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Coomassie blue staining for high sensitivity gel-based proteomics☆

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ABSTRACT

Gel electrophoresis, particularly one- (1DE) and two-dimensional electrophoresis (2DE), remain among the most widely used top-down methods for resolving and analysing proteomes. Detection of the resulting protein maps relies on staining (i.e. colloidal coomassie blue (CCB) or SYPRO Ruby (SR), in addition to many others). Fluorescent in-gel protein stains are generally preferred for higher sensitivity, reduced background, and wider dynamic range. Although traditionally used for densitometry, CBB has fluorescent properties. Indeed, infrared detection of CCB stained protein was comparable to SR, with BioSafe (Bio-Rad) and the Neuhoff formulation (NCCB) identified as potentially superior to SR; a minor sensitivity issue encountered in gel-resolved proteomes; might have been due to the unified staining protocol used. Here the staining protocol for both CCB formulations was optimised, yielding improved selectivity without affecting sensitivity; the resulting linear dynamic range was similar for BioSafe and NCCB and somewhat better than SR. 2D gel-based analyses of mouse brain and *Arabidopsis thaliana* (leaf) proteomes indicated markedly superior spot detection using the NCCB formulation. Thus more sensitive, quantitative in-gel protein analyses can be achieved using NCCB, at a fraction of the cost. This article is part of a Special Issue entitled: From Genome to Proteome: Open Innovations.

1. Introduction

Proteomic analyses employ a number of techniques including liquid chromatography and mass spectrometry (MS). Among the most widely employed are gel-based methods, in particular 2DE, the original and still the highest resolution top-down analytical technique available for proteomic analyses. With this critical breadth of application, 2DE also continues to be refined and enhanced to yield proteomic analyses of the highest calibre [1–10]. In a single analysis, 2DE provides a full visualisation of the proteome including information on protein charge, molecular weight, abundance, isoforms, and post-translational modifications; all this and multiple parallel replicates in a single analysis as opposed to fewer replicates and lower information content in the serial analyses demanded by other methods (i.e. variations on MS-based 'shotgun' approaches). While the latter are useful rapid scanning tools, overall they tend to yield lower quality protein identifications, likely tending to higher false-positive and -negative rates, and the quantitative nature of the analyses remain under debate.

Abbreviations: 1DE, one dimensional gel electrophoresis; 2DE, two dimensional gel electrophoresis; AS, ammonium sulphate; BGAL, beta-galactosidase; CA, carbonic anhydrase; NCCB, Neuhoff colloidal coomassie blue; IPV, inter-protein variability; IRFD, infrared fluorescent detection; LDR, linear dynamic range; LLD, lowest limit of detection; LYS, lysozyme; MS, mass spectrometry; PHOSB, phosphorylase b; SR, SYPRO Ruby; STI, soybean trypsin inhibitor; TYRP, trypsinogen; S^2/BG, signal squared to background ratio.

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Despite 2–3 decades of hackneyed comments in review articles concerning the supposed limitations of 2DE (i.e. poor resolution of hydrophobic proteins, and so forth), refinements and optimisations over the last decade or more, from sample preparation/extraction, through resolving conditions, to image analysis, have resulted in proteomic analyses of the highest calibre [5–7,10,11]. Thus, contrary to decade-old concerns, it is now reasonably well established that the main issue in 2DE is not protein resolution but rather detection [12,13]; additionally, multiple parallel gels in a given analysis are generally sufficient to provide the necessary protein for subsequent identification by MS. However, with the need for visualisation and quantitation, it is essential that a stain provides the highest possible signal to background ratios (S^2/BG) and protein detection sensitivity (i.e. the lowest limit of detection — LLD), deliver the largest possible linear relationship between amount of protein and staining intensity (i.e. linear dynamic range — LDR), the lowest possible staining variability between different proteins (i.e. inter-protein variability — IPV), and be compatible with downstream characterisation techniques, most notably MS. It is also desirable that the stain employed be easy to prepare and use, safe and reasonably cost-effective. The serious need for more rigorous and routine characterisation of protein stains with regard to specific criteria has been reviewed in detail [12].

In-gel protein visualisation has been facilitated by the use of an extensive and diverse array of stains. For 50 years, coomassie blue (CB) has been used to quantitatively assess proteins in-gel by densitometry [14]. Although protein detection down to ~30 ng could be achieved [15–17] it wasn't until 1985 that this was further enhanced with the introduction of a colloidal formulation [18,19]. Creating an equilibrium between CB colloids and free CB molecules meant fewer free dye molecules and thus an overall reduced background staining that resulted in enhanced detection sensitivity [18,19]. The necessary search for ever superior protein detection reagents led to the development of silver stains, negative zinc staining and myriad others [12,20–23]. However, the most significant recent contribution to protein detection was the development of fluorescent dyes as they are typically more sensitive, have a wider LDR, are less prone to background issues, and are compatible with MS. Although an array of fluorescent stains are currently available for in-gel protein detection, SYPRO Ruby (SR) is the most popular commercial reagent for proteomic applications. SR has a suggested in-gel protein sensitivity of ~1–2 ng, and a broad LDR [24,25]; unfortunately, the substantial financial investment required is beyond the capacity of most labs. Sensitivity, selectivity, low IPV, and a wide LDR – at a reasonable cost – are the critical minimal qualities for an in-gel protein detection protocol.

In 2006, protein-bound CB was shown to fluoresce in the near-infrared [26]. Preliminary assessment of CB infrared fluorescence detection (CB-IRFD) of both soluble and membrane proteomes indicated not only detection similar to that of SR but even more importantly, an enhanced signal-to-background relative to SR [11]. These results suggested IRFD following colloidal CB (CCB) staining as a competitive and possibly superior fluorescent alternative. The quantitative capacity of CB-IRFD was thoroughly examined in 2008 [10]. This study showed that the CCB formulation developed by Neuhoff (NCCB) [18,19,27] and a commercial formulation developed by Bio-Rad (Bio-Safe™ Coomassie Stain) yielded the highest performance in terms of sensitivity, selectivity, IPV, and LDR. However, testing on 2D gels revealed slight but important differences between in-gel protein assessment by CB-IRFD vs. SR. While CCB staining provided the more quantitatively trustworthy approach, a significant blunting of SR fluorescence at increasing amounts of protein enabled the detection of a few additional proteins (i.e. amounting to 0.6% of the proteome). The simplest explanation for this difference was that the unified staining protocol used to enable the large-scale comparison of dye formulations in that study diminished the performance of the NCCB staining protocols as originally published [10,17,27,28]. Thus, here we have systematically analysed CB-IRFD of both the BioSafe and NCCB stain formulations using established criteria, including the LLD, S^2/BG, IPV, and LDR. To validate the resulting optimised CCB staining protocols, the detection of gel-resolved mammalian and plant proteomes was assessed relative to SR. Overall, this study establishes that CB-IRFD, in particular with the NCCB formulation used, is a high sensitivity, safe, and cost-effective method for in-gel protein detection that serves as a superior alternative to SR.

2. Materials and methods

2.1. Materials

All consumables were of electrophoresis grade or higher quality. Electrophoresis consumables including acrylamide, tris-glycine SDS buffer, Coomassie Brilliant Blue G-250 dye were purchased from Ameresco Inc. (Solon, OH). Bio-Safe™ Coomassie Stain, SYPRO Ruby protein gel stain, Bio-lyte broad range carrier ampholytes (pH 3–10), and each of four Bio-lyte narrow range ampholytes (pH 3–5, 6–8, 7–9 and 8–10) were purchased from Bio-Rad Laboratories (Hercules, CA). All other consumables, including a number of isolated protein standards were purchased from Sigma (St. Louis, MO). Double distilled water was used throughout.

2.2. Standard proteins

Isolated proteins, beta-galactosidase (BGAL), glycogen phosphorylase B (muscle form; PHOSB), bovine serum albumin (BSA), carbonic anhydrase 1 (CA), pancreatic anionic trypsinogen (TYRP), soybean trypsin inhibitor (STI), and lysozyme C precursor (LYS) were dissolved in distilled water, and insoluble material was removed by centrifugation at 2000 *g* for 5 min. The concentration of each isolated protein was determined using the Beer–Lambert Law; absorbance was measured at 280 nm using the POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Each determination was in triplicate, and the final purity confirmed as previously described [29]. These were then combined into a single standard mixture in which each protein was of the same concentration.

2.3. Native protein preparation

Mouse brains and *Arabidopsis thaliana* leaves were processed by automated frozen disruption [6]. Samples were separated

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