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Improved membrane protein solubilization and clean-up for optimum two-dimensional electrophoresis utilizing GLUT-1 as a classic integral membrane protein

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Abstract

Two-dimensional (2-D) electrophoresis remains a primary resolving tool for proteomic analyses. The final number of proteins resolved by 2-D electrophoresis depends on their respective solubility, size, charge, and isoelectric point. While water-soluble cytosolic proteins have often been well represented in 2-D maps, the same is not true with membrane proteins. Highly hydrophobic in nature, membrane proteins are poorly resolved in 2-D gels due to problems associated primarily with sample preparation. This is of especial concern in neuroscience studies where many proteins of interest are membrane bound. In the current work, we present a substantially improved sample preparation protocol for membrane proteins utilizing GLUT-1 glucose transporter from brain microvessels as an example of a typical membrane protein. GLUT-1 (SLC2A1; Solute carrier family 2 (facilitated glucose transporter), member 1) is a 55 kD glycoprotein that contains 12 membrane spanning alpha helices that impart the protein its characteristic hydrophobicity. GLUT-1 based on its amino acid sequence has a theoretical isoelectric point (pI) of 8.94. Using a combination of the non-ionic detergents, n-dodecyl- β -maltoside (DDM) and amido sulfobetaine-14 (ASB-14) for sample solubilization, and a modification of the Biorad 2-D clean-up protocol involving trichloroacetic acid (TCA)/acetone, we obtained near complete solubilization of GLUT-1 and greater than 90% recovery of this membrane protein in 1-D and 2-D Western blots. The total number of proteins resolved also increased dramatically in Deep Purple[™] total protein stains using our improved protocol.

Keywords

2-D electrophoresis; membrane proteins; solubilization; clean-up; n-dodecyl- β -maltoside; ASB-14; brain microvessels; GLUT-1

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Introduction

Ever since its inception in the 1970s, two-dimensional (2-D) electrophoresis has been a powerful research tool but challenging, due to the issues associated with protein solubilization, aggregation, oxidation, precipitation, as well as interfering substances in isoelectric focusing. These technical hurdles are especially important when using the 2-D approach for analyzing membrane proteins (Braun et al., 2007). Although alternate technologies such as multidimensional protein identification technology (MuDPIT), isobaric tags for relative and absolute quantification (iTRAQTM), protein arrays have proven successful for the identification of membrane proteins, they do not possess the strengths of a 2-D analysis that include excellent quantitation in combination with separation of protein variants (Gorg et al., 2004). Therefore, 2-D is still a preferred method even for the analysis of membrane proteins in many circumstances. The availability of commercial immobilized pH gradient (IPG) strips led different research groups to follow an empirical approach that best suited their protein(s) of interest and solve some of the problems listed above. Recent progress in 2-D electrophoresis has made it much broader in scope with application to proteins that are extremely basic, hydrophobic, or of a large mass that were once absent from the 2-D maps or only present as streaks. Some of these developments include: newer detergents such as ASB-14, capryl sulfobetaine (SB3-10), DDM that have improved the protein solubility together with the introduction of thiourea as an additional chaotrope to urea (Luche et al., 2003); better reducing agents such as tris (2-carboxyethyl) phosphine (TCEP), and β -hydroxyethyl disulfide (HED) (Olsson et al., 2002), and new deuterated alkylating agents such as 4-vinylpyridine (4-VP) (Sebastiano et al., 2003); and also the development of clean-up kits that reduce the ionic contaminants and help in producing clean streak-free 2-D maps. As can be appreciated from above, the most important step in 2-D electrophoresis of membrane proteins is the sample preparation involving solubilization, reduction, alkylation, and clean-up. Solubilization of membrane proteins for 2-D electrophoresis involves careful selection of the detergents that are non-ionic or zwitterionic for isoelectric focusing (IEF) compatibility and result in efficient solubilization of the protein of interest with minimal aggregation. Traditional approaches using 2-D compatible protein extraction protocols (e.g. 7M urea, 2M thiourea, Chaps, or others) have resulted in incomplete solubilization of membrane proteins (Luche et al., 2003, Martins-de-Souza et al., 2007); therefore we first undertook a solubilization experiment to obtain a buffer that is efficient in solubilization of membrane proteins. Our approach in this study was utilizing brain microvascular glucose transporter GLUT-1 that mediates glucose uptake into the brain as a typical membrane protein for neuroscience studies. This transporter was also shown to be absent from 2-D maps of brain endothelial cells when the above traditional solubilization buffer was used (Lu et al., 2007). With our expertise in glucose transporters of the brain (Vannucci et al., 1997, Simpson et al., 2001), GLUT-1 (SLC2A1; Solute carrier family 2 (facilitated glucose transporter), member 1) was chosen as the example membrane protein as it contains 12 membrane-spanning α helices, each containing 20 amino acid residues that impart the protein its characteristic hydrophobicity. For details of the 2-D sample preparation, refer to our previous review article (Freeman and Hemby, 2004), and to (Rabilloud et al., 2007). Solubilization followed by reduction and alkylation is a major challenge for hydrophobic membrane proteins and as such there is an increasing literature directed at this stage of sample preparation (Lundback et al., 2003, Sebastiano et al., 2003, Hopkinson et al., 2005, Rabilloud et al., 2007); however little optimization has been performed on the sample clean-up methods for membrane proteins. Precipitation methods currently available that rely on TCA, acetone, chloroform/methanol etc., do remove the interfering salts, ionic detergents, nucleic acids, lipids but are prone to artifacts such as incomplete quantitative and qualitative sample recovery (Jiang et al., 2004). The qualitative loss of sample proteins is not well appreciated and could be a major flaw in many experimental designs. Several commercial kits do use the above precipitation protocols to

achieve sample clean-up; however there is no information about the extent of sample recovery. In this study, we present a sample precipitation/clean-up protocol modifying the well-known ReadyPrep™ Bio-Rad 2-D clean-up kit and compare the sample recovery of membrane proteins.

Materials and Methods

Rat brain microvessels (~ 100–150 mg) were isolated from three Sprague Dawley rats according to our previously published protocol (Fisher et al., 2007). The final undigested rat brain microvessels (UR; protein content ~ 800–1000 µg) were re-suspended in 10 mM HEPES, 1 mM EDTA, 250 mM Sucrose plus protease inhibitor cocktail with final pH to 7.4 (HES+PI). Rat whole brain homogenate samples (RB) were obtained by homogenizing whole brains from Sprague Dawley rats in HES + PI. Undigested bovine brain microvessels (UD) were obtained from R.A. Hawkins (Finch University of Health Sciences/The Chicago Medical School) (Sanchez del Pino et al., 1995) re-suspended in PBS buffer. Membrane vesicles from brain endothelial cells were prepared as described previously (Sanchez del Pino et al., 1992). Briefly, isolated microvessels from bovine cerebral cortices were digested with collagenase Type IA (180 units collagenase/ml) and homogenized, and the released membranes were separated into five fractions at the interfaces of a discontinuous Ficoll gradient (0, 2, 5, 10, and 20%). The amount of abluminal and luminal membrane in each fraction was determined by the activities of two markers: system A transport of *N*-(methylamino) isobutyric acid for abluminal membrane and g-glutamyl transpeptidase for luminal membrane. Protein estimation for all the samples used in this study was performed with the better Bradford Coomassie Plus assay (Pierce) according to the manufacturer's protocol that has a maximum concentration variation within 5%. The detergents ASB-14 and DDM were obtained from Calbiochem (San Diego, CA), immobililine strips, ampholytes, Destreak™ (Hydroxy ethyl disulphide), urea, thiourea and Deep Purple™ were obtained from GE Healthcare (Piscataway, NJ). Antibodies to GLUT-1 C-terminal 20 amino acids were kindly produced by Hoffman La Roche (Nutley, NJ) (Simpson et al., 2001). Criterion™ precast gels and ReadyPrep™ 2-D clean-up kit was obtained from Bio-Rad Laboratories Inc. (Hercules, CA). Chemiluminescence kit was obtained from Perkin Elmer Inc. (Billerica, MA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO). Water used for all the experiments was de-ionized proteomic grade Milli-Q water (Millipore).

Solubilization/Alkylation

Solubilizing buffer (SB) containing 2% DDM, 1% ASB-14, 5M urea, 2M thiourea, 10 mM TCEP, and 10 mM Tris (pH 8.0) was used to solubilize 200µg total protein of each of the samples for 2 hours at room temperature followed by centrifugation at 100,000×g to obtain a soluble supernatant (S). The pellet (P) obtained here was re-suspended in the same volume of the SB buffer as the supernatant followed by freezing at –80°C. An aliquot of the supernatant was also frozen down at –80°C for later use. The supernatant was then subjected to alkylation with 4-VP by the addition of 10 µl of 200 mM 4-VP to a 100 µl sample in dark for 1 hour at room temperature. The reaction was then stopped by the addition of equimolar amounts of DTT. An aliquot of the alkylated sample was stored at –80°C and the remaining amount was subjected to the 2-D clean-up protocol.

2-D Clean-up

Bio-Rad ReadyPrep™ 2-D clean-up kit was utilized for the protein isolation. This kit is based on TCA/acetone precipitation of proteins for separation of the sample proteins from interfering nucleic acids, salts, lipids etc. The protocol for 100 µl (max. 500 µg total protein) of the sample consists of adding 300 µl of precipitation reagent 1 (TCA based solution), vortexing and incubating on ice for 15 min. 300 µl of precipitation reagent 2 (proprietary) is

then added followed by brief vortexing and finally centrifugation at 20,000×g for 5 min at 4°C. The supernatant is aspirated and a brief centrifugation for 5 min at 20,000×g, 4°C is performed to remove any traces of reagents 1 and 2. The pellet is then washed with 40 µl of wash reagent 1 followed by centrifugation at 20,000×g for 5 min and then discarding the wash by aspiration. Proteomic grade water (25 µl) was then added on top of the pellet followed by vortexing briefly and adding 1 ml of prechilled wash reagent 2 (Acetone) and 5 µl of wash additive. The mixture was vortexed for 1 min and then incubated for 30 min at -20°C. After the incubation period, the tube is centrifuged at 20,000×g for 5 min at 4°C followed by discarding the supernatant. The tube is then centrifuged briefly and remaining wash is discarded followed by briefly air drying (< 5 min) the pellet at room temperature. The final pellet is re-suspended in SB, the volume being the same as that of the starting alkylated sample. In the modified partial protocol, clean-up was stopped after washing the protein pellet with wash reagent 1. This is the pink reagent in the kit, which is a water based alkaline buffer intended to neutralize the trace amounts of the TCA based reagents 1 and 2. The re-suspended samples were vortexed for 3–5 min at room temperature and finally stored at -80°C for at least 1 day before applying to IPG strip (to aid in sample re-solubilization resulting from shear forces of ice crystals).

1-D SDS-PAGE/Western Blotting/Image Quantitation

An equal volume aliquot from pellet and supernatant deriving from the same SB treatment as above (not alkylated for the solubilization experiment) was added to the buffer containing 2.3M Urea, 1.5% SDS, 15mM Tris, 100mM DTT, and 0.01% BPB and solubilized for 2 hours at room temperature followed by SDS-PAGE for 3 hours at 80 Volts constant. For the clean-up experiment, equal volume aliquots from the starting alkylated material, complete clean-up, and partial clean-up samples were treated as above for 1-D SDS PAGE. Here, complete and partial clean-ups were performed on equal amounts of starting alkylated material and finally rehydrated in equal volume of the SB buffer as described in the cleanup protocol above. The gels were then transferred by standard submerged method (192mM glycine, 25mM Tris, 20% Methanol) at 36 Volts constant for 16h at 4°C on to nitrocellulose membrane (NC). This was followed by the Western blotting protocol described previously (Simpson et al., 2001). Briefly, the NC membranes were first blocked with 5% Non-fat milk in PBS/Tween-20 (0.1%) followed by incubation in the GLUT-1 primary Ab (1:10,000) for 3 hours at room temperature. After three 10 min washes with the above buffer, the blots were incubated for 1 hour at room temperature with goat anti-rabbit secondary Ab (1:2,000) conjugated with horseradish peroxidase. After three 10 min washes with PBS/Tween-20 (0.1%), the blots were visualized with the aid of an enhanced chemiluminescence kit, digitized into film images using a Fuji LAS-3000 system and the densitometric images were then analyzed using Multi Gauge software 3.0 (Fuji.Film Life Science, Valhalla, NY). Quantitation was performed from 16-bit linear TIF images by selecting equal area from each sample lane that encompassed the band of interest and gray values obtained after subtracting the background from an empty lane. For the purpose of ease of analysis with dimensionless numbers and to account for gel-gel variation, the values from each lane were normalized to a calibrator sample lane in the same gel by obtaining the percentage of the sample gray values compared to the calibrator gray values. The calibrator sample for the current work is the starting alkylated sample of the undigested cow brain microvessels. Each experiment was repeated three times and quantitation performed on four gels to obtain the mean and the standard error of the mean. All statistical calculations were performed using Graphpad Prism v4.0 (GraphPad Software Inc.).

Strip Rehydration/IEF/2-D SDS PAGE

The 2-D ready protein samples in the SB were added with fresh 2.0 % 3–11 NL IPG buffer and 0.6 % Destreak (Hydroxy Ethyl Disulphide) containing trace of Orange R dye. 200 µl of

each sample containing about 50 µg total protein was loaded onto a 11 cm immobiline pH gradient strip by passive rehydration for 20 hours at room temperature. The proteins were then separated by isoelectric focusing in a 2117 Multiphor II (LKB, Bromma, Sweden) electrophoresis unit. The following gradient program was used – step 1: 300 V 5Vh; step 2: 300 V 1800 Vh; step 3: 3500 V 17.5 kVh, step 4: 3500 V 22.75 kVh; step 5: 100 V 1200 Vh. The strips were then equilibrated with gentle shaking in two subsequent steps for 15 min. each in 10ml of 6M urea, 0.375 M Tris at pH 8.8, 10% SDS, 20% glycerol (McDonough and Marban, 2005). Second-dimensional SDS–polyacrylamide gel electrophoresis was carried out on a Bio-Rad Criterion electrophoresis unit in 10% Criterion Tris-HCl acrylamide precast gels for 2 hours at room temperature, 100V constant.

Deep Purple™ Staining/Western Blotting

The 2-D ready samples were subjected to Western blotting after 2-D SDS-PAGE. To perform 2-D experiments, undigested cow microvessels (UD) sample was selected purely based on high yields. For each 2-D Western blot, a parallel gel was run to obtain the IEF and SDS PAGE patterns by staining with Deep Purple™ total protein. The staining protocol is as described below. For free floating gels ~20 fold excess of the fixation solution containing 7.5% (v/v) acetic acid/10% (v/v) methanol was used to fix the gels for a minimum of 1 hour at room temperature with gentle agitation. The fixation solution was then replaced with wash solution containing 200 mM sodium carbonate and the gels washed with gentle agitation for 30 min. The wash solution was then poured off and the gels incubated with Deep Purple™ stain at 1:200 dilution in proteomic grade Milli-Q water for 1 hour at room temperature with gentle agitation. Finally the gels were washed with 7.5% (v/v) acetic acid twice for 15 min to reduce the background before imaging them on the Typhoon™ 9400 scanner at 500V PMT (Photo Multiplier Tube) and 50 micron resolution by excitation with green laser (532 nm)/emission with 610 bandpass filter. The procedures for Western blotting and image acquisition were as described under 1-D SDS-PAGE/Western blotting.

Results and Discussion

To determine the effectiveness of the solubilization procedure, Western blotting for GLUT-1 was performed on equal volume fractions of supernatant and the pellet following the solubilization protocol described in the Materials and Methods section. The blots presented in Fig. 1a show that solubilization of the brain microvessels with the SB buffer containing 5M urea, 2M thiourea, 2% DDM, 1% ASB-14, 10 mM TCEP, and 10 mM Tris (pH 8.0) achieved near complete solubilization with very little GLUT-1 remaining in the resultant pellet fractions from any of the samples. We have applied the detergent combination of DDM and ASB-14 based on literature indicating their efficacy in solubilizing membrane proteins in general and GLUT-1 in particular (Boulter and Wang, 2001, Luche et al., 2003, Lundback et al., 2003, Taylor and Pfeiffer, 2003). DDM has been shown to be very effective in solubilizing and extracting GLUT-1 from erythrocyte membranes and also prevent GLUT-1 aggregation (Boulter and Wang, 2001). After successful solubilization of GLUT-1 and most proteins (compared by protein assay from pellet and supernatant), we proceeded further in the sample preparation by reducing the sample with TCEP and alkylating with 4-VP (Bai et al., 2005). An optimal 2-D in the basic region is very challenging due to the associated horizontal streaking which is primarily due to a depletion of the reducing agents such as DTT in the basic pH range during the IEF. This leads to a restoration of inter and intra disulphide bridges in the proteins. By utilizing trialkylphosphines such as TCEP that are non-reactive to alkylating agents used in downstream processing and that have highly specific reduction reaction we can achieve complete disulphide reduction. Therefore, we utilized the novel protocol developed by (Bai et al., 2005) to perform reduction and alkylation using TCEP and 4-VP. Vinyl pyridines such as 4-VP are weakly basic molecules containing a double bond that selectively alkylate –SH groups in proteins and prevent their

re-oxidation to disulphide bridges. They exhibit 100% alkylation, along with insensitivity to neutral or zwitterionic surfactants that are present in typical 2-D solubilization cocktails and also have been shown to be efficient even at the unfavorable neutral pH conditions. Having alkylated the thiol groups, the samples were subjected to sample clean-up using the Bio-Rad ReadyPrep™ 2-D clean-up kit to reduce contaminating salts, nucleic acids, lipids etc so as to obtain clean streak-free 2-D maps. It is well known that there are significant protein losses after clean-up and re-suspension associated with the precipitation methods commonly used for sample clean-up (Lundback et al., 2003, Nandakumar et al., 2003). In many cases such losses are acceptable as long as there is detectable amount of the protein of interest. However, qualitative sample losses are unacceptable as they can introduce errors into the analysis and conclusions of the study (Zellner et al., 2005). For example with the brain microvascular GLUT-1, we suggest presence of different isoforms and in this scenario qualitative losses are clearly unacceptable (Simpson et al., 2001). In order to assess qualitative losses, we compared the brain microvessels from rat and bovine sources along with rat whole brain homogenate standard before and after sample clean-up. After complete clean-up (C), a significant depletion of GLUT-1 is seen compared to the alkylated starting sample (A) (Fig. 1b & Fig 1c). Our data suggests these as qualitative losses as the total sample protein recovered as determined by the Better Bradford Coomassie plus protein assay (Pierce), was around 80% (data not shown) whereas the percent recovered for GLUT-1 was only about 60% (fig. 1c). We hypothesized this loss as arising from the acetone wash step that can result in loss of membrane proteins due to the partitioning effect into acetone. Organic solvents, such as acetone precipitate hydrophilic proteins easily, whereas hydrophobic proteins tend to remain in the soluble fraction (Srivastava and Srivastava, 1998, Thongboonkerd et al., 2002). To test this hypothesis and to obtain complete recovery of GLUT-1, we eliminated the acetone wash step in a modification of the original protocol called partial clean-up (P) as described in the Materials and Methods section. The data for the partial clean-up are also shown in the Fig. 1b and Fig. 1c with the GLUT-1 bands for the lane P clearly showing a major recovery (>90%) of this transmembrane protein. To take this further and to see how widespread the effects of qualitative sample losses are and to confirm that the losses are indeed from membrane proteins, we performed 2-D electrophoresis on the plasma membrane fractions (luminal and abluminal membranes proteins) of the undigested cow microvessels and obtained total protein stains. The Deep Purple™ total protein stain of Fig. 2a shows a dramatic increase in the number of protein spots detected on gel using the modified partial clean-up (P) when compared to the complete clean-up (C) supporting our hypothesis that membrane proteins were lost in the complete cleanup. To check if the increase in the number of spots in the partial clean-up also translated to the candidate integral membrane protein GLUT-1 as suggested by our 1-D Western analysis, we performed 2-D Western blots for GLUT-1 on undigested microvessels (Fig. 2b). These results demonstrate that GLUT-1 positive spots increased substantially when the partial clean-up protocol was followed. The spots in the pI range of 8–9 where most of the GLUT-1 has been shown to be concentrated (Englund et al., 1995) were present in much higher intensity when partial clean-up was utilized.

Our solubilization and clean-up protocol clearly detect GLUT-1 from brain microvessels that were absent from the 2-D maps of similar samples in a previous report (Lu et al., 2007). In total, these results demonstrate that optimization of the detergent composition and sample precipitation dramatically increases the recovery of membrane proteins and eliminates qualitative artifacts. This approach has potential for application to other membrane proteins from brain samples. Apart from these, we also report GLUT-1 positive spots at the acidic end of the scale around 3.0 pI and also at 6.0 pI (Fig 2b), potentially resulting from the excessive sialic acid glycosylations of GLUT-1. It is also possible that the diffuse spots at these pI could be a result of oligomerization of GLUT-1 monomers as suggested by Englund and Lundahl, (1995) for spots around 6.0 pI. We are currently assessing the various covalent

modifications by using deglycosylation and dephosphorylation in combination with chymotrypsin digestion and mass spectrometry (MS).

In conclusion, our data shows that solubilization of GLUT-1 is near complete with the detergent combination of ASB-14 and n-DDM and this might be applicable to membrane proteins in general. Additionally, a modified protein purification procedure is important not just for obtaining 2-D compatibility but also to prevent qualitative sample losses particularly membrane proteins in clean-up kits containing acetone based washing steps.

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Fig 1a

Fig 1b

Fig 1c

Fig. 1.

Fig 1a. Western blot for GLUT-1 showing the pellet (P) and supernatant (S) fractions after solubilizing undigested cow brain microvessels (UD), rat whole brain homogenate (RB) and rat brain microvessels (UR) in SB: 5M Urea, 2M Thiourea, 2% DDM, 1% ASB-14, 10mM TCEP, 10mM Tris. The blot indicates near complete solubilization of the samples with negligible GLUT-1 staining in the pellet fraction. Fig 1b. A representative Western blot for GLUT-1 showing the starting alkylated sample (A) along with the completely cleaned-up (C) and partially cleaned-up (P) samples for undigested cow brain microvessels (UD), rat whole brain homogenate (RB) and rat brain microvessels (UR). Fig 1c. The quantitation of the Western blot is shown in the bar graph with the starting alkylated sample set at 100%

and ratios for complete and partial clean-up obtained using Multiguage 3.0 software as described under materials and methods section. Each of the blots has been repeated three times and the error bars indicate standard error of the means (SEM). * Represents significance with one-tailed paired t-test (p-value <0.05) between partial and complete clean-up samples. There was no significant difference between the starting (A) and the partial clean-up (P) sample using the same t-test indicating near complete recovery with partial clean-up. Please note the lower molecular weight of GLUT-1 (45 kD) from rat brain homogenate sample, the lower glycosylation form of GLUT-1 that is present in most cells of the brain except the microvascular endothelial cells. It is also present as a weak lower band in the undigested rat microvessel sample where it is a result of minor contamination of microvessels with non-microvascular tissue.

Fig. 2.

Fig 2a. 2-D SDS PAGE gels stained with Deep Purple™ total protein stain showing a dramatic increase in the number of protein spots with partial clean-up (P) compared to the complete cleanup (C) in the plasma membrane fractions of cow brain microvessels - Luminal Membrane Proteins and Abluminal Membrane Proteins. These fractions were obtained as described under materials and methods section. Fig 2b. Western blots for GLUT-1 after 2-D SDS-PAGE showing GLUT-1 positive spots in the pI range of 8-9 for partial clean-up (P) sample but only weakly in the complete clean-up sample (C) of the undigested cow brain microvessels (UD).