Electrolytic Reduction: Modification of Proteins Occurring in Isoelectric Focusing Electrophoresis and in Electrolytic Reactions in the Presence of High Salts

Der-Yen Lee and Geen-Dong Chang*

Graduate Institute of Biochemical Sciences, College of Life Science, National Taiwan University, P.O. Box 23-106, Taipei 106, Taiwan

Artifacts in two-dimensional electrophoresis (2-DE) caused by the presence of salts in isoelectric focusing (IEF) have been previously described as a result of increasing conductivity and inducing electroosmosis. However, electrolysis induced by the presence of salts should not be disregarded. In this study, electrolytic reduction–oxidation reaction (redox) was found to be enhanced in the presence of salts in IEF. The consequence of the electrolytic redox leads to acidification of the low-pH region and alkalization of the high-pH region within the immobilized pH gradient (IPG) strip. As a result, a breakdown of immobilized pH buffer near the high pH region of IPG strips along with reduction of basic proteins resulted in uncharacterized artifacts in 2-DE. Electrolytic reduction in the presence of alkali and alkaline metal ions was demonstrated to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protein disulfide bonds, and protein carboxylic acids. Importantly, semipreparative electrolytic reduction of proteins can be carried out in the presence of sodium ions in a homemade electrolytic apparatus. These findings give additional explanations to the observed artifacts in 2-DE and reveal the unknown effects of salts in IEF. Moreover, we have provided a method with the potential to convert proteins or peptides to corresponding modified products containing aldehyde groups that can be used for conjugation with amine-containing compounds.

Since its introduction in 1975, two-dimensional electrophoresis (2-DE) has been widely used for the analysis of protein samples ranging from pure preparation to crude tissue extracts.1–3 First dimension electrophoresis, isoelectric focusing (IEF), has been improved by using an immobilized pH gradient (IPG) strip.4–6 To optimize resolution and reproducibility, comprehensive protocol has been subsequently described.7–9 Prior to IEF in the first dimension, interferring compounds, such as salts, nucleic acids, polysaccharides, lipids, and particulate material, need to be removed from the samples to achieve optimal resolution and visualization.10 It is generally believed that increased conductivity due to the presence of high salts would hinder focusing in IEF, and electroosmosis resulting from the rapid transport of water accompanying the movement of ions would aggregate proteins, which are responsible for the artifacts in IEF in the presence of high salts.11,12

However, electrolytic reactions always accompany electrophoresis operating at voltages considerably higher than the redox potential of water, anodic oxidation generating oxygen gas and protons and cathodic reduction generating hydrogen gas and hydroxide ions. The presence of electrolytes would inevitably increase current and thus increase the rate of electrolysis and power output. As a result, the pH of the solution at the anode decreases and that at the cathode increases, and bubbles are formed at both electrodes. In addition to hindrance of focusing and electroosmosis, pH titration from both ends would in theory cause pH gradient shrinkage within the IPG strips. It has been suggested that performing IEF at low voltage for a prolonged time (4 h at 500 V instead of only 1 h at 500 V) can reduce the deleterious effects of high salts.13 Unfortunately, the design of a strip holder used for IEF such as the Ettan IPGphore system or Protein IEF system is a closed buffer system so that the stalled ions would increase the rate of electrolysis. Setting a low voltage at the rehydration stage of IEF did not practically remedy the IEF artifacts in our experiments. To solve this problem, we applied the primed IPG strip to in-gel dialysis against a fresh rehydration buffer for removing salt contaminations and then performed refocusing. This process significantly improved the 2-DE profiles

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but unexpected protein patterns appeared due to protein modifications at the high pI range.

Several protein modifications have been described responsible for possible artifacts in 2-DE.\textsuperscript{11,13–16} However, only carbamylation may occur to some extent due to inappropriate processing of samples.\textsuperscript{14,15} Two unexpected major artifacts, formation of homo- and hetero-oligomers between proteins and β-elimination (desulfuration) of cysteine residues, have been shown to plague 2-DE mapping. Proper reduction and alkylation prior to the IEF process are able to prevent scrambled disulfide bridges formation and β-elimination of cysteine residues during IEF.\textsuperscript{13–16} Unfortunately, none of these modifications explain our preliminary observations. To elucidate our observations, we focused on enhanced electrolytic reaction caused by salts present in IEF. Electrochemistry has been applied in organic synthesis such as anodic oxidation and cathodic reduction of particular functional groups.\textsuperscript{17,18} For protein research, electrochemical reactions have been used to achieve protein modifications, such as iodination of proteins\textsuperscript{19–21} and reduction of protein disulfide bonds.\textsuperscript{22,23} Also, oxidation of the iron moiety of hemoglobin occurs as an artifact in the presence of salts in IEF.\textsuperscript{11}

In this article, we observed that the pH gradient of the IPG strip was affected after electrophoresis in the presence of salts and the basic proteins were massively modified by electrolytic reduction. Using carbonyl-reactive amines and hydrazides, we demonstrated that proteins with higher pI values were prone to electrolytic reduction in the presence of high salts, resulting in formation of complex functional groups. Conversion of carboxylic acids to aldehydes and possibly to alcohols in proteins would predictably increase the pI values of the corresponding proteins. Thus, we have demonstrated a novel protein modification in 2-DE due to the presence of salts in IEF and provided a potential method for protein modification by electrochemical reduction.

### EXPERIMENTAL SECTION

**Materials.** Day 1 zebrafish embryos were collected and stored at −70 °C. Acrylamido buffers, 0.2 M stock solution (Fluka); GelBond PAG film (BioWhittaker); ribonuclease A, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), bovine insulin, and ovalbumin (Sigma); phenol red (Merck); low-range molecular weight markers (Calbiochem); EZ-Link Pentaamine-Biotin (bPA, 1 mM) was included in rehydration buffer for 2-DE streptavidin-peroxidase blot overlay. Ribonuclease A (RNase A) was prepared in 100 mM DTT in rehydration buffer for denatured IEF. Alternatively, rehydration buffer was replaced by 0.5% IGF buffer in distilled water for native IEF. Additional salts prepared as 100× stock solutions were added to the protein solutions to achieve the designated final concentrations. The protein samples were applied by rehydration loading into the IPG strip. After rehydration for 4–10 h, IEF was continued until the total voltage hours reach 8 000–12 000. The IPG strips were transferred to a tray and soaked in 75 mM Tris-HCl, pH 7.8, 0.1% SDS, and 0.002% bromophenol blue buffer for 10 min, twice, before SDS-PAGE.

**In-Gel Dialysis and Refocusing IEF.** IEF was carried out with a starting voltage at a maximal voltage of 500 V for various time intervals, and the IPG strips were desalted by soaking the IPG strips in rehydration buffer or distilled water twice for 5 min, a procedure herein named in-gel dialysis. Then, the IPG strips were applied to a 7 cm IPG strip holder containing 60 μL of rehydration buffer to complete IEF for an additional 8 000–12 000 voltage hours, called refocusing IEF. For 2-DE streptavidin-peroxidase blot overlay, 1 mM bPA was included in the rehydration buffer during refocusing.

**SDS-PAGE.** Tris-Tricine SDS-PAGE was performed as previously described\textsuperscript{24} with minor modifications. The bisacrylamide to acrylamide ratio of acrylamide solution was 0.03. Proteins were separated by 7.5% polyacrylamide gel for 2-DE and stained by silver staining.\textsuperscript{25} Insulin was separated by 10% polyacrylamide gel and stained by GelCode blue dye solution.

**Electrolytic Reduction of Water and MTT in IEF.** IPG strips rehydrated by distilled water with or without salts for 4 h were subjected to IEF. IEF was performed at a maximal voltage of 500 V for 600 voltage hours or 8 000 voltage hours. The strips were

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rinsed in distilled water and stained in 0.5 mg/mL phenol red for 10 s to reveal the acidification and alkalization. Alternatively, IPG strips rehydrated by 0.5 mg/mL MTT in water with or without salts for 4 h were subjected to IEF. IEF was performed at a maximal voltage of 500 V for 600 voltage hours. The presence of reducing power was indicated by the localization of insoluble purple formazan in the IPG strips.

**Electrolytic Reduction of Proteins in an Electrolytic Chamber.** In this homemade apparatus, the anodic chamber (2.5 cm × 3.5 cm × 2.0 cm) was filled with 3 mL of 1% acetic acid containing 0.1 M salt and salt-bridged with a slice of PDVF membrane (1.8 cm × 8.0 cm) wet with methanol, water, and then 1% acetic acid/0.1 M salt. Protein samples were dissolved in 1% acetic acid containing 0.1 M salt and then loaded into the cathodic chamber (2.5 cm × 3.5 cm × 2.0 cm). The use of acetic acid would minimize the migration of proteins toward the anodic chamber due to protonation of the proteins. Bovine insulin was electrolyzed at 50 V for various time intervals. The electrolyzed samples were removed at different time intervals and subjected to SDS-PAGE for determination of disulfide reduction.

Proteins (ovalbumin and RNase A) were electrolyzed in 1% acetic acid containing 0.1 M NaCl at 100 V for 4 h, and the solutions were then adjusted to pH 7.0–8.0 prior to reaction with primary amines or hydrazides. The solution was added by equal volume of 10 mg/mL biotin-pentylamine or 2 mg/mL biotin-LC-hydrazide and allowed to incubate at room temperature for 30 min for Schiff base reaction. The samples were subjected to SDS-PAGE and streptavidin-peroxidase blot overlay. For 2,4-dinitrophenyl (DNP) modification of proteins, 5 mL of the protein solution was added by 5 mL of 12% SDS and 10 mL of IX DNP-hydrazine and incubated at room temperature for 15 min. The reaction was stopped by adding 7.5 mL of neutralizing buffer and 1.5 mL of 2-mercaptoethanol. The samples were subjected to SDS-PAGE and immunoblotting with anti-DNP antibodies. Proteins on the gels were transferred at 50 V for 3 h onto the PVDF membrane in 50 mM Tris base, 40 mM glycine, 0.04% SDS, and 10% methanol transfer buffer.

**Streptavidin Peroxidase Blot Overlay.** The PVDF membrane was then blocked with 3% BSA in PBS containing 0.05% Tween 20 (PBS-T) for 1 h, washed with PBS-T three times for 5 min, and probed with horseradish peroxidase-conjugated streptavidin (0.01 mg/10 mL) in PBS-T containing 3 mg/mL of BSA for 30 min. After a 5 min wash with PBS-T three times, streptavidin reactive bands were detected by the NiCl2 enhancement method26 or using the Immobilon Western ECL substrate solution followed by autoradiography.

**Immunoblotting of DNP-Proteins.** The PVDF membrane was blocked with 1% BSA/PBS-T for 1 h. After incubation with rabbit anti-DNP antibody (1:150 dilution) in 1% BSA/PBS-T for 1 h, the membrane was washed with PBS-T for 5 min, three times, and probed with horseradish peroxidase conjugated goat antirabbit IgG antibody (1:200 dilution) in 1% BSA/PBS-T for 1 h. After a 5 min wash with PBS-T three times, the blots were visualized using Immobilon Western ECL substrate solution followed by autoradiography.

**Sample Preparation and Mass Spectrometric Analysis.** RNase A and electroreduced RNase A were buffer-exchanged into 25 mM (NH4)HCO3 by Millipore Centricron YM-10, and 10 µL of protein sample (0.5 mg/mL) was dried in microcentrifuge tubes by a Speed-vac. Protein samples were dissolved in SDS sample buffer and subjected to SDS-PAGE or dissolved in 100% acetonitrile as analytes and subjected to mass spectrometric analysis. For determination of the mass of the proteins, the analytes were directed to the homemade nanosprayer applied with ~3.5 kV on the QSTAR XL hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). All data were acquired and processed using Analyst QS 1.1 with the Bioanalyst 1.1 extension. Full scan mass spectra (MS) were recorded in the positive ion mode in the range of m/z 400–2000, and the raw mass spectra were deconvoluted using Analyst QS 1.1 protein deconvolution software.

**RESULTS AND DISCUSSION**

**Artifacts in 2-DE.** Contamination of salts in IEF results in high conductivity, prolonged and poor focusing, electroosmosis, and thus poor resolution of proteins. However, the closed system design of the IPGphore strip holder does not permit excessive ions to leave the system and neither accommodates excessive ions accumulating at the electrodes. In theory, an increased rate of electrolytic reactions can be observed, anodic oxidation and cathodic reduction of water generating protons and hydroxide ions, respectively. Thus, large regions at either end of the IPG strip should remain blank where proteins do not focus.

IEF in the presence of 10 mM NaCl induced deteriorating resolution near the cathodal end, resulting in protein clumping and vertical streaking in 2-DE (parts B vs A of Figure 1). Increasing concentrations of NaCl exaggerated the pattern of poor resolution at sites near the cathode and anode resulting in protein clumping, vertical streaking, and horizontal streaking (Figure 1C). As expected, Na2SO4 (Figure 1D) and (NH4)2SO4 (Figure 1E) induced more pronounced protein clumping possibly due to higher solute concentrations upon dissociation. We then designed a procedure for improving 2-DE from salt interference. After protein-loading, priming IEF in the presence of 20 mM NaCl at a maximal voltage of 300 V for 200 voltage hours, the IPG strip was incubated with freshly prepared rehydration buffer for 5 min, twice, to remove excess salt (herein named in-gel dialysis) and IEF was performed again (refocusing IEF). Most artifacts caused by a high concentration of salts in the sample were alleviated (Figure 1F). However, extra protein spots appeared at areas very close to the cathode. In other words, some of the artifacts in 2-DE caused by a high concentration of salts in the sample are reversible and can be removed by in-gel dialysis. However, modified proteins of higher pI values, which are initially masked by the lack of focusing and formation of protein aggregate, are unmasked by in-gel dialysis and refocusing IEF.

In order to closely monitor the artifact products of high pI values, we used purified RNase A in the following experiments. Under regular conditions, denatured RNase A was resolved as a protein spot of pI 8.4 and molecular mass of 21 kDa (Figure 2A). After priming IEF in the presence of 20 mM NaCl at a maximal voltage of 500 V for 200 voltage hours, the IPG strip was subjected to in-gel dialysis and refocusing IEF. RNase A was resolved as protein spots of pI 9.0, 9.4, and ≥11.0, all higher than the original pI value (Figure 2B). Next, we applied native IEF to eliminate the influence of urea which might cause carbamylation of proteins and alter their pI values. Under regular conditions, native RNase
A was resolved as a protein spot of pI 10.2 (Figure 2C). In the presence of 20 mM NaCl throughout IEF, RNase A was resolved as a protein spot of pI 9.3, showing a cathodic drift of pI values that might be caused by electrolysis-generated hydroxides. After priming IEF in the presence of 20 mM NaCl at a maximal voltage of 500 V for 200 voltage hours, the IPG strip was subjected to in-gel dialysis and refocusing native IEF. RNase A was resolved as protein spots of pI 10.5, 10.9, and ≥11.0, all higher than the original pI value, 10.2 (Figure 2E). Obviously, formation of protein products of higher pI values due to the presence of high salts in IEF does occur in the absence of urea, thus eliminating the role of urea in this event.

After priming IEF in the presence of 20 mM NaCl at a maximal voltage of 500 V for 0, 0.5, 1, and 1.5 h, the IPG strip was subjected to in-gel dialysis and refocusing native IEF. The isoelectric point of RNase A gradually changed from 10.2 to 10.5 and ≥11.0 (Figure 3). Apparently, proteins with high pI values such as RNase A can be irreversibly modified during IEF in the presence of high salts, generating proteins of higher pI values.

**Perturbation of the pH Gradient.** Electrolytic reaction may play a key role in the formation of the above-mentioned artifacts including cathodic drift because electrolysis of water should be enhanced in the presence of salt during electrophoresis. Accumulation of protons at the anode and hydroxide ions at the cathode would protonate and deprotonate proteins near the respective electrode and also level off the pH gradient within the IPG gel near both ends. Subsequently, areas near both ends will

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**Figure 1.** Salt-induced artifacts in 2-DE: Day 1 zebrafish embryo protein samples were resolved by 2-DE with pH 4–10, and the IEF was performed with containing (A) no salt, (B) 10 mM NaCl, (C) 20 mM NaCl, (D) 20 mM Na2SO4, (E) 20 mM (NH4)2SO4, or (F) in-gel dialysis and refocusing IEF after priming IEF in the presence of 20 mM NaCl at a maximal voltage of 500 V for 200 voltage hours. Rectangles, anodic or cathodic drift; circles, unexpected artifacts.

**Figure 2.** Irreversible pI shift of bovine RNase A in 2-DE in the presence of NaCl: For denatured IEF, RNase A was resolved by pH 4–11 2-DE under (A) regular conditions or (B) priming IEF in the presence of 20 mM NaCl followed by in-gel dialysis and refocusing IEF in rehydration buffer containing 100 mM DTT. For native IEF, RNase A was resolved by pH 4–11 2-DE, in 0.5% IPG buffer (C) under regular conditions or (D) in the presence of 20 mM NaCl or (E) under priming IEF in the presence 20 mM NaCl followed by in-gel dialysis and refocusing IEF. Before in-gel dialysis, priming IEF was carried out at a maximal voltage of 500 V for 200 voltage hours.

**Figure 3.** Time course of RNase A modifications in the presence of 20 mM NaCl: RNase A was resolved by pH 4–11 2-DE, in 0.5% IPG buffer under priming IEF in the presence of 20 mM NaCl followed by in-gel dialysis and refocusing IEF. Before in-gel dialysis, priming IEF was carried out at a maximal voltage of 500 V for 0, 0.5, 1, and 1.5 h.
be exempt from protein focusing. To determine the pH perturbation, we monitored the pH values across the IPG strip by immersing the strips in a solution of phenol red which detects the pH range of 6.8–8.4 with a color transition from yellow, orange to purple after a brief rinse in distilled water. Accomplishment of IEF in the presence of high salts generated both tides of acidity and alkalinity moving into the center of the strip as indicated by the appearance of a brighter yellow and purple color, respectively, instead of a color transition (data not shown). For various salts, the extent of alkalization was highest in the presence of LiCl, NaCl, and CaCl$_2$ and was lowest in the presence of (NH$_4$)$_2$SO$_4$ with the descending sequence of LiCl $>$ NaCl $>$ CaCl$_2$ $>$ KCl $>$ MgCl$_2$ $>$ (NH$_4$)$_2$SO$_4$. Magnesium metal may form as a gray precipitate near the cathodic end in the presence of 25 mM MgCl$_2$ during the IEF while other alkali and alkaline elements are very reactive in water solution causing electroreduction of water and affecting the pH gradient of the IPG strips. For ammonium ions, because reduction of ammonium ions generates nitrogen and hydrogen gas evaporating into the air, little electrolysis of water occurs. In addition, the rate of ion movement toward the cathode may contribute to the differential extent of alkalization.

Reducing Power Generated in Electrosynthesis. We next examined the extent of the reducing power generated during electrolysis. We included MTT in distilled water and performed IEF in the presence of NaCl at various concentrations. In the absence of NaCl, there was little MTT reduction. In the presence of 6 and 12 mM NaCl, insoluble purple formazan formed after 600 voltage hours of electrophoresis at sites near the cathode. In the presence of 25 and 50 mM NaCl, diffuse formazan product deposited 2–3 cm away from the cathode (data not shown). The results indicate that higher concentrations of NaCl interfere with the electrophoresis of MTT, and cathodic reduction reactions occur away from the cathode. Therefore, localization of MTT at the cathode seems not to be required for its reduction. The extent of MTT reduction was highest in the presence of LiCl, NaCl, and CaCl$_2$ and was lowest in the presence of (NH$_4$)$_2$SO$_4$ with the descending sequence of LiCl $>$ NaCl $>$ CaCl$_2$ $>$ KCl $>$ MgCl$_2$ $>$ (NH$_4$)$_2$SO$_4$. Application of moist filter paper and anion exchanger paper (size 10 $\times$ 3 mm$^2$) between the electrodes and the IPG strip did not alleviate the electrolysis-generated reduction of MTT. However, the presence of cation exchanger paper significantly prevented the reduction of MTT at sites near the cathode indicating freely movable cations are required for the spreading of reducing power for MTT reduction.

We modified a homemade horizontal electrophoresis apparatus with platinum electrodes as an electrolyzer to perform cathodic reduction. Electrolytic reduction of bovine insulin caused disulfide bond reduction and generated insulin A and B chains at 50 V for 1.5 h in the presence of 0.1 M NaCl and LiCl. After 2 h of reduction in the presence of 0.1 M NaCl and LiCl, most insulin molecules were reduced into A and B chains (data not shown). The extent...
of insulin reduction was highest in the presence of LiCl and NaCl and was lowest in the presence of \((\text{NH}_4)_2\text{SO}_4\) with the sequence of \(\text{LiCl} > \text{NaCl} > \text{CaCl}_2 > \text{KCl} > \text{MgCl}_2 > \text{NH}_4\text{SO}_4\). Therefore, alkali and alkaline metal ions as electrolytes are effective to produce the reductive species which can reduce MTT and protein disulfide bonds. The efficiency of reduction is proportional to ion concentration, and the order of activity among different cations is identical in MTT and protein disulfide bond reduction and in electrolysis-induced alkalization. Altogether, freely movable metal ions accumulation at sites near the cathode is required for the generation of reducing power.

One pivotal question remains mysterious to us; what is the nature of the reducing power that reduces MTT and proteins at sites a couple centimeters away from the cathode? It appears that contact with the cathode is not required for the reduction of MTT and proteins at sites a couple centimeters away from the cathode. The extent of MTT reduction is highest in the presence of LiCl and NaCl and was lowest in the presence of \((\text{NH}_4)_2\text{SO}_4\) with the sequence of \(\text{LiCl} = \text{NaCl} > \text{CaCl}_2 > \text{KCl} > \text{MgCl}_2 > \text{NH}_4\text{SO}_4\). Therefore, alkali and alkaline metal ions as electrolytes are effective to produce the reductive species which can reduce MTT and protein disulfide bonds. The efficiency of reduction is proportional to ion concentration, and the order of activity among different cations is identical in MTT and protein disulfide bond reduction and in electrolysis-induced alkalization. Altogether, freely movable metal ions accumulation at sites near the cathode is required for the generation of reducing power.

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amide groups of amino acid residues can theoretically convert a given protein to a more basic form.

As shown in Figures 1F, 2, and 3, protein modification turned given proteins into a more basic form. Expected reduction products of carboxylic acids are the corresponding aldehydes and alcohols. Schiff base or hydrazone formation with primary amines or hydrazides enables us to detect any aldehyde intermediates during and after cathodic reduction. To detect carbonyl groups generated in 2-DE, we introduced biotin-pentylamine (bPA) in IEF to conjugate aldehyde groups. It turned out that streptavidin-peroxidase overlay assay displayed a few signals which merely appeared on the cathodic end of 2-DE in regular IEF (Figure 5A). However, inclusion of 20 mM NaCl in the rehydration buffer not only interfered with the focusing but also caused massive carbonyl formation in proteins which were detected with bPA incorporation. Pentylamine reactive proteins were clustered at the boundary of cathodic drift of 2-DE due to alkalization (Figure 5B). In agreement with above results, localization of proteins at the cathode is not required for the electroreduction to occur. Although in-gel dialysis and refocusing IEF alleviated the extent of protein carbonyl formation, the extent of protein carbonyl formation increased in comparison with regular conditions (Figure 5C). The modified proteins tended to move to the higher pH range and formed protein oligomers after refocusing IEF. The results show that carbonyl groups of the proteins increase during IEF in the presence of NaCl, and the proteins become more basic after refocusing IEF. The data suggest that proteins of higher pI values may result from reduction of carboxyl groups. Moreover, the high-molecular mass protein oligomers may result from formation of a Schiff base between proteins.

Preparative Electrolytic Reduction of Proteins. So far, our results indicate that alkali and alkaline metal ions in electrolysis act as an important mediator for electrochemical reduction. We further used the homemade electrolyzer to carry out electrolytic reduction of proteins and monitor the formation of carbonyl groups by addition of bPA, biotin-LC-hydrazide (bHz), or 2,4-dinitrophenylhydrazine (DNPH). Indeed, RNase A after electrolysis in 1% acetic acid containing 0.1 M NaCl at 100 V for 4 h was reactive to bPA and bHz and detected by the streptavidin-peroxidase overlay assay (Figure 6A). The dose–response relationship was demonstrated with increasing concentrations of bPA resulting in increasing amounts of bPA-ovalbumin adducts and also the bPA-ovalbumin dimer (Figure 6B). Additionally, the OxyBlot protein oxidation detection kit was used to detect any carbonyl groups in the protein side chains which can be derivatized into 2,4-dinitrophenylhydrazone (DNP-hydrazone) adducts by reaction with DNPH. The DNP-conjugated protein samples were subjected to SDS-PAGE and then detected by immublotting with rabbit anti-DNP antibodies provided by the assay kit. Before electrolysis, RNase A was not reactive to the assay and ovalbumin contained a few carbonyl groups. RNase A and ovalbumin after electrolysis

![Figure 7](image-url)
in 1% acetic acid containing 0.1 M NaCl at 100 V for 4 h displayed significant increases in carbonyl groups and were detected by the assay (Figure 6C). Thus, we have scaled up the electrolytic reduction of the proteins to a semipreparative scale. Electrolytic reduction of proteins in the presence of sodium ions can in practice prepare a pseudoprotein whose acidic residues are reduced. We also quantified the amount of amine functional group in RNase A and found that the amine content decreased after electropherased RNase A were analyzed by LC–MS/MS to search for modified peptide fragments from electroreduced RNase A (data not shown). Unfortunately, we could not find peptide fragments with clear modification sites.

CONCLUSION

In this paper, we have disclosed two novel effects of salts in the practice of 2-DE: electrolysis-generated acidification and alkalization tides and electrolytic reduction of proteins as summarized in Figure 8. Acidification tide originating from the anode and alkalization tide from the cathode are responsible for the anodic drift (Figure 8a) and cathodic drift (Figure 8b) along with increased conductivity and electroosmosis during IEF. More importantly, electrolytic reduction of proteins of higher pI values will irreversibly convert acidic amino acid residues into the corresponding aldehydes or alcohols and further increase their pI values (Figure 8c). Electrolytic reduction of proteins causes permanent modifications, and the modifications persist after in-gel dialysis and refocusing IEF. Cation species of the alkali and alkaline metals play key roles in determining the rate and efficiency of electrolytic reduction. The precise intermediates formed by alkali and alkaline metal ions demand further investigation. Nevertheless, it becomes possible to apply electrolytic reduction in developing a pseudoprotein or a pseudopeptide with carbonyl groups which may be used for conjugation with amine-containing molecules and protein cross-linking. One possible application of these carbonyl-containing pseudoproteins is conjugation of amine-containing haptens for immunizations.

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