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ANALYSIS OF THE EFFICIENCY OF POST-ELECTRPHORETIC PROTEIN STAINING USING COLLOIDAL COOMASSIE BLUE G-250

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Abstract. Constant improvements in the technique for the electrophoretic separation of proteins have resulted in development of more sensitive and less time-consuming staining methods. Despite the fact that fluorescent dyes are highly sensitive, classical methods such as silver or coomassie blue still represent very popular staining techniques. However, information concerning the comparison of the same stain but used in different protocols are sparse. Based on the results of many different authors it is difficult to clearly assess the efficiency of staining protocol. Therefore, present study was aimed at evaluating (in respect of sensitivity, simplicity and time of staining procedure and also the degree of environmental hazard) two different staining procedures with Coomassie Blue G-250 for proteins in polyacrylamide gels. A comparison of two CBB-based procedures for staining proteins showed that modified protocol of Pink is much more efficient in protein detection. This staining technique also takes less time to carry out and it is less toxic as it requires no methanol when compared to the Hoving protocol.

Key words: 1-DE, 2-DE, CBB G-250, Hoving protocol, Pink protocol

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INTRODUCTION

Constant improvements in the technique for the electrophoretic separation of proteins have resulted in development of more sensitive and less time-consuming staining methods. Despite the fact that fluorescent dyes are highly sensitive, classical methods such as silver or coomassie blue still represent very popular staining techniques [Candiano et al. 2004, Westermeier and Marouga 2005]. This results from the fact that the fluorescent stains are much more expensive than the classical ones and they require specialised equipment for the image acquisition [Westermeier 2006]. Moreover, improvements in staining protocols resulted in an approximately 20-fold increased sensitivity of prior classical staining techniques [Kang et al. 2002, Candiano et al. 2004, Skrzypczak et al. 2011].

In the available literature, there are studies in which different visualisation procedures and also the efficiencies of various staining methods are compared [Candiano et al. 2004, Rabilloud 2012]. However, information concerning the comparison of the same stain but used in different protocols are sparse [Rabilloud 2012]. Based on the results of many different authors it is difficult to clearly assess the efficiency of staining protocol. Therefore, present study was aimed at evaluating (in respect of sensitivity, simplicity and time of staining procedure and also the degree of environmental hazard) two different staining procedures with Coomassie Blue G-250 for proteins in polyacrylamide gels.

MATERIAL AND METHODS

One-dimensional gel electrophoresis (1-DE)

2 mg $\cdot$ ml^{-1} BSA solution was diluted to achieve 10, 5.2, 1, 0.5, 0.2 and 0.1 ng $\cdot$ ml^{-1} of proteins. Samples were then mixed in a 1:5 ratio with the Laemmli buffer containing: 30 ml 0.5 M Tris (pH 6.8), 10 g SDS, 50 ml glycerol, 20 ml β-mercaptoethanol, bromophenol blue). Next, the samples were heated for 10 minutes in 80°C and loaded to the polyacrylamide gels.

Before the discontinuous electrophoretic separation four polyacrylamide gels were cast as a 12% separating gel topped by a 4% stacking gel. Samples were loaded into the wells and the gels were run at 50V for 20 minutes and subsequently at 110V for 90 minutes. After electrophoretic separation, the gels were stained with two different protocols. The first one according to the method of Howing [Westermeier 2006] and the second one according to the procedure of Pink [Pink et al. 2010].

Two-dimensional electrophoresis (2-DE)

Urine samples were collected from 7-day-old calves, centrifuged (15 minutes, 4°C, 3000 rpm) and then concentrated with 3 kDa molecular weight cut-off filters AmiconUltra (Millipore).

Processed urine samples were dissolved in the 500 µl of lysis buffer containing 5 M w/v urea, 2 M w/v thiourea, 4% w/v CHAPS, 40 mM w/v Tris, 0.2% w/v 3––10 ampholytes and 2 mM TBP. Total protein concentration was estimated by the modified Bradford assay (Bio-Rad Protein Assay, Bio-Rad). Total urine proteins (58 µg) were mixed with the rehydration buffer (9 M urea, 4% (w/v) CHAPS, 100 mM DTT, 0.2% (v/v) Bio-Lyte 3–10 ampholyte) to a total volume of 125 µl and applied to 3–10.7 cm ReadyStrip™ IPG Strips (Bio-Rad). Strips were first rehydrated with rehydratation buffer and samples passively (6 h, 0V, 20°C) and then actively (12 h, 50 V, 20°C).

Isoelectrofocusing (IEF) was run in the Protean® IEF Cell (Bio-Rad) in total 9000 Vh. After IEF, the IPG strips were reduced in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% v/v glycerol) containing 1% (w/v) DTT for 15 minutes and then alkylated with equilibration buffer containing iodoacetamide (2.5% w/v) for 20 minutes.

After equilibration process strips were placed on the top of 12% SDS polyacrylamide gels (20 × 25 cm) and held in position with molten 0.5% (w/v) agarose in running buffer. The gels were run in the second dimension in Protean Plus™ Dodeca Cell™ electrophoretic chamber (Bio-Rad) at 40 V for 1.5 h and subsequently at 100 V for 2 h at 10°C. After 2-DE separation, gels were stained with two different protocols. The first one according to the method of Howing [Westermeier 2006] and the second one according to the procedure of Pink [Pink et al. 2010].

Staining with CBB G-250 according to the protocol of Hoving. The gels were washed with distilled water three times, each time for 5 minutes and then immersed in solution containing: 50% ethanol, 3% phosphoric acid. Following this, the gels were washed with distilled water three times, each time for 20 minutes and then immersed in the buffer consisting of 17% w/v ammonium sulphate, 3% phosphoric acid, 34% methanol. After one hour of incubation 0.035% w/v of CBB G-250 was added to the buffer and incubated for the next three days. After that time the gels were washed with distilled water for 20 minutes.

Staining with CBB G-250 according to the protocol of Pink et al. The gels were washed with distilled water three times, each time for 5 minutes and then immersed in solution containing: 50% ethano l,3% phosphoric acid for 3 hours. Subsequently, the gels were stain in the buffer containing: 0.02% w/v CBB G-250, 10% ethanol, 8% phosphoric acid, 5% w/v aluminium sulphate 18-hydrate for the next 3 hours. After that time, the gels were immersed with destaining buffer: 10%

ethanol, 2% phosphoric acid for 45 minutes. Destained gels were placed in the distilled water overnight in order to change their pH for neutral.

After staining all gels were scanned with the aid of GS-800™ calibrated densitometer (Bio-Rad).

RESULTS

Different concentration of the BSA solution (0.1–10 ng) were separated using SDS-PAGE and stained with Coomassie Brilliant Blue G-250 in order to assess the detection limit of CBB according to the procedures of Hoving (Fig. 1A) and Pink et al. (Fig. 1B). As a result, we have demonstrated that when the Hoving protocol was used, visible effect of staining was observed at the concentrations of 5 and 10 ng of proteins in a band. This allowed as to define the detection limit of 5 ng of proteins in a band. When the gels were stained with CBB according to protocol of Pink et al. visible bands was observed at the concentrations of 2, 5 and 10 ng of proteins in a band. Therefore, the detection limit was estimated to be 2 ng protein/band (Fig. 1B).

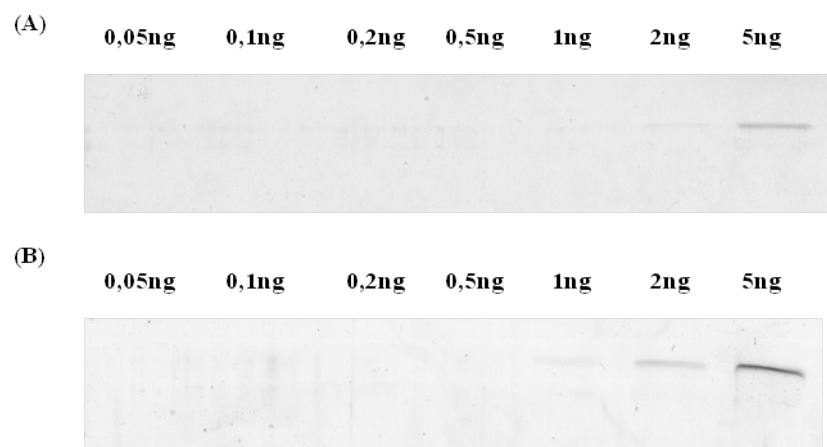

Fig. 1. Sensitivity of CBB G-250 staining (A) Hoving protocol – the lowest detectable BSA concentration was *ca.* 2 ng; (B) Pink et al. protocol the smallest detectable BSA concentration was *ca.* 1 ng

Rys. 1. Czułość barwienia błękitem coomassie G-250 według protokołu (A) Hovinga – najniższa oznaczalna koncentracja BSA wynosiła ok. 2 ng; (B) Pink i in. najniższa oznaczalna koncentracja BSA wynosiła ok. 1 ng

In the present study we have also separated calves urine proteins using two-dimensional electrophoresis (2-DE). Achieved protein profiles were stained with CBB G-250 according to the procedures of Hoving (Fig. 2) and Pink et al. (Fig. 3).

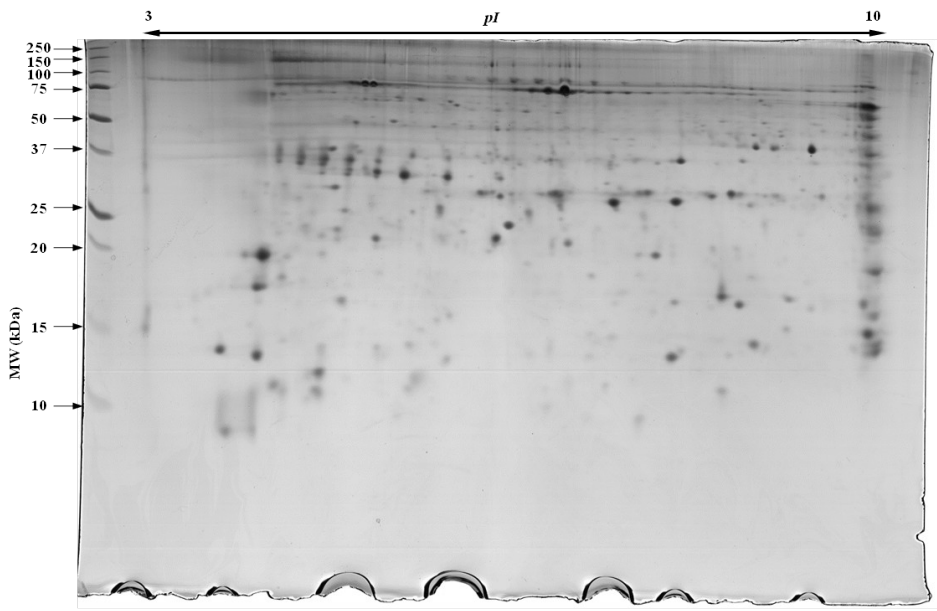

Fig. 2. 2-DE protein profile of calves urine stained with Coomassie brilliant blue G--250 according to Hoving's protocol. 58 µg of protein was applied on 7 cm nonlinear IPG strips (pH 3–10) for the first dimension, the second dimension was performed on a 12% SDS-PAGE

Rys. 2. Profil białkowy moczu cieląt barwiony błękitem coomassie G-250 według protokołu Hovinga. Białka rozdzielono z wykorzystaniem elektroforezy dwukierunkowej. Na poliakrylamidowe immobilizowane paski żelowe długości 7 cm i nieliniowym zakresie pH 3–10 nałożono 58 µg białka i poddano ogniskowaniu izoelektrycznemu. Białka w drugim wymiarze separowane były w 12% żelach poliakrylamidowych z użyciem elektroforezy SDS-PAGE

We have shown that when the Hoving protocol was used, approximately 230 protein spots were detected on the 2-D gels and in case of protocol of Pink et al. around 280 protein spots were seen (1.22 times higher).

DISCUSSION

A comparison of two staining protocols using CBB G-250 revealed that modified protocol of Pink et al. [2010] is much more sensitive (the detection threshold was 2 ng protein/spot), takes less time to carry out and it also environmentally friendly (there is no need of using methanol). In the protocol of Kang [2002] the detection threshold was estimated to be 1 ng BSA/band (1 ng protein/spot). On the

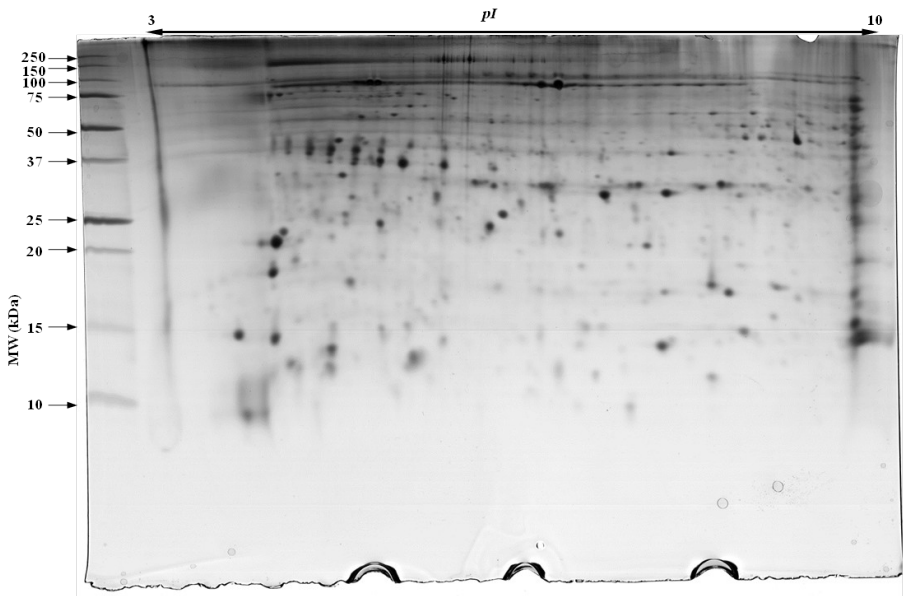

Fig. 3. 2-DE protein profile of calves urine stained with Coomassie brilliant blue G--250 according to Pink et al. protocol. 58 µg of protein was applied on 7 cm nonlinear IPG strips (pH 3–10) for the first dimension, the second dimension was performed on a 12% SDS-PAGE

Rys. 3. Profil białkowy moczu cieląt barwiony błękitem coomassie G-250 według protokołu Pink i in. białka rozdzielono z wykorzystaniem elektroforezy dwukierunkowej. Na poliakrylamidowe immobilizowane paski żelowe długości 7 cm i nieliniowym zakresie pH 3–10 nałożono 58 µg białka i poddano ogniskowaniu izoelektrycznemu. Białka w drugim wymiarze separowane były w 12% żelach poliakrylamidowych z użyciem elektroforezy SDS-PAGE

other hand in the study of Dyballa and Metzger [2009] the sensitivity of this protocol was assessed to be in a range of 4–6 ng/band. Pink et al. [2010] developed a modified CBB staining method of Kang [2002] and achieved the highest detection threshold corresponding to the concentration of 0.75 ng · ml^{-1} of BSA. Additionally, these authors defined the sensitivity of this method at a level of 2 ng protein/spot what was consistent with the results of the present study [Pink et al. 2010]. Pink et al. [2010] also modified the staining time by shortening it to 24 hours. However, it seems that incubation the gels in the staining buffer overnight instead of 3 hours would rather increase the detection threshold. On the other hand, it would considerably prolong the overall staining time what may result in embedding the CBB G-250 particles on the surface of the gels.

We have achieved a lower detection threshold (approximately 5 ng protein per spot) when the protocol of Hoving was applied. Surprisingly we have achieved a higher detection when compared to this in the source material that was estimated at the level of 1 ng protein/spot [Westermeier 2006]. The observed phenomenon may be attributed to differences in the purity of chemical reagents and the quality of the water that were used during staining process. Nevertheless, this method is time-consuming, it takes 4–5 days to carry it out. Another important disadvantage of this method is the presence of methanol in the buffer applied prior main staining, what makes it less environmentally friendly and less safe for the person who perform the staining when compared to the method developed by Pink et al. [Dyballa and Metzger 2009]. The results of the present study clearly indicate that the protocol published by Pink et al. [2010] is much more sensitive, takes less time to carry out and it also environmentally friendly when compared to the Hoving procedure.

Fluorescent stains are considered as the most sensitive method for detecting protein spots [Westermeier and Marouga 2005]. However, improvements of existing staining protocols using CBB resulted in detection comparable to commonly used fluorescent stain – Sypro® Ruby (approximately 1 ng protein/spot) [Candiano et al. 2004]. For example, Candiano et al. [2004] developed the Blue Silver method based on Neuhoff method. In this staining technique the authors used solution containing: 0.12% w/v CBB G-250, 10% w/v ammonium sulphate, 10% phosphoric acid and 20% methanol.

CONCLUSIONS

In conclusion, a comparison of two CBB-based procedures for staining proteins showed that modified protocol of Pink et al. [2010] is much more efficient in protein detection. This staining technique also takes less time to carry out and it is less toxic as it requires no methanol when compared to the Hoving protocol.

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ANALIZA SKUTECZNOŚCI POST-ELEKTROFORETYCZNEJ DETEKCJI BIAŁEK Z UŻYCIEM KOLOIDALNEGO ROZTWORU BŁĘKITU COOMASSIE G-250

Streszczenie. Ciągłe udoskonalanie elektroforetycznych metod rozdziału białek wymusiło konieczność poszukiwania bardziej czułych i mniej czasochłonnych metod ich wizualizacji. Pomimo pojawienia się na rynku, charakteryzujących się wysoką czułością, barwników fluorescencyjnych, nieustającą popularnością cieszą się barwniki klasyczne, takie jak sole srebra czy błękit coomassie. Bardzo rzadko spotyka się publikacje, w których porównywane są protokoły wykorzystujące ten sam barwnik. Na podstawie wyników badań różnych autorów trudno jest jednoznacznie określić skuteczność danego protokołu barwienia. W związku z powyższym podjęto badania, których celem była ocena (pod względem czułości barwienia, czasochłonności, prostoty wykonania oraz stopnia zagrożenia środowiska) dwóch różnych procedur barwienia białek w żelach poliakrylamidowych z wykorzystaniem Coomassie Brilliant Blue G--250. Przeprowadzona w doświadczeniu analiza porównawcza metod barwienia z użyciem błękitu coomassie G-250 wykazała, wyższą skuteczność detekcji białek z wykorzystaniem protokołu Pinka. Metoda ta jest również mniej czasochłonna od protokołu opracowanego przez Hovinga, a także nie wymaga zastosowania obciążającego dla środowiska metanolu.

Słowa kluczowe: 1-DE, 2-DE, CBB G-250, protokół Hovinga, protokół Pinka

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