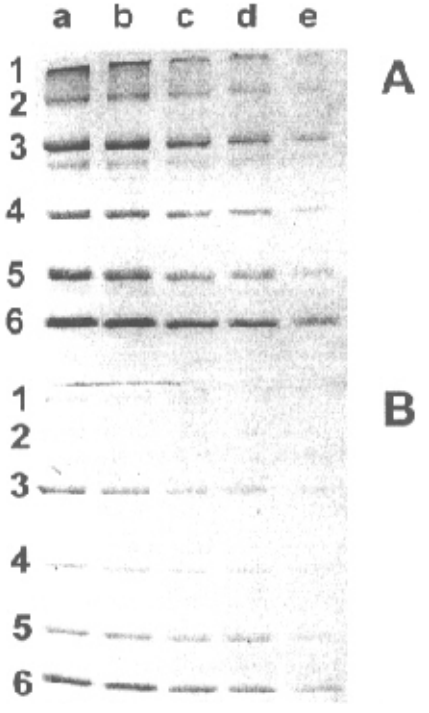

Fig.1 The six marker proteins stained by the improved CBB staining and the typical CBB staining procedure. The six marker proteins: 1. rabbit phosphorylase b (97,000), 2. bovine serum albumin (66,200), 3. rabbit actin (43,000), 4. bovine carbonic anhydrase (31,000), 5. trypsin inhibitor (20,000), 6. hen egg white lysozyme (14,400). The loading amounts (μg) of each protein: (a) 2.0, (b) 1.5, (c) 1.0, (d) 0.8, (e) 0.4. Panel A: the improved CBB staining; Panel B: the typical CBB staining

chloro groups (on the alpha-carbon atom) in the acetic acid molecule was important for the protein-precipitating action. On the other hand, the protein-precipitating action of TCA was independent of the nature of proteins and was not pH-dependent.[15]

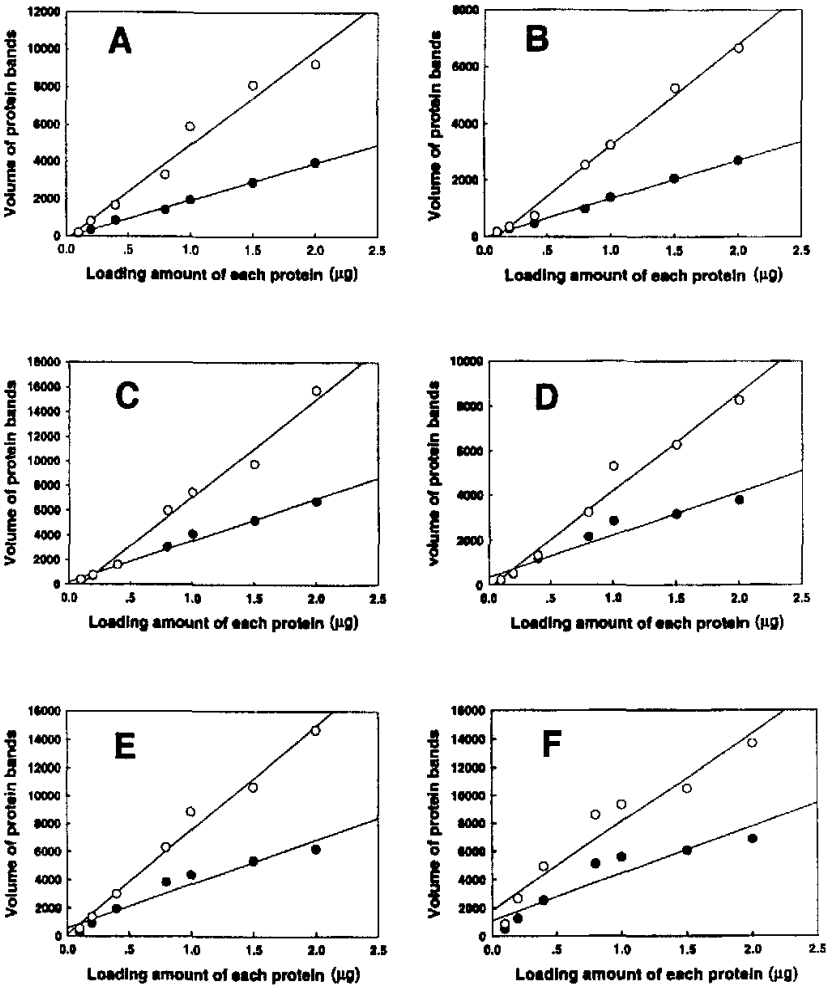

Fig.2 The linear relationship between the volumes of protein bands (vertical axes) and the protein loading amounts (horizontal axes) for the improved CBB staining and the typical CBB staining. Panel A: rabbit phosphorylase b, Panel B: bovine serum albumin, Panel C: rabbit actin, Panel D: bovine carbonic anhydrase, Panel E: trypsin inhibitor, Panel F: hen egg white lysozyme.
• – • the typical CBB staining, o – o the improved CBB staining

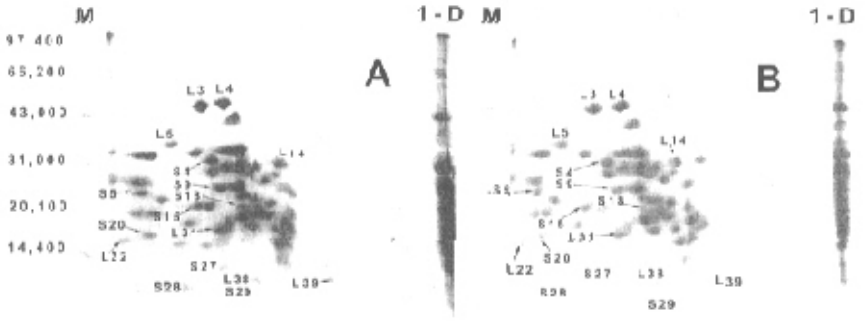

Fig.3 80 S rat liver ribosomal proteins separated by 2-D electrophoresis and stained with different CBB staining procedures. The loading amount of each ribosomal protein was approximately 1 μg/per protein. Panel A: the improved CBB staining, Panel B: the typical CBB staining

In this paper, it was reported that the sensitivity of CBB staining was enhanced by fixing proteins rapidly with 25% TCA before carrying out CBB staining. It was found that under the same loading amounts, the six marker protein bands stained by the improved CBB staining were clearer than those stained by the typical CBB staining (Fig.1). It could be deduced that, different from the improved CBB staining, part of proteins in gels might be lost during staining and destaining process of the typical CBB staining. The preceding TCA fixation in the improved CBB staining might be able to retain most of proteins in gels without disturbing the subsequent staining procedures. The quantitative and statistic analysis indicated that the difference in staining sensitivity between two staining procedures was significant. For the six marker proteins studied, sensitivity of the improved CBB staining was about twice as high as that of the typical CBB staining (Fig.4D). It was also found that the improved CBB staining complied with the linearity between staining depth of band color (represented as volume of protein bands) and the loading amounts of protein samples, indicating that the improved staining procedure conformed to the staining property of CBB dye (Fig.2). Furthermore, for strong basic proteins such as ribosomal proteins separated by 2-D electrophoresis, the improved CBB staining had a higher sensitivity than the typical CBB staining. Some basic low molecular weight ribosomal proteins, such as S28 and S29, could not be clearly visualized by the typical CBB staining, while these proteins under the same loading amounts could be clearly stained by the improved CBB staining (Fig.3). For basic high and middle molecular weight ribosomal proteins, such as L3, L4, S4, L22, L31 and S20, sensitivities of the improved CBB staining were about twice as high as those of the typical CBB staining (Fig.4A,B). For basic low molecular weight ribosomal proteins, sensitivity of the improved CBB staining was about 3.5–28 times higher [S27 (7 times),

S28 (28 times), S29 (23 times)] than that of the typical CBB staining (Fig.4C). Thereby, a conclusion can be reached that the improved CBB staining would be much suitable for staining of basic low molecular proteins in polyacrylamide gels.

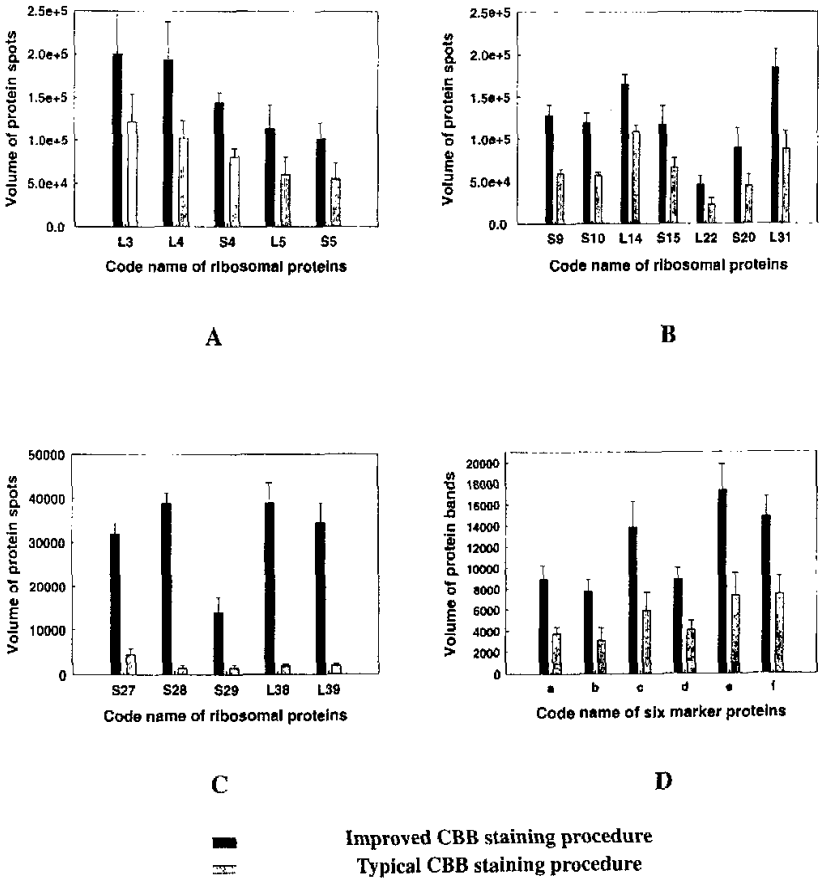

Fig.4 Comparison of sensitivities of the improved CBB staining and the typical CBB staining for six marker proteins and ribosomal proteins. Panel A: basic high molecular weight ribosomal proteins (more than 30 KDa), Panel B: basic middle molecular weight ribosomal proteins (10 30 KDa), Panel C: basic low molecular weight ribosomal proteins (less than 10 KDa), Panel D: six marker proteins (14.4 KDa-97 KDa). (a) rabbit phosphorylase b, (b) bovine serum albumin, (c) rabbit actin, (d) bovine carbonic anhydrase, (e) trypsin inhibitor, (f) hen egg white lysozyme

In this paper, a new improved CBB staining procedure with higher sensitivity for staining of proteins in SDS polyacrylamide gels was reported. Due to the rapid and efficient fixation of proteins in gels by TCA, the improved CBB staining might be suitable

for more exact quantitative analysis of proteins in polyacrylamide gels, especially for basic low molecular weight proteins. On the other hands, this modified procedure might be applied to multidisciplinary studies, such as biological researches in combination with nuclear sciences.

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