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ORIGINAL RESEARCH PAPER

A modified Coomassie Brilliant Blue staining method at nanogram sensitivity compatible with proteomic analysis

Xuchu Wang · Xiaofang Li · Yinxin Li

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Abstract A more sensitive and convenient Coomassie Brilliant Blue (CBB) staining method for visualizing proteins was developed. Compared with the modifications include the supplement of 10% (v/v) methanol into the fixing solution, an increase of an additional sensitization step and CBB raised from 0.1 to 0.125%. The improved method can detect proteins at nanogram level. The improved method is more sensitive than Blue Silver and more convenient than the Silver protocol. Mass spectrometry results confirmed that it is suitable for subsequent proteomic research.

Keywords Coomassie Brilliant Blue · Mass spectrometry · Plant proteomics · Protein staining method · Two-dimensional electrophoresis

Introduction

Visualizing proteins after SDS-PAGE is a prerequisite to succeeding in subsequent proteomic research. Coomassie Brilliant Blue (CBB), silver and fluorescence are three classes of protein staining methods widely used (Patton 2000). Recently, SYPRO family dyes have been used due to their convenience and high sensitivity (Steinberg et al. 1996; Lopez et al. 2000; Patton 2000; Steinberg et al. 2000). Combined with different fluorescence dyes, a 2-D-fluorescence difference gel electrophoresis technology was devised by GE Healthcare, which is currently unrivaled for its ability to visualize and quantify relative changes in abundances of proteins (Patton 2000). However, the fluorescence used in this method can quench rapidly and the dyes are expensive. Additionally, special hardware and software for protein assessment are needed (Patton 2000).

Silver staining is usually considered as the most sensitive, non-radioactive protein visualization method. It can detect proteins at the nanogram level (Yan et al. 2000; Candiano et al. 2004). However, it is not convenient because of its laborious multiple steps, as well as a high background. A large number of silver staining techniques also have poor compatibility with mass spectrometry (MS) due to the use of glutaraldehyde, which can irreversibly cross-link with polypeptide chains (Candiano et al. 2004).

Coomassie dyes, which were originally introduced to visualize protein bands in 1963, are classical dyes used for protein staining. Today, CBB staining is still a favorite method for its reproducibility, clear background, reasonable sensitivity, and the excellent compatibility with MS (Candiano et al. 2004). Many modification versions of this staining method were reported (Patton 2000). Recently, the sensitivity of CBB staining was enhanced to 30 ng *per* band by

X. Wang · X. Li · Y. Li (✉)
Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, P.R. China
e-mail: yxli@ibcas.ac.cn

Neuhoff and colleagues (Neuhoff et al. 1988; Candiano et al. 2004). Based on the Neuhoff stain, Candiano et al. (2004) developed a CBB staining method, named Blue Silver, giving a detection limit of 1 ng. However, the Blue Silver technique requires a longer time to destain the gel in water, thus making the gel extend remarkably and very crisp.

Here, we describe an improved CBB staining protocol, which has a similar sensitivity to the improved silver protocol, but more sensitive than the Blue Silver.

Materials and methods

The proteins from *Salicornia europaea*, a succulent euhalophyte, were extracted by the phenol method according to Carpentier and colleagues (Carpentier et al. 2005) with minor modifications. The 1-DE, 2-DE, image analysis and mass spectrometry were performed according to Yan et al. (2000) and the protocol of the manufacturer (2-DE manual, GE Healthcare).

After electrophoresis, the gels were stained with Blue Silver according to the protocol of Candiano et al. (2004) and the improved Silver staining method according to Yan et al. (2000) or our modified CBB staining method.

The protocol of our method is as follows. After electrophoresis, the gel was removed from electrophoresis apparatus and transferred into 20 volumes of fixing solution (10% v/v acetic acid, 10% v/v methanol, and 40% v/v ethanol), and fixed for 1 h. It was then put the gel into the sensitization solution (1% v/v acetic acid, 10% v/v ammonium sulfate) and stirred for an additional 2 h. The staining solution consists of 5% v/v acetic acid, 45% v/v ethanol and 0.125% (w/v) CBB R-250. The gel was then placed into 20 volumes of staining solution and stirred for more than 4 h or overnight. The gel was transferred to the destaining solution I (5% v/v acetic acid, 40% v/v ethanol) and stirred for 1 h, then into the destaining solution II (3% v/v acetic acid, 30% v/v ethanol) until the background was clear. The final gel can be preserved in 5% (v/v) acetic acid for several months.

Results and discussion

The sensitivity of this modified CBB staining is shown by staining one-dimensional electrophoresis (1-DE) gels. As seen in Fig. 1, this modified CBB stained gel (Fig. 1, A) showed distinct protein bands with clear background, whereas the gels stained with Blue Silver and the improved Silver protocol had a little background (Fig. 1, B and C, respectively). In the CBB stained gel, the protein bands spanned from over 94 kDa to less than 14 kDa. However, in the Blue Silver staining gel, some minor bands, especially in the low molecular weight (MW) regions, were not detectable (Fig. 1, B). The similar result was also obtained by staining 2-DE gels (Fig. 2, B). As for silver staining, although all the main bands can be visualized, some bands can hardly be distinguished from the deep background (Fig. 1, C).

Furthermore, the sensitivity of the aforementioned three staining methods was confirmed by 1-DE using BSA. As shown in Fig. 1, in the CBB stained gel, the main BSA band (MW 66.2 kDa) was still visible when 10 ng protein was loaded (Fig. 1, D, lane 10), indicating a sensitivity of 1 ng/mm^2. The Blue Silver method had a lower sensitivity at 3 ng/mm^2 (Fig. 1, E, lane 9). Compared to the other protocols, the Silver method exhibited a higher sensitivity, which reached 0.6 ng/mm^2 (Fig. 1, F, lane 12). These results indicated that the modified CBB staining protocol can detect proteins at nanogram level and this method is more sensitive than the Blue Silver.

Fig. 2 shows the comparative staining results of 2-DE gels. In general, the modified CBB (Fig. 2A) and the Silver method (Fig. 2C) produced more protein spots than the Blue Silver (Fig. 2B). The modified CBB protocol thus approaches the sensitivity of the Silver and is more sensitive than the Blue Silver. Most importantly, the background of the modified CBB stained gel was clear (Fig. 2A). Substantial quantitative and qualitative differences of spots in some representative regions were observed among the gels stained with these three protocols as highlighted by rectangular boxes (Fig. 2, regions a, b, c, d and e). Some protein spots, which were visualized clearly on the modified CBB and the Silver stained gels, disappear in the selected regions of the Blue Silver stained gel (Fig. 2, regions c, d and e).

It cannot be denied that silvering is the most sensitive methods for permanently staining proteins in polyacrylamide gels (Yan et al. 2000; Candiano et al. 2004). Our results confirmed that the Silver method is more sensitive than both the Blue Silver and our CBB method (Fig. 1 and 2). However, due to

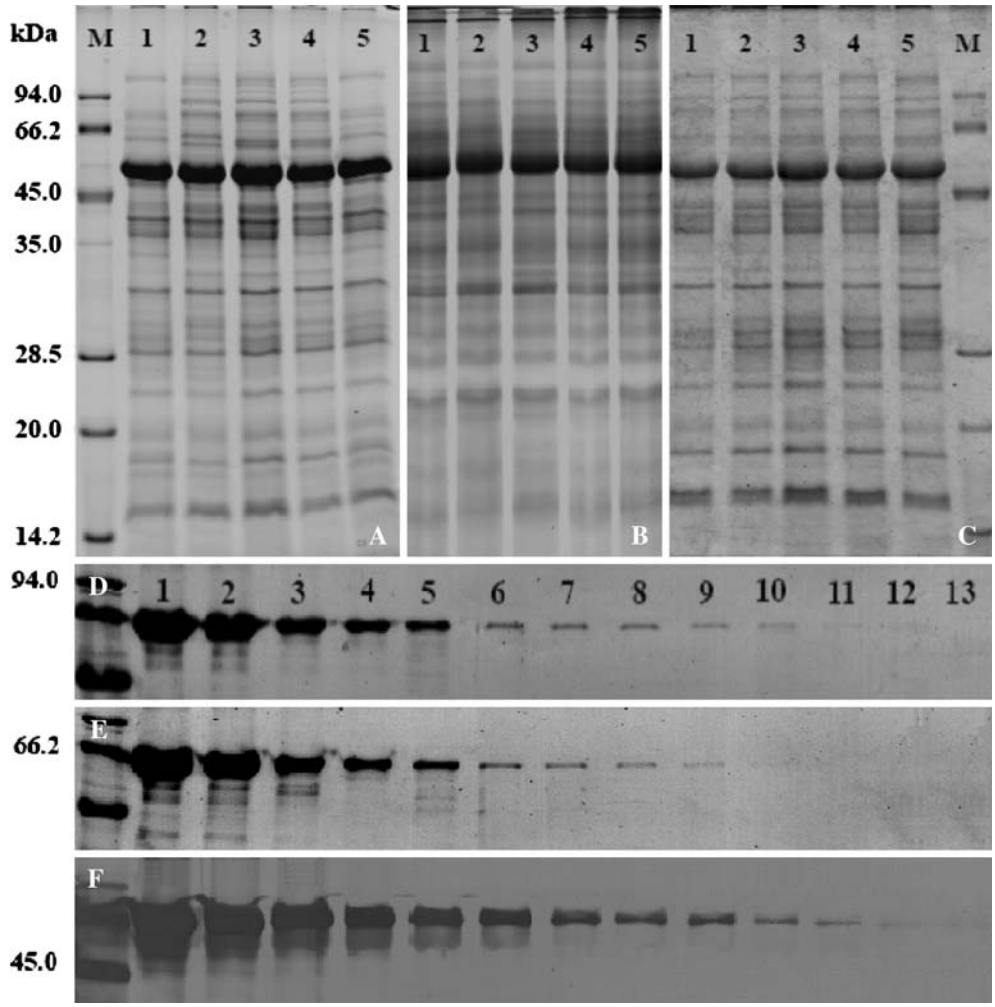

Fig. 1 Comparison of three staining methods on one dimensional gel. Total proteins, 20 μg, extracted from shoots of *S. europaea* treated with 0, 200, 400, 600 and 800 mM NaCl were loaded into the wells of lanes 1–5 and stained with modified CBB (gel **A**), Blue Silver (gel **B**) and silver (gel **C**), respectively. The sensitivity of the modified CBB, Blue Silver and Silver methods upon SDS-PAGE of BSA was presented on the gels (**D**, **E** and **F**), respectively. Lanes 1–13, BSA in decreasing amounts loaded *per* lane (10, 3, 1, 0.5, 0.3 and 0.1 μg, 80, 50, 30, 10, 8, 6 and 5 ng, respectively). M, molecular weight markers

the drawbacks of the silvering method (poor staining reproducibility, laborious multiple steps, high background, and incompatibility with MS), most scientists working on proteomic analysis prefer classical organic dyes of the Coomassie family. CBB dyes are insensitive to many things, such as quality of solvents, temperature, and developing time (Candiano et al. 2004). The characteristics of CBB dyes make the CBB staining efficiently avoid the risk of under- or over-staining and have excellent stain reproducibility. Although CBB staining methods need a longer staining time (more than 4 h) than silvering methods, they are convenient. Compared with the Blue Silver, this improved CBB method showed a higher sensitivity (Fig. 1 and 2), although the additional sensitization step requires more time (2 h) than the Blue Silver. In short, the aforementioned comparison staining results over Blue Silver, improved Silver and our method showed that the improved CBB method reported here is more sensitive than Blue Silver and is more convenient than the Silver protocol.

In order to determine whether the proteins stained by the modified CBB staining method are compatible

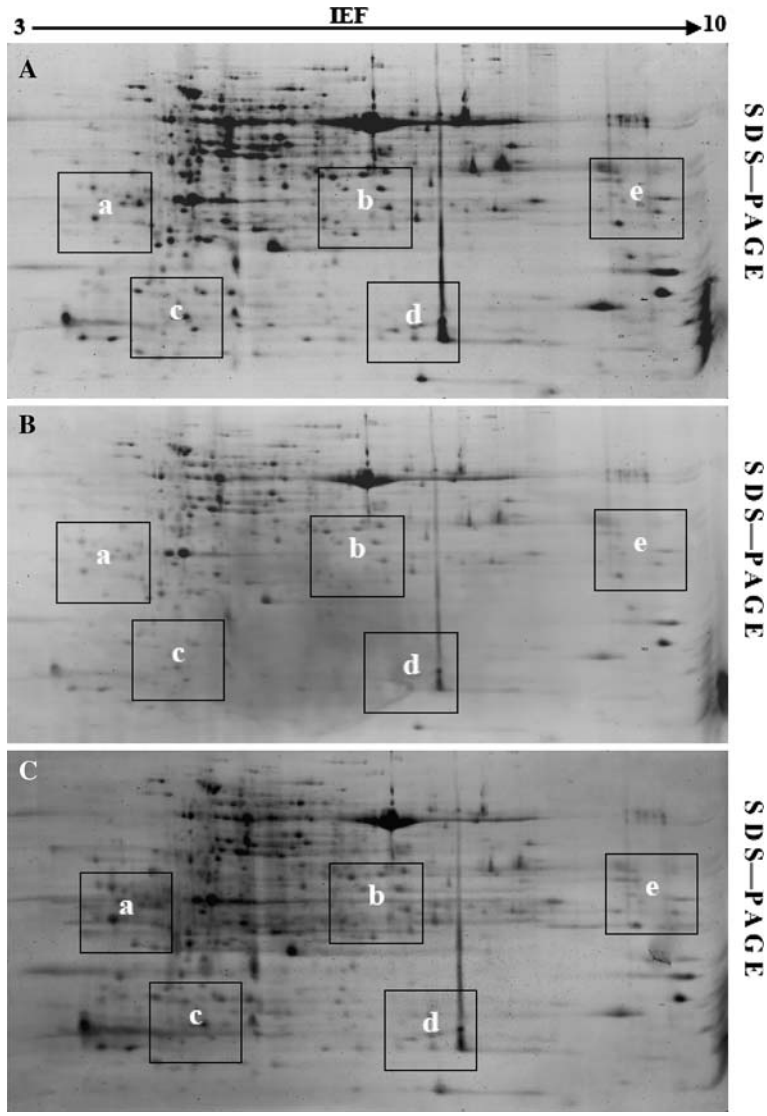

Fig. 2 Evaluation of the sensitivity after 2-DE over three staining methods. Proteins, 700 μg, isolated from shoots of *S. europaea* treated with 200 mM NaCl were separated on a 24 cm IPG strip with linear pH from 3 to 10 in the first dimension. Three similar 2-DE gels were stained with modified CBB (**A**), Blue Silver (**B**) and the Silver protocol (**C**), respectively. Representative regions of the gels indicating particularly different distributions of protein spots are highlighted with letters by rectangular boxes

with MS, the protein band (0.1 μg protein) of BSA in the 1-DE gel (Fig. 1, D, lane 6) and the most abundant protein spot in the 2-DE gel (Fig. 2A) were excised manually and identified by MALDI-TOF MS according to the protocol of Yan et al. (2003). The Mascot searched results in databases (http://www.ncbi.nlm.nih.gov) showed that these proteins are homologous to the albumin (Accession number: gi|162648) and the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Accession number: gi|34576735) with Mascot scores 143 and 126, respectively. Furthermore, about 100 spots were eluted, analyzed by MS and identified from databases with satisfactory results (data not shown). These results demonstrated that the modified CBB staining method is compatible with MS and can be used in the subsequent proteomic research.

Conclusion

We have developed a simple and sensitive CBB staining protocol. It is compatible with MS and can detect protein at the nanogram level. This method was developed from the latest modified CBB staining method, which was described in detail by Guo (1991)

and was recently introduced to stain 2-DE gels for proteomic research by GE Healthcare (2-DE manual, GE Healthcare). Some modifications were made to improve the staining sensitivity on both the reagents and staining procedure. Firstly, the 10% (v/v) methanol was added into the fixing solution to fix the proteins in the gel efficiently. Secondly, the sensitization step was added to facilitate the dyes into the gel and cross-link with the proteins. Finally, the concentration of CBB was increased from 0.1 to 0.125%. Compared with the other two methods, this improved method is more sensitive than Blue Silver, and more convenient than the Silver protocol. Therefore, this modified CBB protocol can be an alternative staining method in future proteomic research.

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