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A Modified CBBR-250 Microwave-Assisted Staining and Destaining Method on SDS-PAGE Gels

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Abstract: The application of microwave-assisted technologies has drastically reduced overall staining and destaining times in CBB R-250 staining on SDS-polyacrylamide gels but accurate temperature control and reproducibility for conventional commercial microwaves are not satisfactory. The objective of this study is to improve the existing procedures and develop an optimal method for using microwave-assisted technologies. Researchers adjusted the component of staining and destaining solutions and attempted to control the microwave temperature by shortening time, the staining and destaining could be completed within 30 min and resulted in dark purple-blue protein bands. In addition, the SDS-polyacrylamide gel displayed a transparent gel background. The detection limit for bovine serum albumin and fetuin-A on the SDS-polyacrylamide gels was 0.01 μg. These results showed that the modified method required less time. Furthermore, the modified procedure minimized the use of harmful organic solvents due to ethanol instead of methanol, methanol is more toxic than ethanol yet it maintained a high sensitivity for protein staining on SDS-polyacrylamide gels.

Key words: Coomassie brilliant blue R-250, microwave-assisted, SDS-PAGE, commercial microwaves, technologies

INTRODUCTION

Researchers in molecular biology spend a significant amount of time tending to the staining and destaining of electrophoresis gels. Coomassie Brilliant Blue (CBB) G-250, CBB R-250 and silver dyes are commonly used in staining procedures for polyacrylamide gels (Adam, 2003; Altikatoglu and Celebi, 2011; Chen *et al*., 1993). CBB R-250 was first introduced for protein staining on cellulose acetate paper by Groth *et al.* (1963) and Chrambach *et al.* (1967) and on polyacrylamide gels by Meyer and Lambert (1965) and Duhamel *et al.* (1980). The process of staining and observing protein bands in polyacrylamide gels with CBB R-250 involves fixation and staining of the protein bands within the gel and removal of the excess dye from the gel background using a suitable destaining solution. The main drawback of this method is the long staining time required for the dye to stoichiometrically bind to proteins because the diffusion of the dye into the gel is so slow. Similarly, removing excess dye from the gel during the destaining process can be very slow.

Different combinations of fixative/staining and destaining solutions have been used with CBB R-250 to simplify and speed up the staining and destaining cycles. Enhanced stability and decolorization of CBB R-250 was achieved by using dextran aldehyde-modified horseradish peroxidase (Groth *et al*., 1963). In another study, elimination of the destaining cycle was achieved, albeit with excessively long staining times by using very low concentrations of the dye, e.g., 0.0004% in ethanol/acetic acid/water (10/5/85) (Ji *et al*., 2007) or 0.003% in methanol/acetic acid/water (45/10/45) (Juan *et al*., 2005) a different report showed that performing staining/fixation for 18 h in 0.025% dye in isopropanol/acetic acid/water (25/10/65) and destaining in 7.5% acetic acid allowed a continuous clearance of the dye from the destaining solution by recirculating charcoal (Lill *et al*., 2007). One group performed fixation in 12.5% w/v Trichloroacetic Acid (TCA) for 30 min, stained with 0.05% dye in 12.5% TCA for 1 h and then destained in 10% TCA for 48 h (Lill and Nesatyy, 2012). Sreeramulu and Singh (1995) and Lin *et al.* (2008) uses dilute sodium chloride solutions to destain CBB R-250 stained gels and salt destaining gives a clear gel background in 2-3 h without causing fading of specific prolamin bands.

Recently, microwave irradiation has been applied in a variety of protocols related to the biological sciences (Marchetti-Deschmann *et al*., 2009; Meyer and Lambert, 1965; Nesatyy *et al*., 2002; Neuhoff *et al*., 1985;

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Osset *et al*., 1989; Pramanik *et al*., 2002; Rabilloud, 2000). One of the first microwave-assisted applications used in biology was for fixing, staining and destaining Sodium Dodecyl Sulfate (SDS) polyacrylamide gels. In 2002, a study on non-glycosylated proteins showed that the temperature increase during microwave irradiation resulted in increased diffusion rates of the applied solvents which quickened staining processes (Sreeramulu and Singh, 1995). It was found that microwave treatment drastically reduced the duration of the staining protocols required to visibly separate proteins by both one and two dimensional electrophoresis. Microwave-assisted protein staining methods were found to be rapid and compatible with mass spectrometry. However, the accurate temperature control and reproducibility of conventional commercial microwaves was not satisfactory, the absorption of microwave radiation was found to be uneven. Household microwaves typically induce concentrated areas of intense heat, known as hot spots and therefore cannot ensure that each sample is exposed equally to microwave energy.

MATERIALS AND METHODS

All chemicals used in this study were electrophoresis or analytical grade and all solutions were prepared in deionized water. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using the Bio-Rad (Hercules, CA, USA) Mini-PROTEAN Tetra Cell Gel Electrophoresis System. Microwave-assisted staining and destaining were performed in a household Galanz (Foshan city, China) WP700L17 microwave oven that allowed a maximum power of 700 W. For a wider application of this method, two types of standard proteins were prepared by combining known quantities of individual pure proteins-bovine serum albumin (fatty acid free BSA, sigma, representing nonglycosylated proteins) and fetuin-A (Mouse Fetuin-A,US-Biological, representing glycosylated proteins). The detection of glycoproteins on SDS-PAGE gels is a very challenging task because glycan moieties can inhibit the protein-dye interaction or protein-silver reaction, slowing down or completely preventing the staining process. Therefore, staining glycoproteins is a critical and time-consuming step because efficiency primarily depends on protein structure. CBB dye molecules interact with hydrophobic amino acid side chains near the amide bonds of proteins (Swatkoski *et al*., 2008). Carbohydrate moieties inhibit this mechanism by restricting the stain's access to the protein core, significantly slowing down the process or decreasing the method's sensitivity (Westermeier, 2006).

Therefore, the study was concerned with the detection limits of glycoproteins. A BSA concentration series (6.00, 5.00, 4.00, 3.00, 2.00, 1.00, 0.50, 0.10, 0.05 and 0.01 μg) was prepared by diluting a stock solution of BSA and a fetuin-A concentration series (1.50, 1.00, 0.50, 0.40, 0.30, 0.20, 0.10, 0.05, 0.01 and 0.001 μg) was prepared by diluting a stock solution of fetuin-A. SDS-PAGE of purified myostatin protein (glycosylated proteins, extracted from mouse skeletal muscle) was also used to detect this modified method. The sample quantity of 1-10 corresponding lanes is respectively approximately 6.00, 5.00, 4.00, 3.00, 6.00, 5.00, 4.00, 3.00, 2.00 and 1.00 μg and each corresponding lane as the sample quantity in three methods. Researchers complete electrophoresis of three pieces of SDS-polyacrylamide gels in the same electrophoresis tank.

Each dilution was mixed in a ratio of 1:2 with 1×sample buffer (+10% α-mercaptoethanol), heated (99°C, 5 min) and centrifuged (5000 r min^{-1}, 2 min). From each solution, 15 μL was loaded into each well of a 1.0 mm thick 10% SDS-polyacrylamide (Acr:Bis = 29:1) gel using 1×Tris-glycine electrophoresis buffer. The constant voltage was first set to 80 V for 30 min and was then raised to 120 V for 1 h.

After electrophoresis, the gel was picked up (gloves were used to prevent making fingerprints) and transferred to a small container containing deionized water. If removing the gel from the glass on the electrophoresis apparatus was difficult, researchers gently agitated the glass plate in deionized water until the gel separated from the plate and then the gel was rinsed with deionized water for several seconds at room temperature. Then, gels were stained and destained with method 1, 2 and modified method. In modified method, the deionized water in the container was discard and replaced with about 100 mL of CBB R-250 solution (0.1% w/v CBB R-250 in ethanol/acetic acid/water (25/10/65, v/v/v)) to submerge the gel. The container and the gel were heated for 1 min with microwave irradiation (700 W). After removing the gel from the microwave, it was agitated in the hot staining solution for 5 min without further microwave irradiation. Next, the gel was rinsed 2-3 times with deionized water at room temperature and then the deionized water was discarded. About 100 mL of destaining buffer (0.5 M NaCl) was poured into each container to submerge the gel and then the container and the gel were heated for 1 min with microwave irradiation (700 W). After removing the gel from the microwave, it was agitated in the hot destaining solution for 10 min without further microwave irradiation then this destaining process was repeated.

Table 1: Comparing the modified method with two literature methods of CBB R-250 protein staining on SDS-polyacrylamide gels

Steps	Method 1	Method 2	Modified method
Rinse	H_2O; twice	H_2O; twice	H_2O; twice
Fixing	Methanol/acetic acid/water (9/1/10, v/v/v), 2 min		
Staining	0.1% w/v CBB R-250 in methanol/acetic (9/1/10, v/v/v); incubation with microwave irradiation (170 W) 5 and 60 min RT	0.025% w/v CBB R-250 in methanol/acetic acid/water (18/7/75, v/v/v) in the presence of 6% w/v TCA; overnight at RT	0.1% w/v BB R-250 in ethanol/acetic acid/ water (25/10/65, v/v/v); incubation with microwave irradiation (700 W) 1 and 5 min RT
		H_2O; twice	
Rinse	H_2O; twice	0.5 M NaCl; 3 h at RT	H_2O; twice
Destaining	Methanol/acetic acid/water (40/7/53, v/v/v); incubation with microwave irradiation (170 W) 5 and 20 min RT		0.5M NaCl; incubation with microwave irradiation (700 W) 1 min and 10 min RT; twice

Table 2: Comparing the results of our modified method with two literature methods of CBB R-250 protein staining on SDS-polyacrylamide gels

Steps	Method 1	Method 2	Improved method
Used time	1 h 32 min	14~16 h	30 min
Background	Transparent	Clear	Transparent
Protein bands	Purple-blue complicated	Pale blue	Dark purple blue
Procedures	0.01 μg (BSA)	Complicated	Simplest
Sensitivity	0.01 μg (Fetuin-A)	0.05 μg (BSA) 0.05 μg (Fetuin-A)	0.01 μg (BSA) 0.01 μg (Fetuin-A)
Chemicals	Methanol and acetic acid	Methanol, acetic acid and TCA	Ethanol, acetic acid and NaCl
Harmful to operator	More	More	Less

Method 1 and 2 were cited in Marchetti-Deschmann *et al.* (2009), Zehr *et al.* (1989), Sreeramulu and Singh (1995) and Lin *et al.* (2008)

Finally, the gel was rinsed twice with deionized water for several seconds and was either photographed or placed in long-term storage.

In Table 1 and 2, researchers present the comparison of the improved method and two different procedures reported in the literature (Lin *et al.*, 2008; Zehr *et al.*, 1989) (method 1 and 2) for CBB R-250 protein staining on SDS-polyacrylamide gels. In method 1, the gel was fixed (2 min, 45% MeOH and 5% acetic acid in water) and subsequently incubated in the CBB R-250 solution (5 min, 0.1% CBB R-250 in 45% MeOH and 5% acetic acid in water, microwave assisted). After removing the gel from the microwave, it remained in the hot staining solution for 1 h without further microwave irradiation. In order to remove the CBB R-250 stain, the gel was put into the destaining solution (5 min, 40% MeOH and 7% acetic acid in water, microwave assisted) and was kept in the hot destaining solution for an additional 20 min without further microwave irradiation. In total, method 1 required 1 h and 32 min. In method 2, a gel was stained overnight with 0.025% w/v CBB R-250 in methanol/acetic acid/water (18/7/75, v/v/v) in the presence of 6% (w/v) TCA. For destaining, the gel was placed in a 0.5 M sodium chloride solution in water for about 3 h at room temperature. In Table 1 these three staining and destaining procedures are summarized.

RESULTS AND DISCUSSION

CBB R-250-stained protein bands on SDS-polyacrylamide gels were identified easily and resulted in clear electrophoresis bands and a transparent background. The band intensity after performing the modified protocol was comparable to and in some cases exceeded, the intensity of the bands obtained from the other two staining methods. From Fig. 1 and 2, it is clear that the protein sensitivity associated with the two microwave-supported methods was higher than the other method. The results of the modified method compared with the two literature methods for CBB R-250 protein staining on SDS-polyacrylamide gels is summarized in Table 2. From Table 2, researchers see that the modified method required less harmful chemicals in the staining solution while the other two procedures required more harmful chemicals that were used repeatedly in method 1. This modified method was also used with purified myostatin proteins (glycosylated proteins) with excellent results (Fig. 3). In the experiment, the samples were correspondingly loaded onto an SDS-polyacrylamide gel whereby after electrophoresis, the gel was cut and each gel piece (containing identical sample loadings) underwent one of the three staining/destaining methods described above. Electrophoresis of the corresponding samples was carried out on a single SDS-polyacrylamide gel in the electrophoresis tank. After performing the gels with different staining/destaining methods, researchers found that the improved method resulted in a transparent gel background, evidenced by observing the disposable PE gloves that were placed under the gel as a cushion (Fig. 3c). These results demonstrate that the improved method was more efficient and sensitive for SDS-polyacrylamide protein analysis. In addition, it is conducive to the physical health of the operator because of less harmful organic solvents.

The conventional CBB R-250 protein staining procedure often takes a very long time to perform (4-6 to

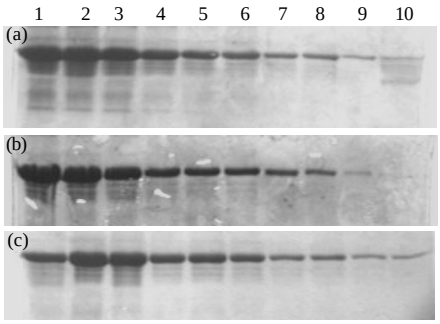

Fig. 1: SDS-PAGE of bovine serum albumin (fatty acids free, 66 kDa), the sample quantity of 1-10 lanes is respectively 6.00, 5.00, 4.00, 3.00, 2.00, 1.00, 0.50, 0.10, 0.05 and 0.01 μg. Gels were stained and destained with method 1, 2 and modified method. The same sample is added to 1-10 corresponding lanes of SDS-polyacrylamide gels in three methods and each corresponding lane as the sample quantity. Researchers complete electrophoresis of three pieces of SDS-polyacrylamide gels in the same electrophoresis tank

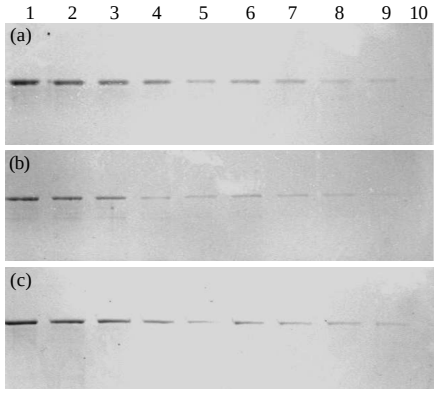

Fig. 2: SDS-PAGE of recombinant human Fetuin-A (55-60 kDa), the sample quantity of 1-10 corresponding lanes is respectively 1.50, 1.00, 0.50, 0.40, 0.30, 0.20, 0.10, 0.05, 0.01 and 0.001 μg and each corresponding lane as the sample quantity in three methods. Gels were stained and destained with method 1, method 2 and modified method. Researchers complete electrophoresis of three pieces of SDS-polyacrylamide gels in the same electrophoresis tank

12-18 h). The improved procedure researchers developed takes <30 min. Moreover, the resulting protein bands did not disperse during the destaining step. Even when the stained gel was soaked in water for 1 month, the protein bands remained distinct and orderly. The CBB

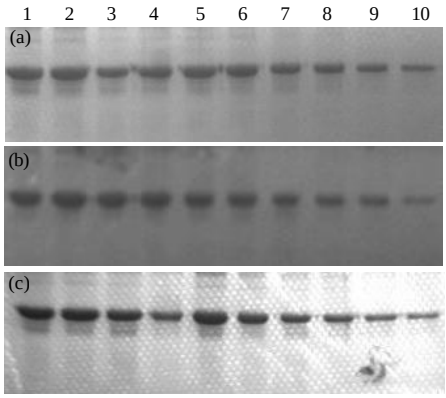

Fig. 3: SDS-PAGE of purified myostatin protein. Gels were stained and destained with method 1, 2 and modified method. The sample quantity of 1-10 corresponding lanes is respectively approximately 6.00, 5.00, 4.00, 3.00, 6.00, 5.00, 4.00, 3.00, 2.00 and 1.00 μg and each corresponding lane as the sample quantity in three methods. Researchers complete electrophoresis of three pieces of SDS-polyacrylamide gels in the same electrophoresis tank

R-250 stained SDS-polyacrylamide gel could be stored long term at 4°C. After storing the gel for 1 year at 4°C in a sealed plastic bag following a 5 min UV treatment to prevent microbial growth, researchers found that the gel remained intact and unchanged. The conventional CBB R-250 staining procedure often requires the operator to change the destaining solution and agitate the gel until the gel background is clear. This step may result in the leaching out of many prolamin bands. Salt destaining seemed to prevent such leaching in the improved procedure. Furthermore, using a salt solution reduced the exposure to these harmful organic solvents: methanol, acetic acid and TCA. With an appropriate extension of the destaining time, the gel background can become even clearer which will further increase the sensitivity of the procedure. Another advantage of the procedure was that the staining solution could be recycled 3-5 times without affecting protein staining results. However, researchers did find that the container should not be sealed with a lid or plastic wrap during the staining and destaining procedures. Also, it is critical to control the amount of staining and destaining solutions because these volumes are related to the precise control of temperature in the microwave and can affect the repeatability of the results. Although, the microwave apparatus plays an important role in the design of microwave-assisted experiments, the biggest challenges faced when using conventional commercial microwaves are reproducibility and accurate

temperature control. Microwave-assisted proteomics is still in its infancy. An ongoing discussion among synthetic chemists has focused on the mechanism of microwave catalysis. Some advantages of using microwave power are that the associated high temperatures promote the free diffusion of molecules and it speeds up reagent diffusion and penetration into the gel. SDS is an anionic surface-active agent that can open the hydrogen bonds and hydrophobic bonds during electrophoresis and combine with proteins. While SDS is some what stable at 80°C, temperatures can reach 100°C during microwave heat treatment. Therefore, SDS is decomposed under these circumstances and lifted the SDS curb on Coomassie blue staining. As a result, Coomassie blue dye can more easily combine with proteins. Another advantage of using microwave power is that proteins are rapidly fixed when heated so fixing, staining and destaining times are significantly reduced which may also reduce the chances of gel contamination. Many experts now believe that these microwave effects are the result of microwaves' ability to superheat solvents beyond their normal boiling points. For example, water reaches 105°C before boiling in a microwave oven whereas acetonitrile boils at 120°C instead of its usual 82°C (Zehr *et al*., 1989). Until the true mechanisms by which microwave irradiation mediates this alternative catalysis are better understood, it remains difficult to predict which biochemical and analytical processes will benefit from microwave assistance.

CONCLUSION

In the present study, researchers developed an optimal protocol for CBB R-250 protein staining on SDS-polyacrylamide gels in which the procedure was simplified without affecting its sensitivity or the storage of the SDS-polyacrylamide gels. Researchers attempted to control the microwave temperature by shortening time with the assistance of microwave irradiation. This modified microwave-supported staining and destaining protocol was investigated in terms of duration, sensitivity and toxicity.

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REFERENCES

Adam, D., 2003. Microwave chemistry: Out of the kitchen. Nature, 421: 571-572.

Altikatoglu, M. and M. Celebi, 2011. Enhanced stability and decolorization of coomassie brilliant blue R-250 by dextran aldehyde-modified horseradish peroxidase. Artif. Cells Blood Substitutes Biotechnol., 39: 185-190.

Chen, H., H. Cheng and M. Bjerknes, 1993. One-step Coomassie brilliant blue R-250 staining of proteins in polyacrylamide gel. Anal. Biochem., 212: 295-296.

Chrambach, A., R.A. Reisfeld, M. Wyckoff and J. Zaccari, 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem., 20: 150-154.

Duhamel, R.C., E. Meezan and K. Brendel, 1980. Metachromatic staining with Coomassie Brilliant Blue R-250 of the proline-rich calf thymus histone, H1. Biochim. Biophys. Acta (BBA)-Protein Struct., 626: 432-442.

Groth, D.S.F., R.G. Wbster and A. Datyner, 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. J. Biochem. Biophys Acta., 14: 377-391.

Ji, Y.T., C.Q. Qu and B.Y. Cao, 2007. An optimal method of DNA silver staining in polyacrylamide gels. Electrophoresis, 28: 1173-1175.

Juan, H.F., S.C. Chang, H.C. Huang and S.T. Chen, 2005. A new application of microwave technology to proteomics. Proteomics, 5: 840-842.

Lill, J.R. and V.J. Nesatyy, 2012. Microwave-assisted protein staining, destaining and in-gel/in-solution digestion of proteins. Methods Mol. Biol., 869: 521-532.

Lill, J.R., E.S. Ingle, P.S. Liu, V. Pham and W.N. Sandoval, 2007. Microwave-assisted proteomics. Mass Spectrom. Rev., 26: 657-671.

Lin, S., D. Yun, D. Qi, C. Deng, Y. Li and X. Zhang, 2008. Novel microwave-assisted digestion by trypsin-immobilized magnetic nanoparticles for proteomic analysis. J. Proteome Res., 7: 1297-1307.

Marchetti-Deschmann, M., J. Kemptner, C. Reichel and G. Allmaier, 2009. Comparing standard and microwave assisted staining protocols for SDS-PAGE of glycoproteins followed by subsequent PMF with MALDI MS. J. Proteomics, 72: 628-639.

Meyer, T.S. and B.L. Lambert, 1965. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim. Biophys. Acta (BBA)-Gen. Subj., 107: 144-145.

Nesatyy, V.J., A. Dacanay, J.F. Kelly and N.W. Ross, 2002. Microwave-assisted protein staining: Mass spectrometry compatible methods for rapid protein visualisation. Rapid Commun. Mass Spectrum., 16: 272-280.

Neuhoff, V., R. Stamm and H. Eibl, 1985. Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: A systematic analysis. Electrophoresis, 6: 427-448.

Osset, M., M. Pinol, M.J. Fallon, R. de Llorens and C.M. Cuchillo, 1989. Interference of the carbohydrate moiety in Coomassie Brilliant Blue R-250 protein staining. Electrophoresis, 10: 271-273.

Pramanik, B.N., U.A. Mirza, Y. Hain, Y.H. Liu, P.L. Bartner, P.C. Weber and A.K. Bose, 2002. Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: A new approach to protein digestion in minutes. Protein Sci., 11: 2676-2687.

Rabilloud, T., 2000. Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods. Springer, Berlin, Germany, ISBN: 9783540657927, Pages: 248.

Sreeramulu, G. and N.K. Singh, 1995. Destaining of Coomassie Brilliant Blue R-250-stained polyacrylamide gels with sodium chloride solutions. Electrophoresis, 16: 362-365.

Swatkoski, S., P. Gutierrez, C. Wynne, A. Petrov, J.D. Dinman, N. Edwards and C. Fenselau, 2008. Evaluation of microwave-accelerated residue-specific acid cleavage for proteomic applications. J. Proteome Res., 7: 579-586.

Westermeier, R., 2006. Sensitive, quantitative and fast modifications for Coomassie Blue staining of polyacrylamide gels. Proteomics, 6: 61-64.

Zehr, B.D., T.J. Savin and R.E. Hall, 1989. A one-step, low background Coomassie staining procedure for polyacrylamide gels. Anal. Biochem., 182: 157-159.