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Comparision of staining methods for two dimensional electrophoresis gel resolved with *Puntius javanicus* liver proteome

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Abstract

The aim of this study was to compare the various staining methods based on commassie briliant blue and silver nitrate stain for the two dimensional gel electrophoresis resolved with *Puntius javanicus* liver proteome. The staining methods were selected base on the previous reportabout their compatiblitywiththe mass spectrometry analysis. Silver staining methodis known as the most sensitive method to visualize the maximum number of protein spots resolved in 2D gel but it is less sensitive(incompatible) toward mass spectrometry detection. Results of this study showed that a modifiedstaining method using colloidal coomassie blue G-250 (CCB) is roughly similarly sensitive but lower protein spot detected compared with silver staining (SS) as indicated at the number of 303±26 and 693±14of proteinresolved in both types of stained gels. The conventional methods of staining using commassie brilliant blue G-250 and R-250 only detected less number of protein spots(128±17and 78±11, respectively) compared to modified CCB staining method. As the commasie brilliant blue stain wasis known to be a very sensitive for mass spectrometry detection, the modified method of CCB was selected for further study on *Puntius javanicus* liver proteome.

INTRODUCTION

Proteomic is the study of the large scale of the entire set of protein in living organism which play role in the of the physiological metabolic pathways of cells (Wilkins et al., 1996). Study on proteome based on two dimensional electrophoresis (2DE) were carried out by previous studies to evaluate a number of unique protein called protein expression species (PES) cause by environment factor (Culwell et al., 2008; Vineis et al., 2009; Zhou et al., 2014). The most critical part in proteomic is to obtain the highest number of abundant protein resolved in 2DE gelthrough the selection of sample extraction or/and buffer preparation for protein solubilisation (Shaw and Riederer, 2003; Khoudoli et al., 2004; Carpentier et al., 2005). Then, after optimisation of gel electrophoresis was performed, the protein need to be visualised using various staining methods to obtain the highest number of detected protein spot associate with mass spectrometry (MS) detection compatiblity. However each of the staining method have their own advantages and disadvantages (Table 1).

In this study, several staining methods were compared in order to choose the best method to stain protein spots resolved on 2DE gels of *Puntius javanicus* liver proteome. The resolved *P. javanicus* liver proteome on 2DE gels were visualised using conventional method of CBB blue R-250 and G-250, modified colloidal CBB G-250 (Dyballa and Metzger, 2009) and silver staining(Yan et al., 2000).

MATERIALS AND METHODOLOGY

Sample preparation

Our previous work showedthat the best extraction method wasdone by using TCA-acetone precipitation which shows the highest number of protein spots resolved on 2DE gelsas compared to other selected methods of extraction (Data not shown). Only chemical and solvent with analytical reagent grade were used. All of the solutionswere prepared using high purity deionized water (18 MΩ/cm).*P. javanicus* liver was ground to a fine powder form with mortar and pestle under liquid nitrogen flow.

Table 1: Various staining method with their own advantages and disadvantages.

Staining methods	Detection	Cost	MS compatibility	Referred protocol
Sypro ruby	High	Very expensive	Yes	Lamanda et al., 2004
CBB R-250	Low	Cheap	Yes	Choi et al., 1996
CBB G-250	Low (Higher compared to R-250)	Cheap	Yes	Anderson et al., 1991
CBBlue G-250 – Modified	High	Cheap	Yes	Dyballa and Metzger, 2009
Silver nitrate	Very high	Expensive	No	Switzer et al., 1979
Silver nitrate –Modified	Very high	Expensive	Yes but less	Yan et al., 2000

CBB= Coomassie brilliant blue

P. javanicus liver was ground to a fine powder form with mortar and pestle under liquid nitrogen flow. The sample was then mixed with TCA-acetone at the ratio of 1:10 (w/v) and protein extraction was done by vortexingthe mixture for three minutes. The extractant was then stored overnight at -25^{o}C. On the next day, the extract was centrifuged at 12,000xg for 10 minutes, the pellet was recovered and washed with ice cool acetone then again centrifuged at 12,000xg for another 10 minutes. This washing step was repeated twice. The pellet was recovered again and mixed with acetone containing 2 % of DTT, mild vortexed for three minutes and then stored for two hours at -25^{o}C. After centrifugation at 12,000xg foranother 10 minutes, the pellet was let to air dry and then dissolved with rehydration buffer (7M urea, 2 M thiourea and 4% CHAPS). The sample was gently vortexed for a minute and centrifuged at 10,000xg for 10 minutes. The supernatant was recovered and the total soluble protein concentration wasdetermined based on Bradford et al 1976 method using a series of BSA concentrationsas a protein standard.

Isoelectric focusing (IEF) and 2DE

Prior to IEF, 0.7 mg of protein sample (except for silver staining, 0.3 mg) was added to the final volume of 300 µl rehydration buffer containing freshly prepared 20mM DTT and 0.2% biolyte 3-10. This protein sample mixture was then introduced onto IPG strip (17 cm, pH 3–10) (BioRad, Singapore). IEF was then performed using PROTEAN IEF cell by initially set Rapid-gradient voltage at 250 V for 15 minutes, followed by a linear-gradient voltage settingat 10,000 V for three hours and finallyat 60,000V/h. Equilibration of IPG strip for reduction and alkylation was performed for 10 minutesin each step, then laid onto a 12.5% polyacrylamide slab gel and electrophoresis was performed at 16mA for 30 minutes and 24 mA for 2 hours and 30 minutes. After the electrophoresis was completed, the gel was washed with water for 10 minutes and repeated twice to eliminate SDS contamination which may interfere the staining process later on.

Table 2: Solution preparation and staining steps of CBB R-250 on the first gel (Gel A)

Procedure name	Solution	Protocol
Staining	40% of methanol, 10% of acetic acid and 0.025% CBB R-250 in deionized water.	Overnight incubation with gentle shaking.
Destaining • Fast	• 40% of methanol and 10% of acetic acid in water.	Incubate by shaking until the background is transparent.
Or • Slow	• Several changes of water.	
Storage	1% of acetic acid.	Incubate in 4^{o}C for a long term use.

Solution preparation and gel staining

Each gel was stainedwiththe different staining solution as shown inTable 2-5. Silver staining method was based on Yan et al., 2000. This was selected due to its compatibility report with MS.

Table 3: Solution preparation and staining steps of CBB G-250 on the second gel (Gel B)

Procedure name	Coomassie solution	Step
Staining	20% of methanol, 1.6 % of ortho-phosphoric acid, 8 % ammonium sulfate and 0.08% CBB G-250 in deionized water.	Overnight incubation with gentle shaking.
Destaining	Several changes of water.	Incubate by shaking until the background is transparent.
Storage	1% of acetic acid.	Incubate in 4^{o}C for a long term use.

Table 4: Solution preparation and staining steps of modified CBB G-250 based on Dyballa and Metzger, 2009 method on the third gel (Gel C)

Procedure name	Coomassie solution*	Step
Staining	10% of ethanol, 2 % of ortho-phosphoric acid, 5 % aluminium sulfate-(14-18)-hydrate and 0.02 % CBB G-250 in deionized water.	Overnight incubation with gentle shaking (First protein spots were appearing after10 minutes incubation).
Destaining	10% of ethanol and 2% of ortho-phosphoric acid in water.	Incubate by shaking until the background is transparent.
Storage	1% of acetic acid.	Incubate in 4^{o}C for a long term use.

*****Not filtered

Table 5: Solution preparation and staining steps of modified silver stain based on Yan et al., 2000 method with slight modification on the forth gel (Gel D)

Procedure name	Coomassie solution	Step**
Fixation	40% of methanol, 10% of acetic acid glacial in water.	Overnight incubation
Sensitization	30 % of methanol, 4% of sodium thiosulfate (prepared with 5%), and 6.8 % (w/v) of sodium acetate in water.	30 minutes incubation.
Washing	Water	Five minutes for three times
Silver reaction	10 % of silver nitrate (prepared with 2.5%) in water.	20 minutes incubation.
Washing	Water	One minutes for twice
*Developement staining	2.5 % (w/v) of sodium carbonate and 0.04% of formaldehyde in water.	Incubate until reach to the desire intensity or before the background turn to dark.
Stopping	1.46 % (w/v) in water	10 minutes
Washing	Water	Five minutes for three times
Storage	1 % of acetic acid.	Incubate in 4^{o}C for a long term use.

*Freshly prepared and add formaldehyde just before used

**Gently shake except for gel storage

All the stained gel was then photographed using 16.1 megapixel digital camera (Cybershot DSC-W570, Sony,Japan)

Results and discussion

Pink et al., 2010 mentioned that protein load more than 0.5 mg may caused overlapping of protein spot and reduced the sharpness of the spots. However, in this study we have obtained the maximum loading concentration at 0.7 mg which showsa good resolution and higher number of detection compared to the concentration of lower than 0.7 mg.The conventional staining method using CBB R-250 and G-250, a modified colloidal CBB G-250 and a modified silver nitrate staining were carried outin the present study (Figure 1). The lowest number of protein spots(78±11) was detected in Gel A after stained with CBB R-250 whereby more spots (128±17) was detected inGel B,which was stained withG-250. There was no doubt that silver staining shows the highest number (693±14) of detection but unfortunately this method exhibited less sensitive towards MS detection caused by the ε-formylation of lysine thatinduced by the presence of formaldehyde (Shevchenko et al., 1996; Pink et al., 2010). The almost similar pattern of protein spots (303±26) with in silver staining was detected in modified staining method using CBB G-250 but significant lower compared to the number of protein spot in silver stain.The sensitivity of this method is enhanced by the replacement of ammonium sulfate in conventional preparation of CBB G-250 with aluminium sulfate and ethanol which is less toxic compared to methanol. Kang et al., 2000 reported that by incorporating of aluminium ion in CBB solution, itwill enhance the binding of stain molecule to protein spots and accelerate the staining processes. Thus, the modified CBB G-250 staining method is the best method selected in this study due to its less background stained, high detection of protein spotsas well as convincing compatibility with MS.

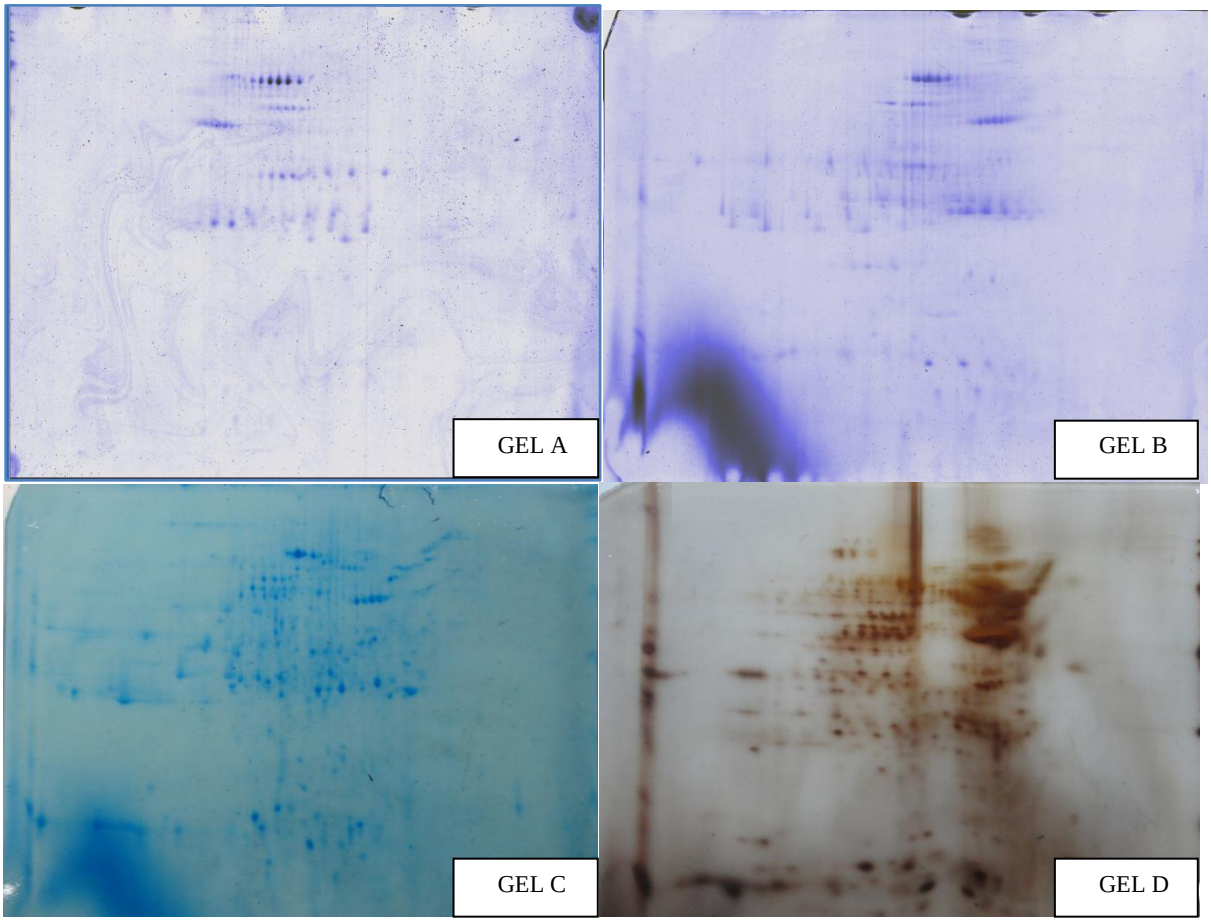

Figure 1: Different staining methodsfor 2DE gel resolved with *P. javanicus* liver proteome. Gel A: stained with CBB R-250; Gel B: stained with CBB G-250; Gel C: stained with modified CBB G-250; Gel D: stained with silver staining method. All of the gels were stained under room temperature.

Conclusion

The present study indicated that the different number of protein spotswere detected in different types of staining methods evaluated. The increasing order of number of protein spots detected is asfollowing:CBB R-250<CBB G-250< Modified CBB G-250<Silver staining. We have decided to select the modified CBB G-250 staining method for further our study on isolation and identification of *P. javanicus* liver proteome due to its comparable sensitivity with silver staining andcompatibility with MS.

However, silver staining is still would be ultilised as the reference gel with the highest number of protein spots compared to all the CBB staining methods.

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