1 A 'green' approach to fixing polyacrylamide gels

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Abstract

Handling chemicals that require specific safety precautions and protections generates the need for hazardous waste removal and transportation costs. With the growing effort to reduce both cost per analysis and the environmental footprint of research, we report an effective alternative to the widely used methanol/acetic acid gel fixation solution. 1.0M citric acid dissolved in 5% acetic acid (C3A) provides comparable results following both SDS-PAGE and two-dimensional gel electrophoresis, while also eliminating waste removal costs.

Keywords

Fixation
Proteomics
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Proteomes
Eco-friendly

Non-standard abbreviations

1DB – SDS-PAGE sample buffer
1DE – One-dimensional gel electrophoresis
2DB – 2DE sample buffer
2DE – Two-dimensional gel electrophoresis
AU – Arbitrary units
C3A – Citric acid + acetic acid
cCBB – Colloidal Coomassie Brilliant Blue
nIRFD – Near-infrared fluorescence detection

High sensitivity proteome analysis is critical to top-down proteomics, with optimized two-dimensional gel electrophoresis (2DE) being the most mature and widely used approach to effectively resolve the constituent proteoforms [1,2]. This capacity to routinely resolve proteoforms - the functional protein species - is thus central to understanding molecular mechanisms, and identifying meaningful biomarkers and therapeutic targets. Here we continue a long-standing objective to assess, refine and optimize the process of 2DE [2–4].

Gel fixation after the electrophoretic resolution of proteoforms serves to precipitate the species within the gel matrix at their point of resolution via an acidic wash, which also enhances their subsequent interaction with stains by removing SDS [5,6]. Currently, a methanol or ethanol and acetic acid combination is routinely used as a fixative, particularly prior to staining with colloidal Coomassie Brilliant Blue (cCBB) [4,7–12]. Unfortunately, when methanol is used for gel fixation following SDS-PAGE, it induces methylation at the side chains of aspartate and glutamate; substitution with ethanol results in ethylation of these same residues. In both instances the reactions are promoted by acetic acid [13,14]. Additionally, due to the chemical properties, there is a need for specific safety precautions and protection, as well as the introduction of rather substantial hazardous waste removal and disposal costs. Notably, this comes with the increased risk of handling and transporting hazardous waste, and the additional risks posed to the environment [15]. While methanol has negative effects on the natural environment it is also hazardous to human health and at the high concentrations used, ethanol is also considered hazardous [16–18]. There has thus been a growing effort to improve the environmental footprint of research, where possible and appropriate, by replacing current chemical components with

‘greener’ alternatives, that also reduce the overall cost of research [19,20]. The purpose of this study was to identify a greener, cost-effective alternative fixation solution that eliminates the need to handle and dispose of hazardous chemicals and thus also reduces risk to the environment. Additionally, we have sought to reduce nonspecific modifications of protein species that is unavoidable with the use of methanol and ethanol. As previously, we used one-dimensional SDS-PAGE (1DE; the second dimension of 2DE) to test a potential refinement and then quantitatively confirmed improvement using a full 2DE protocol [7,8]. Here, citric acid was used to replace methanol as it is low-cost, non-volatile and has the EPA Safer Chemical/Safer Choice/Green Circle rating [21–23]. Compared to methanol (and ethanol), citric acid is rapidly degraded in wastewater treatment facilities, surface waters and soil [24–26]. While many combinations were tested (0.3M – 1.5M), here we report that a combination of 1.0M citric acid dissolved in 5% acetic acid (or commercial vinegar) yielded results consistent with the routine mixture of 10% methanol/7% acetic acid.

All chemicals, except for commercial vinegar, were of ultra-pure or analytical grade. Samples of previously snap-frozen whole rat brain (*Rattus norvegicus*) and green lentils (*Lens culinaris*) were homogenized via automated frozen disruption as previously described [8,27]. The resulting powdered total rat brain and total lentil samples were each solubilized in 1D buffer (1DB; 25mM Tris [pH 8.8], 2% (w/v) SDS, 50mM DTT, 7.5% (w/v) glycerol, 0.001% (w/v) bromophenol blue, and 1x protease inhibitors); aliquots of the homogenized rat brain were also solubilized in 2DB (8M urea, 2M thiourea, 4% CHAPS, and 1x protease inhibitors) [27]. Unstained broad range (10-200 kDa) protein standards (New England Biolabs) were solubilized in 1DB. Total protein concentration was assessed as described [28]. Aliquots were snap-frozen and stored at -80°C until needed.

1DE and 2DE were carried out as previously described [8] with minor modifications. SDS-PAGE was carried out using 1.0 mm thick 12.5%T resolving gels overlaid with a 5%T stacking gel with 5 mm wide wells. Samples in 1DB were vortexed, sonicated for 10 min, heated at 100°C for 10 min, and cooled to room temperature prior to loading. After resolution, gels were cut vertically down the middle (equal sample loading on either side) and fixation was in either 10% (v/v) methanol/7% (v/v) acetic acid (*control*) or 0.3-1.5M citric acid (CA; w/v) in either 5% glacial acetic acid (AA; v/v) or commercial vinegar (5% [v/v] AA) for 1 hour at RT with gentle rocking. Following fixation, gels were washed with 15-20 mL volumes of ddH₂O with gentle rocking at room temperature 3 x 20 min. Notably, with 5% acetic acid alone, the signals of low molecular weight species in both the brain and lentil samples were significantly smaller than in control gels ($t[10] = 8.438, p < 0.05$; $t[10] = 5.051, p < 0.05$). In contrast, with 1.0M citric acid alone, the signal-to-noise ratio of the protein standards was significantly lower than in the control ($t[4] = 3.655, p < 0.05$). The CA+AA (C3A) fixative and cCBB staining solution were made fresh immediately prior to use. With minor modifications from Neuhoff et al., cCBB stain consisted of 2% (v/v) phosphoric acid, 10% (w/v) ammonium sulfate, 0.1% (w/v) CBB, and 20% (v/v) methanol, made to volume with ddH₂O, combined in this order with constant stirring [8,9,28,29]. Gels were stained for 20 hours at room temperature with gentle rocking. Gels were subsequently washed 5 x 15 min with 0.5M NaCl destain solution and imaged immediately by nIRFD on an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare, Buckinghamshire, UK) using 685/≥750 nm ex/em [8]; pixel size was set to 50µm and PMT gain to 600 V. Deep imaging was then carried out as previously described [30].

Intensity of bands on 1DE gel images (16-bit tiff) were analyzed using Image Lab (v. 6.0.1; Bio-Rad Laboratories, Inc.) and data processed using Excel (v. 16.31) and Prism (v. 8.3.0;

GraphPad Software). Only well-resolved protein bands were measured (i.e. dark bands with minimal streaking and no leading or trailing edges; **Figure 1A**). Signal intensity was quantified as volume (the sum of the pixel volumes within the specified areas with background subtracted) in arbitrary units (AU; lane profile option, Image Lab v. 6.0.1). Signal-to-noise ratio (S/N) was calculated as the ratio of lane signal intensity to local background intensity. Errors are reported as standard error of the mean (SEM). Statistical analyses were with Prism (v. 8.3.0; GraphPad Software); $p < 0.05$ was considered significant.

Quantitatively, there were no obvious differences in band intensities between replicate gels using 0.3M and 1.0M C3A however, some variability was seen with 0.6M C3A (**Figure 1B**). Inter-gel protein signal was highly reproducible with all C3A fixatives, yielding the same sensitivity as the control (i.e. methanol/acetic acid) (**Figure 1C**). S/N for 0.3M C3A was significantly lower than the control for both the commercial protein standards ($t(66) = 2.773$, $p < 0.05$) and the lentil sample ($t(66) = 4.714$, $p < 0.05$) along with 0.6M C3A S/N being significantly lower for the lentil sample ($t(66) = 3.212$, $p < 0.05$). There were no significant differences seen in S/N with 1.0M C3A (**Figure 1D**). Notably, 1.2M and 1.5M C3A were also tested but did not perform better than 1.0M C3A (data not shown). Thus, considering the variability seen with 0.3M and 0.6M, 1.0M C3A was tested as a fixative for 2D gels.

Figure 1. 1DE data. (A) Representative 1D gel (1.0M C3A) of extracted proteins from rat brain and green lentil with assessed bands indicated. Band intensity signals with SEM for (B) unstained protein standards, brain and lentil. (C) Inter-gel protein signal (intensity) for all

experiments. **(D)** Signal-to-noise ratio for all sample types across conditions. In each case, control represents standard fixation with methanol/acetic acid. There were $n = 2$ technical replicates (i.e. lanes) per sample on 3 separate gels per experiment, and experiments were independently replicated 2-3 times. ‘*’ indicates statistically significant differences ($p < 0.05$).

Qualitatively, all 2D gels yielded comparable spot patterns indicating resolution of proteoforms across the full pI and MW ranges (**Figure 2A and B**). All 2DE gel images were analyzed using Delta2D (v. 4.0.8; DECODON), as previously described [8]. Images were warped and fused followed by automated spot detection; artefacts and molecular weight markers were manually excluded from analysis using the spot editing tool. The final spot ‘pattern’ was then transferred to all images to obtain absolute spot volumes. The generated quantitation table provided average normalized spot volumes across both conditions, coefficient of variation, and difference in protein abundance ($p < 0.05$; false discovery rate = 1%). Spot differences with a relative standard deviation of $\leq 30\%$ and fold changes of ≥ 2 (increase or decrease) relative to the control, were considered genuine differences. No spots were found to be significantly different between the control and 1.0M C3A conditions. To determine individual gel spot count, automated spot detection was carried out on raw *unfused* images and there were no significant differences seen between the control (503 ± 13 spots) and 1.0M C3A (509 ± 11) conditions (**Figure 2C**). Average normalized spot volumes were found to be linearly related in comparing the fixation conditions, with no outliers (**Figure 2D**). The few data points slightly deviating from the linear relationship changed by ≤ 0.3 -fold and were thus not considered significant. No all-or-none or significant spot abundance changes were detected. Additionally, there were also no significant spot differences between the control (643 ± 19 spots) and 1.0M C3A (641 ± 21 spots), following deep imaging, confirming identical quality and ensuring fixation of even very low abundance proteoforms [4,30].

Figure 2. 2D data. Representative 2D gel images of proteins extracted from rat brain after fixation under (A) control or (B) 1.0M C3A conditions. (C) Total spot count; $n = 9$ gels. Error is SEM. (D) Scatter plot of average normalized spot volumes. There were $n = 3$ technical replicates (i.e. separate gels) per experiment, and experiments were independently replicated 3 times.

In conclusion, 1.0M citric acid in 5% acetic acid (C3A) is an effective alternative to fixation with methanol and acetic acid for 2D gels. Relative to methanol, citric acid is internationally accepted as a ‘green’ chemical alternative [22,23]. Thus, in addition to being less harmful to humans and the environment, C3A also reduces cost, is ‘green’ (i.e. can be disposed of as wastewater), and thus eliminates hazardous handling and waste disposal [16,17,22,23,31,32]. Notably, commercial vinegar was as effective as dilute research-grade glacial acetic acid (not shown); nonetheless we continue to use the latter to ensure quality and reliability. These results contribute to our ongoing goal of accurately and quantitatively retrieving proteome information from diverse sample types. We do note though that there is no single method, of any type and application, that is ‘perfect’ for every proteoform in every sample type. However, with quantitative data from two disparate sample types as well as established protein standards, we are confident that the results are broadly applicable. Minimally, the current approach retains established standards but as a green alternative.

Although many refinements and modifications have been made to this 2DE-based top-down proteomic methodology over the last 20 years [1–4], protocols should nonetheless be continually re-assessed and updated to ensure the most stringent analyses possible. As there is currently a substantial focus on reducing environmental footprints, this work aimed to establish a ‘greener’ alternative to the traditional fixative mixture of methanol/acetic acid; of the multiple solutions tested, 1.0M C3A yielded results identical to the methanol/acetic acid solution. Our goal was to reduce the risk of nonspecific modifications of protein species and reduce the rather large volumes of hazardous waste produced by large-scale gel-based protein analyses. As this has been accomplished, we have introduced this as a formal refinement of our current 2DE protocol.

Declaration of interest

None.

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