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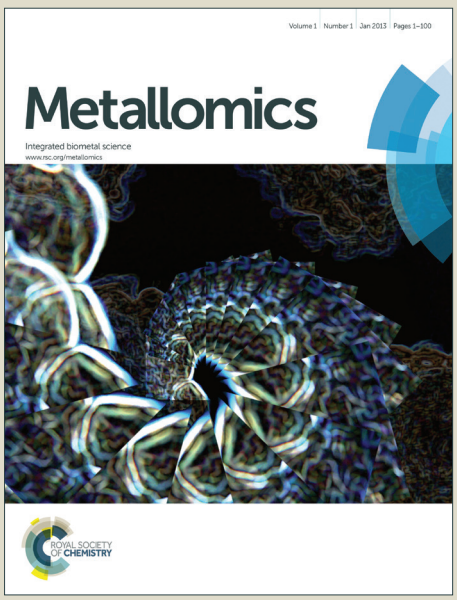

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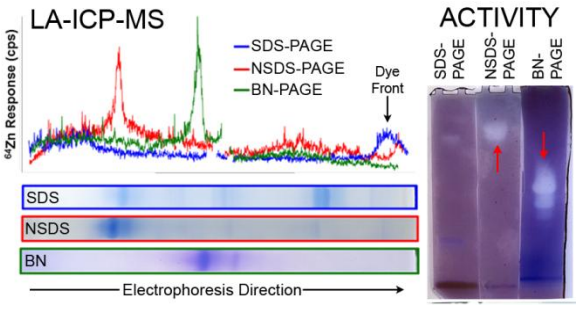
Systematical modifications of traditional gel electrophoresis have yielded a method to separate soluble proteins with high resolution while retaining metal cofactors and enzymatic function



Native SDS-PAGE: High Resolution Electrophoretic Separation of Proteins With Retention of Native Properties Including Bound Metal Ions

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Abbreviations ADH, alcohol dehydrogenase; AP, alkaline phosphatase; β-GAL, β-galactosidase; BN-PAGE, blue-native PAGE; CA, carbonic anhydrase; dd, double distilled; LA-ICP-MS, laser ablation-inductively coupled plasma-mass spectrometry; LDS, Lithium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTT, 3(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NBT, Nitro Blue Tetrazolium; NSDS-PAGE, native SDS-PAGE; PAGE, polyacrylamide gel electrophoresis; PMS, Phenazine Methosulfate; SDS-PAGE, sodium dodecyl sulfate PAGE; SOD, superoxide dismutase

Abstract

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to obtain high resolution separation of complex mixtures of proteins. The method initially denatures the proteins that will undergo electrophoresis. Although covalent structural features of resolved proteins can be determined with SDS-PAGE, functional properties are destroyed, including the presence of non-covalently bound metal ions. To address this shortcoming, blue-native (BN)-PAGE has been introduced. This method retains functional properties but at the cost of protein resolving power. To address the need for a high resolution PAGE method that results in the separation of native proteins, experiments tested the impact of changing the conditions of SDS-PAGE on the quality of protein separation and retention of functional properties. Removal of SDS and EDTA from the sample buffer together with omission of a heating step had no effect on the results of PAGE. Reduction of SDS in the running buffer from 0.1% to 0.0375% together with deletion of EDTA also made little impact on the quality of the electrophoretograms of fractions of pig kidney (LLC-PK_1) cell proteome in comparison with that achieved with the SDS-PAGE method. The modified conditions were called native (N)SDS-PAGE. Retention of Zn^{2+} bound in proteomic samples increased from 26 to 98% upon shifting from standard to modified conditions. Moreover, seven of nine model enzymes, including four Zn^{2+} proteins that were subjected to NSDS-PAGE retained activity. All nine were active in BN-PAGE, whereas all underwent denaturation during SDS-PAGE. Metal retention after electrophoresis was additionally confirmed using laser ablation-inductively coupled plasma-mass spectrometry and in-gel Zn-protein staining using the fluorophore TSQ.

Introduction

The most commonly used technology to obtain high resolution analytical separation of mixtures of proteins is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[1, 2] The procedure involves initial denaturation of component proteins with an anionic detergent that also binds to them, imparting to all proteins a negative charge proportional to their molecular mass. This step is followed by electrophoresis through a porous acrylamide gel matrix that separates proteins with excellent resolution on the basis of molecular mass. Largely unchanged since its introduction the early 1970s, this method works well in applications that do not require retention of native features of protein structure or function.[1, 3] Thus, assessment of purity of protein samples, evaluation of protein expression, and immunochemical identification and quantification of proteins (western blotting) are methods that utilize SDS-PAGE.[1, 2, 4]

An obvious limitation of SDS-PAGE resides in its deliberate denaturation of proteins prior to electrophoresis. Enzymatic activity, protein binding interactions, detection of protein cofactors, etc. generally cannot be determined on proteins isolated by SDS-PAGE. Instead, other methods must be employed to separate native proteins for investigations of structure-function relationships.[1, 5] One such alternative is the blue-native PAGE technique.[6] This method has been used in the determination of protein-protein interactions, in which proteins in the sample are separated as oligomers in first dimension BN-PAGE, followed by a denaturing second dimension SDS-PAGE to identify the monomers within the oligomers.[7-11] However, as a one-dimensional separation method, it faces the opposite problems of SDS-PAGE. While, BN-PAGE retains the native state of proteins, it falls short of the high resolution of proteomic mixtures that is attained with SDS-PAGE and can add ambiguities to successful molecular weight determinations.[12-14]

A welcome advance would be a procedure that finely resolves individual proteins and does so in their native state. More specifically, such a method would allow for proteins to be adequately separated while still retaining their metal partners. This would alleviate some of the current limitations and concerns in using PAGE in conjunction with metalloprotein analysis.[13, 15, 16] This paper describes a method, termed native SDS-PAGE (NSDS-PAGE), that accomplishes excellent resolution of components of the proteome with retention of native enzymatic activity and/or metal cofactors in most examples that have been investigated. Because of the particular interests of the authors and its applications to the field of metallomics, attention has been focused on the behavior of the Zn-proteome and model Zn-metalloproteins during NSDS-PAGE.

Materials and Methods

Chemicals Pre-casts PAGE gels and corresponding buffers were purchased from Invitrogen. All chemicals were obtained in the highest available purity from Sigma-Aldrich or Fisher Scientific. Several Zn-proteins were utilized in the study, including yeast alcohol dehydrogenase (Zn-ADH), bovine alkaline phosphatase (Zn-AP), superoxide dismutase (Cu,Zn-SOD), and carbonic anhydrase (Zn-CA). Each was purchased from Sigma-Aldrich and used without further purification. Lyophilized enzymes (10 mg/mL) were dissolved in degassed 20 mM Tris-Cl pH 7.4, stored at 4 °C, and used within one month of rehydration. Other enzymes and proteins were also obtained from Worthington Biochemical.

Cell Culture and Partial Protein Purification Pig Kidney Epithelial Cells (LLC-PK_1) were grown under level 1 culture conditions in M199 media (Sigma) supplemented with 4% FCS, 50 mg/L Streptomycin, and 50 mg/L Penicillin G in an atmosphere containing 5% CO_2 at 37 °C. Once cells had reached confluence, media was decanted and culture plates were washed

3X in cold Dulbecco's phosphate buffered saline (DPBS). Cells were scraped using a rubber cell scraper into DPBS and collected via centrifugation at 680 x g before resuspension in 1 mL cold ddH_2O per 10 plates. The cell suspension was sonicated and 500 μM phenylmethylsulfonyl fluoride (PMSF) and 1000 U Benzonase© nuclease (Sigma) were added to inhibit proteolysis and increase nuclease activity, respectively. The sonicate was centrifuged at 47,000 x g for 30 minutes at 4 °C to remove cellular debris and the supernatant loaded onto a Sephadex G-25 (GE Healthcare) gel filtration column and eluted with degassed 5 mM Tris-Cl pH 8.0. Fractions in the high molecular weight region exhibiting absorbance at 280 nm were pooled as the proteome (lacking low molecular weight species) and subsequently loaded onto a Macro-Prep DEAE anion exchange column (Bio-Rad) for further separation. The column was eluted with 5 mM Tris-Cl pH 8.0 with a 50 mM NaCl stepwise gradient from 0-500 mM NaCl. Select fractions were concentrated using a Millipore centrifuge filter (3,000 Da molecular weight cut-off) that was spun at 12,000 x g for 20 minutes at 4 °C. Samples were desalted by repeating the concentration three times, washing the protein sample with fresh 5 mM Tris-Cl pH 8.0 for each concentration.

Polyacrylamide gel electrophoresis Denaturing SDS-PAGE was performed according to the Invitrogen NuPAGE® specifications. In brief, 7.5 μL of protein sample (5-25 μg protein) were mixed with 2.5 μL of 4X LDS sample loading buffer (Invitrogen) and heated at 70 °C for 10 min. Samples were then loaded into precast NuPAGE Novex 12% Bis-Tris 1.0 mm minigels (Invitrogen). Then, 5 μL of Pre-stained SDS-PAGE Standards (Bio-Rad) were loaded in each gel run. Electrophoresis was performed at room temperature for approximately 45 min using a constant voltage (200V) in 1X solution of NuPAGE MOPS SDS running buffer (Invitrogen) until the dye front reached the end of the 60 mm gel. Buffer compositions were obtained from

the manufacture's technical guide and are listed in **Table 1**.

Blue Native (BN)-PAGE was also performed according to the manufacture's protocol. In this procedure, 7.5 µL of protein sample were mixed with 2.5 µL of 4X BN-PAGE sample buffer (Invitrogen) and loaded into precast Native-PAGE Novex 4-16% Bis-Tris 1.0 mm minigels (Invitrogen) with 5 µL of NativeMark™ unstained protein standards (Invitrogen). Electrophoresis was carried out at a constant voltage (150V) and at room temperature, employing 1X solutions of Anode and Cathode Running Buffers (Invitrogen) and run until the dye front reached the end of the gel (60 mm) after 90-95 minutes. Buffer compositions were obtained from the manufacture's technical guide as shown in **Table 1**.

In NSDS-PAGE, 7.5 µL of protein sample were added to 2.5 µL of 4X NSDS sample buffer (100 mM Tris HCl, 150 mM Tris base, 10% v/v glycerol, 0.0185% w/v Coomassie G-250, 0.00625% w/v Phenol Red, pH 8.5, **Table 1**). Precast NuPAGE Novex 12% Bis-Tris 1.0 mm mini-gels (Invitrogen) were run at 200V for 30 minutes in double distilled (dd) H_2O to remove storage buffer as well as any unpolymerized acrylamide. During this time, the NSDS-PAGE running buffer (50 mM Tris Base, 50 mM MOPS, 0.0375% SDS, pH 7.3, **Table 1**) was chilled to 4° C. Samples (including 5 µL of Pre-stained SDS-PAGE protein standards) were loaded into the gel and electrophoresis was conducted at constant voltage (150 V) at 4^o C until the dye front reached the end of the 60 mm gel after 80-85 minutes.

Protein Staining SDS-PAGE and NSDS-PAGE gels were stained using SimplyBlue™ Safe Stain (Invitrogen). Gels were washed 3X in ddH_2O for 5 minutes, incubated in 100 mL of SimplyBlue™ Safe Stain for 1 h, destained twice in 100 mL of ddH_2O for 1 hour. BN-PAGE gels were microwaved for 45 seconds in fixing solution (40% methanol and 8% acetic acid) and

placed on a shaker for 30 minutes. Gels were then stained in 30% methanol, 10% acetic acid containing 0.02% Coomassie R-250, microwaved again for 45 seconds, and placed on a shaker for 30 minutes. Gels were destained twice in microwaved 8% acetic acid for 1 hour.

Enzyme activity assays In-gel enzyme activity assays were performed based on protocols from Manchenko.[17] In brief, model proteins or proteomic fractions from LLC-PK_1 extracts were run in duplicate—one for assaying activity and one for protein staining—using SDS-PAGE, NSDS-PAGE and BN-PAGE as described above. Gels were washed 3X in ddH_2O for 5 minutes before assays were performed.

(a) To detect alcohol dehydrogenase (Zn-ADH) activity, gels were incubated in 50 mL of 50 mM Tris-Cl pH 8.5 buffer containing 10 mg of NAD, 10 mg of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.2 mg of phenazine methosulfate (PMS) and 2 mL of 100% ethanol. Gels were incubated on a shaker in the dark until blue bands appeared, then fixed in 25% ethanol.[18]

(b) To measure β-galactosidase (β-GAL) activity, gels were incubated for 15 min in 50 mL of 100 mM Na_2HPO_4 buffer, pH 7.4 containing 10 mg of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) and 5 mg of Nitro Blue Tetrazolium (NBT). Gels were placed on a shaker in the dark until blue bands appeared (approximately 15 minutes). The reaction was halted by fixing the gel in 25% ethanol.[19]

(c) For the detection of superoxide dismutase (Cu,Zn-SOD), gels were submerged in 50 mL of 50 mM Tris-Cl pH 8.5 containing 10 mg of MTT, 6 mg of PMS, and 10 mg of anhydrous $MgCl_2$. The gel container was placed on a shaker exposed to light for 2 hours. Evidence of enzyme activity was the development of clear bands on a dark blue background.[20]

(d) For the observation of alkaline phosphatase (Zn-AP) activity, gels were soaked in 50 mL of 100 mM Tris-HCl, pH 9.0, containing 10 mg of anhydrous $MgCl_2$, 20 mg of 5-Bromo-4-Chloro-3-Indyl Phosphate, and 10 mg of NBT. After incubation on a shaker for 15 minutes in the dark, gels were fixed in 3% acetic acid. Activity was visualized by the appearance of dark blue bands.[21]

(e) To determine urease activity, gels were placed in 50 mL of 25 mM citrate buffer pH 6.0 containing 240 mg of urea, 10 mg of NBT, and 5 mg of dithiothreitol. The gel container was wrapped in plastic wrap and incubated on a gel shaker for 1 hour. The assay solution was replaced with 20 mM HCl and activity was visualized by the appearance of light purple bands.[22]

(f) To measure peroxidase activity, gels were incubated with 50 mL of 50 mM Na_2HPO_4 pH 7.0 containing 5.5 mg of NADH, 4 mg of phenol, 3 mg of NBT and 0.02% hydrogen peroxide. The gel container was sealed and incubated on a shaker for 30 minutes. The reaction ceased after the addition of 25% ethanol. A light brown band characterized the reaction product.[23]

(g) For the determination of carbonic anhydrase (Zn-CA) activity, gels were soaked in 50 mL of 100 mM Tris-Cl pH 9.0 containing 0.1% bromothymol blue for 15 minutes on a gel shaker. The solution was decanted and the gel was lightly blotted with a paper towel to remove excess solution. Carbon dioxide gas from dry ice in water was streamed over the gel surface and active carbonic anhydrase was visualized as faint yellow bands on a blue background.[24]

(h) For L-amino oxidase activity, gels were incubated in 75 mL of 65 mM NaH_2PO_4 pH 6.8 containing 10 mM L-Lysine, 5 mg NBT, and 2.5 mg PMS on a rotary shaker for 30 minutes. Gels were fixed in 25% ethanol and oxidase activity was detected by the appearance of blue

bands.[25]

(i) For glucose-6-phosphate dehydrogenase activity, 30 mg of D-glucose, 10.6 mg of adenosine triphosphate, 10 mg of $MgCl_2$, and 20 U of hexokinase were dissolved in 50 mL of 100 mM Tris-Cl pH 8.4 and the solution was incubated at 37° C. After 2 hours, 10 mg of NAD, 10 mg of MTT, and 1 mg of PMS were added and the solution was poured over the gels. After incubating the gels on a shaker for 30 minutes, the reaction was halted using 25% ethanol. Dark blue bands signified glucose-6-phosphate dehydrogenase activity.[26]

TSQ-Staining and visualization of PAGE gels Gels were electrophoresed using NSDS-PAGE as described above and washed twice in 100 mL of ddH_2O for 5 minutes. The washed gels were transferred to a UVP EpiChemi II Darkroom UV transilluminator gel box and excited at 365 nm (long wavelength UV setting), the excitation wavelength of TSQ. Fluorescent images were recorded with a digital camera with a 470 nm emission filter using a three second exposure time. For TSQ staining, gels were soaked in 50 mL of ddH_2O containing 25 μM TSQ on a rotary shaker for 40 minutes and washed twice with fresh ddH_2O for 5 minutes before fluorescent images were recorded. Gels were then additionally incubated in 50 mL of ddH_2O containing 100 μM TPEN for 30 minutes before subsequent images were captured.

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) The UW-Milwaukee Department of Chemistry and Biochemistry maintains a Micromass Platform quadrupole ICP-MS outfitted with a hexapole collision cell. The unit is linked to a CETAC LSX-213 Laser Ablation attachment. Gels to be analyzed using LA-ICP-MS were washed in 100 mL of ddH_2O and vacuum-dried onto Whatman filter paper and secured in the large format ablation cell (Cetac Technologies). Lanes were ablated using a 200 μm spot size scanned at 60

μm sec^{-1} with 30% laser energy set at 20 Hz and the ablated aerosol was carried to the ICP-MS at a flow rate of 1.0 L He min^{-1}. The inductively coupled plasma was maintained using 1350 W forward power with the argon cool gas and intermediate gas flow rates set at 13.0 L min^{-1} and 1.3 L min^{-1}, respectively. The hexapole collision cell gas flows were set at 2.5 mL He min^{-1} and 1 mL H min^{-1} and metal isotopes ^{64}Zn and ^{63}Cu were measured using peak hopping acquisition mode. ^{13}C was also monitored to assess signal drift over time.

Results

Experiments were aimed at developing a protein separation method that maintained the protein resolution characteristic of SDS-PAGE as well as the retention of protein functional properties and metal cofactors characteristic of BN-PAGE. The approach was to systematically modify SDS-PAGE in graded steps and to compare the results with those obtained with SDS- and BN-PAGE.

SDS-PAGE of LLC-PK_1 cell supernatant and model metalloproteins The commercial kit for SDS-PAGE offered by Invitrogen includes sample and running buffers shown in **Table 1**. Both contain SDS and EDTA. According to the recipe, the protein mixture is added to the sample buffer and heated to 70^{o} C with the explicit intent to denature the constituent proteins through the combined exposure to 2% LDS detergent and heat. Due to its high metal binding affinity, the presence of 0.51 mM EDTA greatly increases the likelihood that metal ions will be extracted from resident metalloproteins during this preparative step.[27-29] Once electrophoresis is undertaken, the sample migrates in a run buffer containing 0.1% SDS and 1 mM EDTA.

LLC-PK_1 cells were used as the source of proteome. The proteome was obtained from

the gel filtration of the cellular supernatant of 5 x 10^8 LLC-PK_1 cells and was further separated by DEAE ion exchange chromatography. Several desalted fractions from the elution profile were subjected to SDS-PAGE using the protocol provided by Invitrogen. **Figure 1a** illustrates the quality of separation of 3 DEAE fractions as visualized by Coomassie R-250 staining. Many distinct protein bands were distinguishable in each of the fractions.

Three Zn-proteins (Cu,Zn-SOD, Zn-ADH, and Zn-CA) were also subjected to SDS-PAGE.[30-32] As seen in **Figure 1c**, the extent of migration of each protein differed during electrophoresis and was related to its monomer molecular mass. Both Cu,Zn-SOD and Zn-CA electrophoresed to positions that corresponded to their approximate molecular weights.[30, 32] Importantly, Zn-ADH that normally exists as a 141 kDa tetramer migrated as individual 35 kDa monomers under denaturing conditions.[33] Testing each protein for enzymatic activity (see methods) showed that none of these enzymes maintained their functionality after electrophoresis (**Figure 3**).

Blue-native PAGE of LLC-PK_1 cell proteome and model metalloproteins. The same experiments that revealed the properties of SDS-PAGE were carried out to describe the behavior of proteins during BN-PAGE**. Table 1** lists the buffers applied in the BN-PAGE method. LDS, SDS, and EDTA are absent and Coomassie Blue is included as a replacement for the negatively charged SDS detergent. Unlike the denaturing method, the 3 proteomic fractions were poorly separated by BN-PAGE (**Figure 1b**). In the case of the model proteins, all moved to the same region of the gel, failing to separate well, in contrast to their migration pattern with SDS-PAGE (**Figure 1d**). Moreover, each protein migrated to a distance inconsistent with its expected molecular weight. However, each displayed enzymatic activity (**Figure 3**), that was maintained

in the absence of the denaturating conditions present during SDS-PAGE, namely SDS, EDTA, and incubation at elevated temperature.

Native SDS-PAGE of LLC-PK$_1$ cell proteome and model metalloproteins Neither SDS-PAGE nor BN-PAGE was able to accomplish the goal of acceptable protein separation with retention of enzymatic activity. A new method or effective changes to these protocols were needed to achieve both. We chose to modify SDS-PAGE. Initially, EDTA, a powerful chelator of many metal ions, was removed from both sample preparation buffer and electrophoretic running buffer to prevent direct sequestration of metal cofactors. Its deletion did not impact the quality of protein separation by SDS-PAGE. In addition, the omission of the initial heating step of the protein sample in the LDS-containing loading buffer did not affect the outcome of SDS-PAGE. Attention was then turned to the detergent concentration (SDS) in the sample buffer. It was lowered stepwise from 2% to 0% for a series of proteome samples. When they were electrophoresed in the standard run buffer, the results were identical to those observed with normal SDS-PAGE conditions for the sample buffer (**Figure 2a**). These data indicated that detergents are not essential in the sample buffer to obtain desirable protein resolution.

Next, the amount of SDS in the run buffer was lowered to determine the minimal amount needed for effective resolution of protein bands. Testing BN-PAGE and the following concentrations of SDS, 0.0125%, 0.025%, 0.0375%, 0.05%, and 0.1% with a DEAE fraction of cell supernatant, it was evident again that BN-PAGE resulted in poor protein separation (**Figure 2b**). Furthermore, the lowest concentration of SDS was ineffective. Upon progressively increasing the SDS concentration in the run buffer, it was shown that 0.0375% SDS was the minimal concentration that supported refined proteome separation, closely approaching that

obtained with SDS-PAGE. In addition, the pre-stained SDS-PAGE protein markers were run under these conditions (**Figure 2c**). Again, at 0.0375% SDS in the run buffer, the 8 proteins in the protein ladder were well separated with no significant improvement as the amount of SDS was increased in the run buffer. These modified buffer conditions were used to perform NSDS-PAGE and are listed in **Table 1**.

The electrophoretic migration of model proteins Zn-ADH, β-Gal, Zn-AP, and Cu,Zn-SOD was also examined utilizing NSDS-PAGE in tandem with SDS- and BN-PAGE (**Figure 3a-d**). Unlike samples run using traditional SDS-PAGE, proteins electrophoresed in NSDS moved a distance consistent with the molecular weights of the intact multimeric forms of the enzymes with β-GAL migrating as dimeric species and Zn-ADH as a tetramer.[33] In the case of Cu,Zn-SOD, the majority of the protein migrated as a multimer near 125 kDa. This high molecular weight form of SOD has been observed previously after gel filtration chromatography, corresponding to an approximate molecular weight of 130-140 kDa.[34, 35] Unfortunately, proper evaluation of protein resolution using Zn-AP could not be made with any of these PAGE methods, most likely stemming from the presence of contaminants in the commercially purchased enzyme. In general, the protein samples migrated in tighter bands when compared to BN-PAGE and to distances more consistent with their native molecular weights.[33, 36, 37] When each of the proteins subjected to NSDS-PAGE was assayed for enzymatic activity, all displayed significant functionality, comparable to their catalytic activity after BN-PAGE (**Figure 3a-d**, right). As expected, these enzymatic functions were not observed after subjecting the enzymes to denaturing SDS-PAGE. The results supported the conclusion that a modification of SDS-PAGE conditions could be made that satisfied the important need for high resolution separation of the

proteome while still maintaining native properties of proteins.

The reduction in concentration of SDS in the NSDS running buffer presumably lowers the extent of binding of negatively charged SDS to proteins undergoing electrophoresis. In turn, for proteins with high pI values, it is possible that they might retain a net positive charge in the presence of SDS and not migrate in NSDS-PAGE as they do in SDS-PAGE. This question was addressed by comparing the behavior of cytochrome c from bovine heart (pI 10-10.5) in each mode of electrophoresis.[38] **Figure 3e** shows that the protein moves as a low molecular mass molecule in both SDS- and NSDS-PAGE methods. Thus, with this model protein, NSDS facilitates the migration of a positively charged protein that is qualitatively similar to that observed with SDS.

The capability of NSDS-PAGE to separate native proteins as judged in the last experiment led to an analysis of the cellular proteomic alkaline phosphatase activity after electrophoresis. Coomassie staining of protein in **Figure 3f** showed that SDS- and NSDS-PAGE resulted in similar resolutions and patterns of distinct bands, while proteins subjected to BN-PAGE were not resolved. On a separate gel, a test for alkaline phosphatase catalytic activity was positive for both NSDS- and BN-PAGE (arrows in **Figure 3f**) while no activity was found after SDS-PAGE. Although the observed activity occurred in a part of the gel that was consistent with AP catalysis, the identity of the enzyme was not confirmed. Nevertheless, in experiments involving either proteomic fractions of cell supernatant or model proteins, conditions of NSDS-PAGE retained enzymatic activity as well as provided excellent protein band resolution.

Maintenance of enzymatic function after NSDS-PAGE Several other proteins besides Zn-AP, Zn-ADH, β-GAL, and Cu,Zn-SOD were subjected to the three forms of PAGE and then

assayed for enzymatic activity. As shown in **Table 2**, with the exceptions of urease and Cu,Zn-SOD, which displayed trace activity, none of the enzymes retained functionality after SDS-PAGE. All did so after BN-PAGE and seven of nine enzymes tested were active after NSDS-PAGE. This survey suggests that many proteins, though not all, are functionally active after exposure to conditions of reduced SDS concentration during and after electrophoresis.

Comparison of metal binding to SOD after SDS-, BN-, and NSDS-PAGE Cu,Zn-SOD was subjected to the three types of PAGE to assess their impact on retention of metal ions. The protein was electrophoresed and then subjected to LA-ICP-MS for metal analysis. In denaturing SDS-PAGE (blue), Zn^{2+} was completely mobilized from SOD and recovered at the gel front (**Figure 4a**, front near 65 mm). Cu^{2+} was also liberated, although a minor fraction remained bound to the multimeric form of the enzyme (**Figure 4b**, band near 18 mm). In contrast, both Zn^{2+} and Cu^{2+} were fully retained in comparable amounts during BN-PAGE (green) and NSDS-PAGE (red) (**Figure 4a-b**). According to **Figure 3d,** multimeric Cu-SOD that existed after SDS-PAGE showed much diminished enzymatic activity, whereas Cu,Zn-SOD present after the other electrophoretic procedures displayed robust catalysis. Since there was no Zn^{2+} associated with the Cu-SOD band after SDS-PAGE, the faintly observed activity was likely due to the powerful redox capability of Cu ion reacting in the enzyme assay solution and not to a functioning enzyme.[39, 40]

Stability of Zn-proteome in SDS-PAGE and NSDS-PAGE run buffers To further evaluate the detrimental effects of SDS and EDTA on the metal binding capacity of the metallome, proteome from LLC-PK_1 cells, which includes as a sub-set the Zn-proteome, was incubated with SDS-PAGE run buffer containing 0.1% SDS and 1 mM EDTA and NSDS-PAGE buffer with

0.0375% SDS and no EDTA. After an hour's reaction—a time consistent with the duration of typical gel electrophoresis—each proteomic sample was separated from buffer by centrifugal filtration using a 3,000 Da molecular weight cut-off filter. Zn-proteome exposed to the standard SDS-PAGE running buffer lost 74 ± 2 % of its Zn^{2+} to the sample buffer. In strong contrast, NSDS mobilized only 2.5 ± 1 % of its complement of Zn^{2+} from the sample (**Figure 4c**). Thus, exposing the proteome to standard SDS run buffer labilizes much of its bound Zn^{2+}, whereas negative effects on metal binding are minimized in the NSDS-PAGE run buffer.

Formation of TSQ-Zn-protein adducts among NSDS-PAGE separated proteins Recent data have shown that quinoline-based Zn-sensors such as TSQ form fluorescent adducts with many cellular Zn-proteins.[41, 42] Since Zn^{2+} remains bound to proteins during NSDS-PAGE separations, such fluorescent TSQ-Zn-protein adducts should be observable within polyacrylamide gels. Incubating two proteomic fractions subjected to NSDS-PAGE with TSQ resulted in the resolution of numerous fluorescent bands in each fraction, corresponding to TSQ-Zn-protein adducts (**Figure 4d)**. To verify that this staining pattern was Zn^{2+} dependent, the gels were subsequently soaked in a solution containing TPEN, a high affinity Zn^{2+} chelator typically used in Zn-sensor experiments.[43-45] As shown elsewhere, reacting TSQ-Zn-protein adducts with TPEN results in a decrease in fluorescence through either ligand substitution or competitive adduct formation with TSQ-Zn-proteins.[29] Thus, the resulting depletion of TSQ-fluorescence after treatment with TPEN provides validity to the claim that Zn-proteins broadly maintained their metal cofactors after electrophoresis using NSDS-PAGE. Overall, these data support the conclusion that NSDS-PAGE offers a viable method for detecting protein-bound metals after electrophoresis.

Discussion

The methodology to obtain high resolution protein separation of complex mixtures by SDS-PAGE has remained essentially unchanged since its introduction four decades ago.[3] It is routinely used to probe the presence, relative concentration, and purity of proteins, their approximate molecular mass, and in conjunction with immunochemical methods or mass spectrometry, their identity and possible covalent modification.[1, 2] Because the method relies on protein denaturation prior to electrophoresis, it cannot be used in the determination of non-covalent components of native proteins such as metal ions or to study features of protein function. Importantly, proteomic-level studies directed at uncovering properties of native protein structures, including non-covalent cofactors, cannot be conducted with SDS-PAGE.

For example, technology to obtain detailed information about cellular distribution and trafficking of biologically essential, toxic, and therapeutic metals and metalloids has been emerging during the past decade. The evolution of elemental detection methodology has begun to make possible the identification of metal ions associated with biomolecules that are isolated from cell extracts and ultimately separated electrophoretically within the solid matrix of polyacrylamide gel.[46-48] Once metals are located in association with protein bands, protein identity can be determined by mass spectrometry.[4] Nevertheless, these methods have not been widely or effectively utilized. The limiting problem in applying LA-ICP-MS or other analytical means to detect elements in metallo-proteomic studies has been recognized as the lack of reliable methods to separate the proteome with high resolution while retaining bound metal ions, particularly in the final electrophoresis preceding metal analysis.[13, 49-58]

Others have described methods with modified modifications of conditions of protein

electrophoresis that seek to ameliorate the denaturing feature of SDS-PAGE.[15, 59-62] In regards to the field of metallomics, Linney et. al. showed the deletion of EDTA to the electrophoretic buffers of SDS-PAGE helped maintain a stable chromium-bovine serum albumin complex as measured by synchrotron X-ray fluorescence.[15] We report here a systematic study to optimize retention of native protein structure with an emphasis on preserving metal cofactors. The NSDS-PAGE method evolved from titration experiments meant to test whether denaturing conditions in the SDS-PAGE procedure might be modified so that properties of native proteins could be preserved even as complex protein mixtures were cleanly separated. It had been assumed previously that denaturation is a prerequisite for binding of negatively charged SDS to proteins that results in their electrophoretic migration at rates directly proportional to molecular mass.[63] Yet, the present work revealed that the preliminary denaturation step in the sample buffer involving exposure to 2% LDS (or SDS), EDTA, and high temperature can be omitted without loss of protein resolving power in the PAGE step. Moreover, EDTA may be removed from the electrophoretic running buffer and the SDS concentration lowered from 0.1% to 0.0375% with little if any loss in protein band resolution during NSDS-PAGE of a proteomic sample from LLC-PK_1 cells in comparison with SDS-PAGE (**Figure 2b**). BN-PAGE was not able to achieve observable separation of constituent proteins within this sample. Importantly, since the protein fraction was washed via multiple runs through centricon filters, high salt and low molecular weight compounds (<3 kDa) were not the causes of the poor separation of the protein mixture as noted elsewhere with BN-PAGE.[7, 8]

Decades ago, Takagi et. al. demonstrated that the binding of SDS to model proteins follows a biphasic isotherm, with the transition occurring at about 0.0375% SDS, the

concentration of detergent in the NSDS-PAGE electrophoresis run buffer (**Table 1**).[64] In conjunction with the present findings, this result suggests that at low concentration SDS binds to proteins without grossly altering their structures, whereas at higher concentrations SDS binds in larger stoichiometry, causing protein denaturation.

A potential pitfall of the modified method was an inability to overcome a substantial positive charge on proteins with large pI values. **Figure 3e** shows that cytochrome C, with a pI of 10-10.5, moves similarly in NSDS- and SDS-PAGE. Thus, in this initial experiment, sufficient SDS binds under the conditions of NSDS to achieve a negatively charged structure that migrates with a similar negative charge to mass ratio observed in the SDS buffer.

The retention of native properties of selected proteins was then compared following SDS, BN-, and NSDS-PAGE. As seen in **Figure 3,** several enzymes were subjected to electrophoresis and then tested for residual enzymatic activity. SDS-PAGE denatured the proteins such that they migrated as monomers that lost all of their catalytic power except in the case of SOD, which retained a small fraction of activity observed at the position of a multimeric species. In contrast, after both BN- and NSDS-PAGE, proteins migrated as native oligomers and displayed qualitatively similar enzyme activities. In the case of NSDS-PAGE, the locations of the protein bands corresponded in all cases except Cu,Zn-SOD to the molecular weights of the native proteins. **Table 2** summarizes these results as well as information on several other proteins. NSDS-PAGE conditions maintained the functional activity of 7 enzymes. Two others which remained active after BN-PAGE lost their functionality. Thus, in this initial survey, NSDS-PAGE was highly, though not completely, effective in stabilizing features of native protein structure. Alkaline phosphatase activity was also demonstrated in proteome separated by NSDS-

PAGE, showing that native enzymatic properties can also be observed in cell isolates as well as with individual proteins (**Figure 3f**).

Retention of functionally active Zn^{2+} in several proteins was inferred from the maintenance of enzymatic activity of Zn-ADH, Zn-AP, Cu,Zn-SOD, and Zn-CA shown in **Figure 3**. Likewise, LA-ICP-MS analysis of Zn and Cu following electrophoresis of Cu,Zn-SOD in **Figure 4a,b** revealed directly that the enzyme retained metals and catalytic activity following NSDS- and BN-PAGE but lost both during SDS-PAGE. Therefore, NSDS-PAGE offers a promising means to achieve needed resolution without loss of metal ions. Indeed, NSDS buffer with 0.0375% SDS hardly released Zn^{2+} from the proteome, whereas SDS-PAGE running buffer, containing 0.1% SDS and 1 mM EDTA mobilized 74% (**Figure 4c**). Of this total, EDTA, alone, accounts for a significant fraction, about one-third, according to previous studies.[28, 29]

It has been well recognized that the proteomic scale detection of protein-bound metals using LA-ICP-MS has been blunted by the lack of means to separate the proteome without loss of bound metal ions.[13, 65] **Figure 4a,b** demonstrates that LA-ICP-MS can be combined with NSDS-PAGE to reveal the metal contents of a model metalloprotein, Cu,Zn-SOD. We are currently developing the method for proteomic-level analyses of metals.

Figure 4d illustrates that the NSDS-PAGE method both resolves protein mixtures and maintains bound Zn^{2+} that forms fluorescent ternary adducts with the Zn^{2+} sensor, TSQ.[41] This result, in particular, emphasizes that *in situ* reactions of a subset of proteins within the proteome can be observed by using an electrophoretic method that retains native structural properties. Moreover, it is anticipated that this type of experiment can be expanded to include other small

molecules such as drugs, toxic agents, or macromolecules to determine novel binding interactions within the manifold of native electrophoresed proteins.

The standard SDS-PAGE method provides an excellent way to resolve proteins within proteomic mixtures under denaturing conditions. In order to assay native protein properties such as catalytic activity after protein separation, other electrophoretic methods have been developed such as BN-PAGE. BN-PAGE retains native protein activities but does so without physically separating them well. NSDS-PAGE shows promise of combining the attractive features of both methods-sensitive protein resolution and retention of native properties-so that proteomic studies can be undertaken simply that probe native structures and their functions.

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Figure Captions

Figure 1. SDS- and BN-PAGE of representative DEAE ion-exchange separated fractions of LLC-PK_1 supernatant and model proteins. Representative fractions from a DEAE separation of the LLC-PK_1 proteome were run using traditional SDS-PAGE (a) and BN-PAGE (b) techniques. Model proteins Cu,Zn-SOD, Zn-ADH, and Zn-CA were also electrophoresed using SDS-PAGE (c) and BN-PAGE methods. Electrophoresis and protein staining conditions are described in methods.

Figure 2. Effects of modifying SDS concentrations in the electrophoresis buffers. a) DEAE fractions of LLC-PK_1 proteome (15 and 5 μg) were incubated in sample buffers with varying concentrations of SDS. Gels were electrophoresed at 200V in run buffer containing 50 mM Tris-Cl, 50 mM MOPS, and 0.1% SDS and stained using SimplyBlue™ Safe Stain. b) DEAE fraction of purified LLC-PK_1 proteome (20 μg) was prepared in NSDS-PAGE sample buffer and electrophoresed in run buffer (**Table 1**) containing varying concentrations of SDS. Results were compared to denaturing SDS-PAGE (right) and BN-PAGE (left). Prestained SDS-PAGE protein markers (c) were electrophoresed in the same gels and buffers as described in (b). Protein staining conditions are described in methods.

Figure 3. Protein migration and residual enzymatic activity after SDS-, NSDS-, and BN-PAGE. 10 μg of Zn-ADH (a), β-GAL (b), Zn-AP (c), and Cu,Zn-SOD (d) were electrophoresed under SDS-, NSDS- and BN-PAGE conditions in duplicate and assessed for protein migration (left) and enzymatic activity (right). Protein staining and enzymatic assay conditions are described in

the Methods. e) 20 μg of cytochrome c (pI = 10.5) were electrophoresed using SDS-, NSDS- and BN-PAGE. f) DEAE fractions of LLC-PK_1 proteome were run using SDS- NSDS- and BN-PAGE methods and then tested for phosphatase activity (see Methods).

Figure 4. Protein metal retention after gel electrophoresis. 7.5 μg of Cu,Zn-SOD were electrophoresed with the three protocols and analyzed for ^{64}Zn (a) and ^{63}Cu (b) using LA-ICP-MS (see methods for ablation parameters). c) Cytosolic Zn-proteome from LLC-PK_1 cells was incubated in both the SDS-PAGE run buffer and the NSDS-PAGE run buffer for one hour before being centrifuged through 3,000 Da molecular weight cut-off centrifugal filters. The results represent Average ± standard deviation, n=3. d) DEAE fractions of LLC-PK_1 proteome (20 μg) were electrophoresed using the NSDS-PAGE method. (Coomassie) Separated proteome fraction stained with Coomassie Blue. (TSQ) Separated fraction stained with 25 μM TSQ for 40 min; (TPEN) TSQ treated proteins incubated with 100 μM TPEN for 30 minutes to quench TSQ-Zn-protein fluorescence.

Table 1: Electrophoresis Buffers

	Electrophoretic Method		
	SDS-PAGE (Invitrogen™)	**BN-PAGE** (Invitrogen™)	**NSDS-PAGE**
Sample Buffer	106 mM Tris HCl 141 mM Tris Base 0.51 mM EDTA 0.22 mM SERVA Blue G-250 0.175 mM Phenol Red 2% LDS 10% Glycerol pH 8.5	50 mM BisTris 50 mM NaCl 16 mM HCl 10% Glycerol 0.001% Ponceau S pH 7.2	100 mM Tris HCl 150 mM Tris Base 0.01875% Coomassie G-250 0.00625% Phenol Red 10% Glycerol pH 8.5
Run Buffer	50 mM MOPS 50 mM Tris Base 1 mM EDTA 0.1% SDS pH 7.7	Cathode 50 mM BisTris 50 mM Tricine 0.02% Coomassie G-250 pH 6.8 Anode 50 mM BisTris 50 mM Tricine pH 6.8	50 mM MOPS 50 mM Tris Base 0.0375% SDS pH 7.7

Table 2. Enzyme Activity after SDS-PAGE, BN-PAGE, and NSDS-PAGE

		Activity		
Protein	**Source**	**SDS-PAGE**	**NSDS-PAGE**	**BN-PAGE**
Alkaline Phosphatase	Bovine Intestinal Mucosa	-	++	++
Alcohol Dehydrogenase	*Saccharomyces cerevisiae*	-	++	++
β-Galactosidase	*Aspergillus oryzae*	-	++	++
Peroxidase	Horseradish	-	++	++
Carbonic Anhydrase	Bovine Erythrocyte	-	++	++
Urease	Jack Bean	Trace	++	++
Superoxide Dismutase	Bovine Erythrocyte	Trace	++	++
L-amino oxidase	Rattlesnake Venom	-	-	++
Glucose-6-Phosphate Dehydrogenase	*Leuconostoc mesenteroides*	-	-	++

Figure 1.

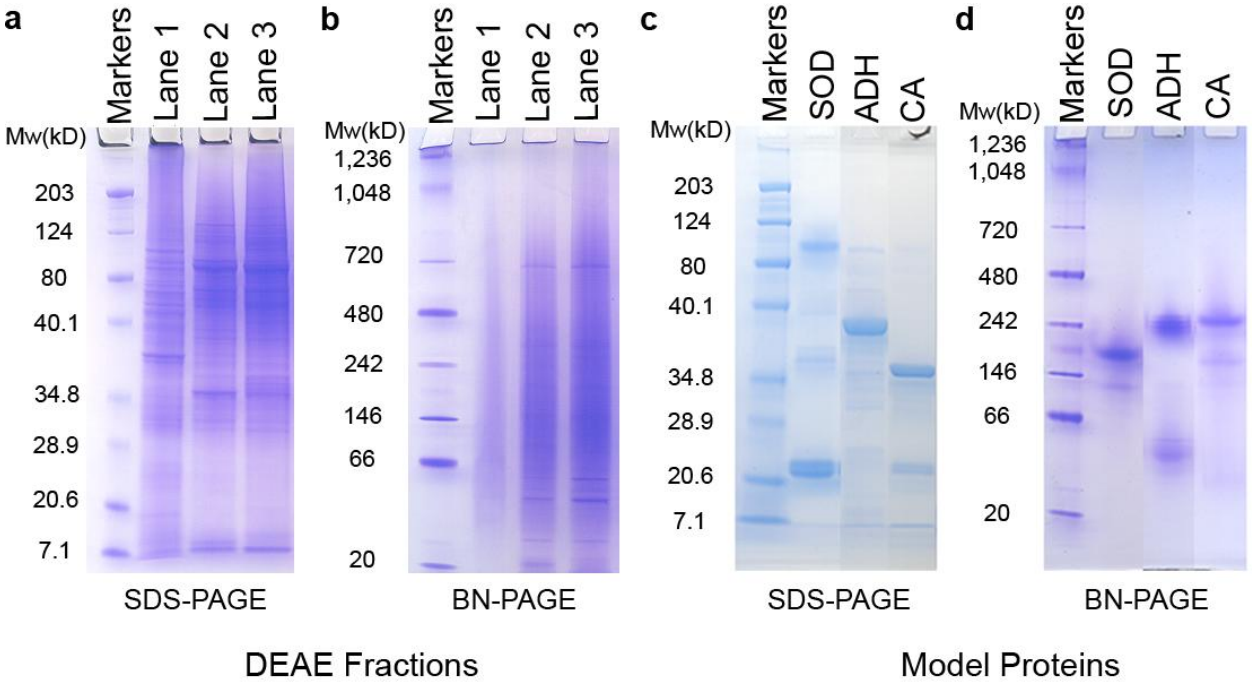

Figure 2.

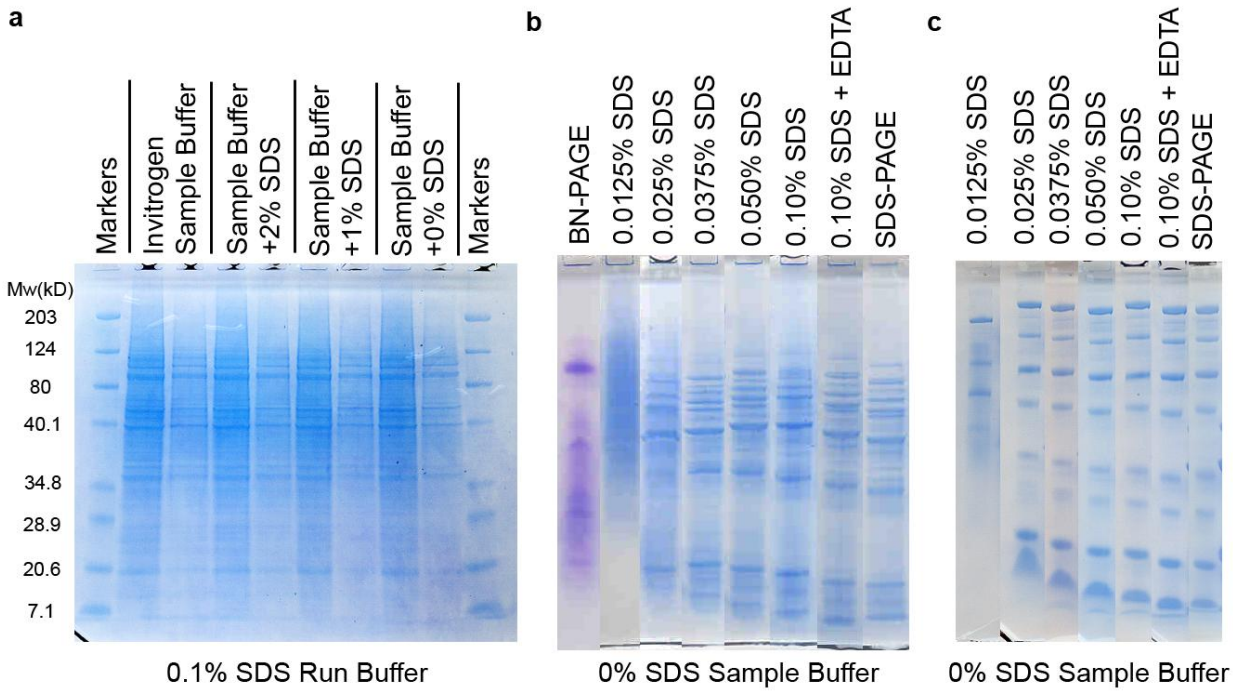

Figure 3.

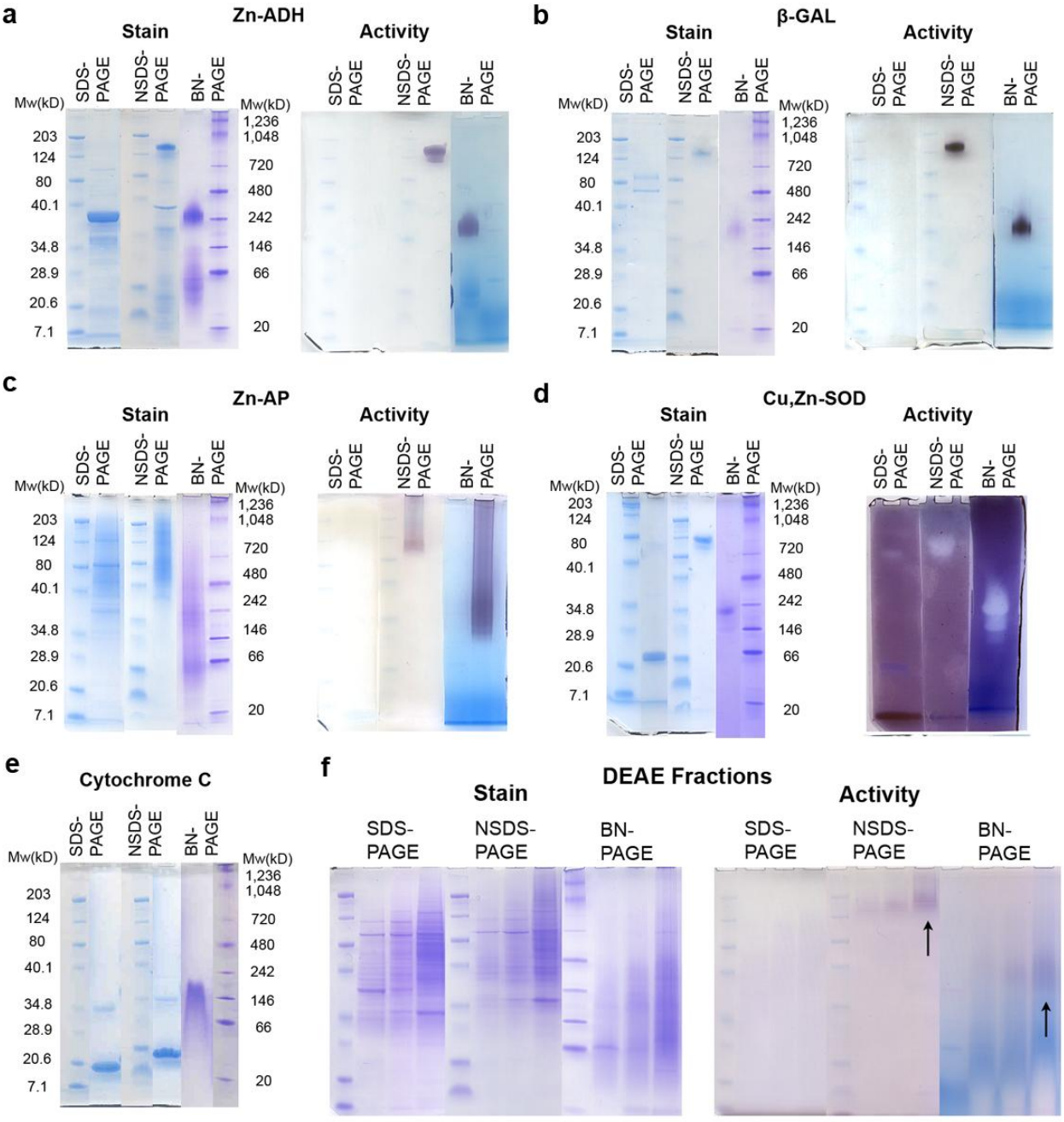

Figure 4.