Experion™ Automated Electrophoresis System and the Experion Pro260 Analysis Kit: Accurate and Reproducible Protein Sizing and Quantitation in the Presence of High Salt Concentrations

Karen Zhu and William Strong, Bio-Rad Laboratories, Inc.,
6000 James Watson Drive, Hercules, CA 94547 USA

Introduction

Ion exchange and affinity chromatography methods are frequently used for protein separation and purification, and these methods commonly employ high concentrations of salt to effect elution of proteins from resins. Once proteins are collected, determinations of protein size (sizing) and concentration (quantitation) are carried out. Initial sizing estimates are nearly always performed using SDS-PAGE, while quantitation can be performed using gel electrophoresis, any one of a number of dye-based quantitative assays, or UV spectroscopy. The varying amounts and high concentrations of salt (generally 0.01–1.0 M) that are incorporated into many chromatographic elution buffers can be problematic for sizing and quantitation using these methods; high salt concentrations may cause band distortion or gel artifacts when the proteins are analyzed by SDS-PAGE, or they may alter background staining with protein assays, making them more variable and more cumbersome to use and necessitating careful planning and controls. Desalting prior to SDS-PAGE or a protein assay is desired in these cases, but it is sometimes not practical owing to limited sample volumes or target protein levels.

The Experion automated electrophoresis system, based on Caliper Life Sciences' LabChip microfluidic separation technology, performs rapid, reproducible, and accurate protein separation, sizing, and quantitation within a single platform. Protein analysis is performed with the Experion Pro260 analysis kit, which contains the reagents, microfluidic chips, and other supplies required for the separation and analysis of 10–260 kD proteins. Though the sizing and quantitation performance of the Experion system and Pro260 analysis kit matches or even surpasses that of SDS-PAGE (Zhu et al. 2005), this automated system must also prove reliable when the protein sample is dissolved in buffers containing high salt concentrations. Inasmuch as the Experion system relies on electrokinetic sample injection, salt concentrations can influence the amount of sample that is injected and analyzed; hence, the sensitivity of the system can be notably influenced by the ionic strength of the sample solution. In this tech note, we demonstrate the accuracy and reproducibility of protein sizing and quantitation using the Experion Pro260 analysis kit over a broad range of protein and salt (NaCl) concentrations.

Methods

Protein Samples

Purified *E. coli* β-galactosidase (116 kD), rabbit muscle phosphorylase b (97 kD), bovine liver glutamate dehydrogenase (55 kD), chicken egg ovalbumin (45 kD), rabbit muscle lactate dehydrogenase (36.5 kD), bovine milk β-lactoglobulin (18.4 kD), and chicken egg white lysozyme (14.3 kD) were purchased from Sigma-Aldrich, Inc. Bovine serum albumin (BSA, 66 kD) was purchased from the National Institute of Standards and Technology (NIST). Rabbit muscle triosephosphate isomerase (26.6 kD) was purchased from Boehringer Mannheim. Sodium chloride was purchased from VWR International, and sodium phosphate (dibasic) was purchased from EMD.

An array of 42 samples, each containing different protein and NaCl concentrations in 10 mM phosphate buffer (pH 7.2), was generated for testing. Each sample contained all nine purified proteins listed above, each at one of six concentrations (50, 100, 200, 400, 800, or 1,600 ng/µl) and one of seven NaCl concentrations (0.01, 0.05, 0.15, 0.30, 0.50, 0.75, or 1.0 M). The 0.15 M NaCl concentration, considered the optimal ionic strength for protein analysis with the Pro260 analysis kit, was used as the control.

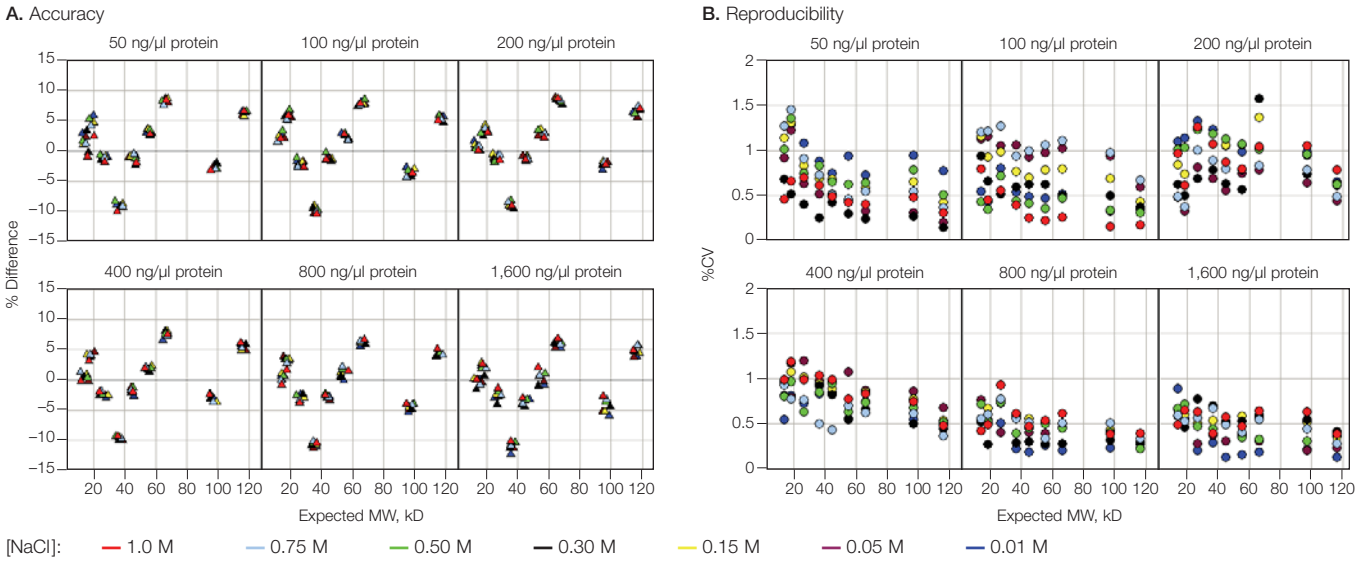

Fig. 1. Scatter plot comparisons of protein sizing accuracy (A) and reproducibility (B) for nine proteins at various concentrations and in the presence of 0.01–1.0 M NaCl. Each panel represents data for a different fixed concentration of all nine proteins, as indicated. Each colored spot represents the average of nine measurements at each experimental salt concentration (three chips with three replicate wells/chip); three chips with four replicate wells/chip were used for 0.15 M NaCl controls. Each color represents a different salt concentration.

Experion Pro260 Analysis

Experion Pro260 analysis kits include Experion Pro260 protein ladder, sample buffer, gel solution, fluorescent stain, spin filters, and microfluidic chips. Samples were prepared by mixing 2 µl Pro260 sample buffer (containing 3.2% β-mercaptoethanol) with 4 µl protein solution. Samples were heated at 95°C, diluted with 0.2 µm-filtered water, and loaded onto chips that were primed according to the protocol provided in the Pro260 analysis kit instruction manual. At least three chips (total number of wells ≥9) were run for each protein and salt combination. Statistical analysis of the sizing and quantitation data was performed using JMP software, version 5.1 (SAS Institute, Inc.). Scatter plot data representations were generated using Spotfire DecisionSite software, version 8.1 (Spotfire, Inc.).

Results and Discussion

Influence of Salt on the Accuracy and Reproducibility of Protein Sizing

Experion software automatically calculates the molecular weight (MW) for each resolved protein in a sample. To accomplish this assessment, the software first generates a calibration curve based on the migration time and known MW of each protein in the Pro260 ladder. Next, it normalizes the migration times of each sample protein by aligning each separation to the ladder lane using internal upper and lower markers that are present in each sample and that bracket the sizing range. Subsequently, the software uses the calibration curve to calculate the size of each sample protein. Sizing results are displayed in real time in an electropherogram, Results table, and simulated gel view.

To determine the impact of salt on the accuracy and reproducibility of protein sizing, we prepared and analyzed a collection of 42 samples comprised of nine proteins covering broad MW (14.3–116 kD) and concentration (50–1,600 ng/µl) ranges, and including a series of salt concentrations (0.01–1.0 M NaCl). The sizes of the nine proteins in each sample were measured, reported by Experion software, and compared to their expected MW. Accuracy, defined by the percent difference between the calculated and expected protein size, was determined using the formula: [(calculated size – expected size)/expected size] x 100. Values close to zero indicate parity between the estimated and known sizes, and a negative or positive value indicates an underestimation or overestimation, respectively. Reproducibility was evaluated using the coefficient of variation, or CV ([standard deviation/mean] x 100), as a statistical measure; CV was expressed as a percentage, with small CV values indicating a small degree of variation in replicates and good reproducibility of the quantitative data.

The data presented in each panel of Figure 1 represent the average interchip (across multiple chips) accuracy and reproducibility of protein sizing. In Figure 1, the data are divided such that at each of the six protein concentrations (50–1,600 ng/µl), a colored triangle or circle represents the % difference (Figure 1A) or %CV (Figure 1B) of a sample protein at a particular NaCl concentration. For most conditions represented, the individual data points nearly overlap, demonstrating that regardless of the protein concentration tested, increases in salt concentration from 0.01 M to 1.0 M had minimal effect on the accuracy and reproducibility of protein sizing. Despite the presence of high salt, the sizing data generated by the Experion Pro260 analysis kit were reasonably accurate, with most proteins deviating by less than 5% from expected sizes (Figure 1A); however, certain proteins, such as lactate dehydrogenase (36.5 kD) and BSA (66 kD), exhibited greater deviations from their expected MW

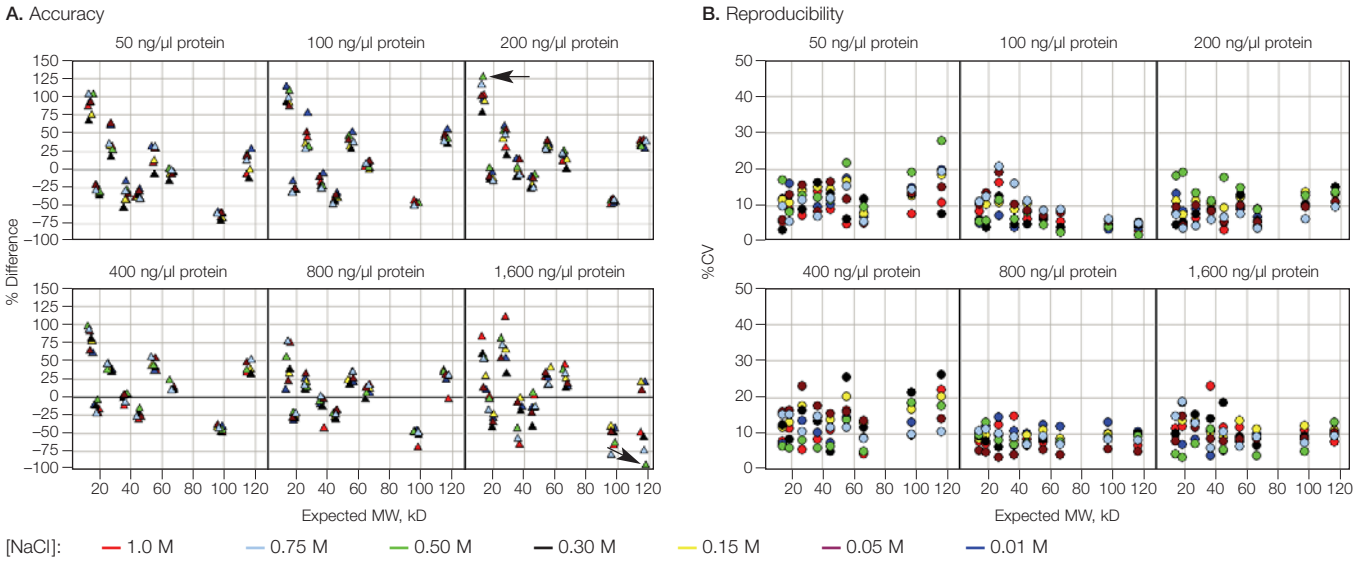

Fig. 2. Scatter plot comparison of protein quantitation accuracy (A) and reproducibility (B) for nine proteins at various concentrations and in the presence of 0.01–1.0 M NaCl. Each panel represents data for a different fixed concentration of all nine proteins, as indicated. Each colored spot represents the average of nine measurements at each experimental salt concentration (three chips with three replicate wells/chip); three chips with four replicate wells/chip were used for 0.15 M NaCl controls. Each color represents a different salt concentration, and arrows indicate maximum over- and underestimations of quantitation accuracy.

(–11.5% and +9.1%, respectively). The results shown in Figure 1B illustrate that the sizing estimates were also highly reproducible (CV <1.6%) for all six protein concentrations and seven salt concentrations tested.

Influence of Salt on the Accuracy and Reproducibility of Protein Quantitation

The Experion Pro260 analysis kit acquires quantitative information about a protein sample by comparing the peak area of each sample protein to that of an internal standard, the 260 kD upper marker, which is present at a known concentration in each sample. Because this internal standard is present in the protein sample throughout preparation and analysis, it is presumed that any effects on quantitation that stem from differences in sample injection or separation will be experienced equally by all the proteins comprising the sample.

To demonstrate this concept, we compared the accuracy and reproducibility of quantitation of each protein species in the samples described previously at each of the seven NaCl concentrations (Figure 2). Depending upon the protein, deviations from expected concentrations as great as –93% (underestimate, 1,600 ng/µl β-galactosidase) to +128% (overestimate, 200 ng/µl lysozyme) were observed. Such deviations are not uncommon in other dye-based quantitation methods and are likely due to differences in the staining efficiencies of proteins (Nguyen and Strong 2005, Sapan et al. 1999). These differences indicate that certain proteins stain differently from the 260 kD upper marker since the concentration of each protein is normalized to this protein by Experion software.

Importantly, however, the level of salt in the sample appeared to have little effect on quantitation at protein concentrations up to 400 ng/µl (3.6 mg/ml total protein), as evidenced by the clustering of data points and the similar % difference values for each protein over this range of protein concentrations (Figure 2A). With protein concentrations of 800 ng/µl and greater (>7.2 mg/ml total protein), a salt-dependent effect first appeared on the quantitation accuracy of lysozyme (14.3 kD, Figure 2A), where the % difference value dramatically changed at many of the salt concentrations, as well as for the 36.5, 97, and 116 kD proteins at the 1.0 M salt concentration (red triangles). This effect on quantitation accuracy appeared to extend to the other proteins in the mix when each of their concentrations was 1,600 ng/µl (14.4 mg/ml total protein). There was no clear correlation in the data supporting the idea that the salt concentration in the sample alone reduced quantitation accuracy; rather, decreased accuracy appeared to be related to the solubility of the proteins when combined at elevated salt and total protein concentrations. In fact, several of the proteins were susceptible to precipitation at this high total protein load and at the higher salt concentrations (>0.50 M); during sample preparation, insoluble matter was visible at these protein and NaCl concentrations.

When the reproducibility of quantitation was examined, the CV for most proteins at the various protein and salt concentrations tested was generally ≤20% (Figure 2B).

Influence of Salt on Sensitivity

The results above show that by using the upper marker as an internal standard, the effect of differences in sample composition on injection and separation can be controlled, so they normally will not affect Pro260 sizing and quantitation performance. However, elevated salt concentrations can cause changes to assay sensitivity due to less sample being injected into the separation channel, resulting in visually perceptible changes in the electropherogram and gel views (Figure 3).

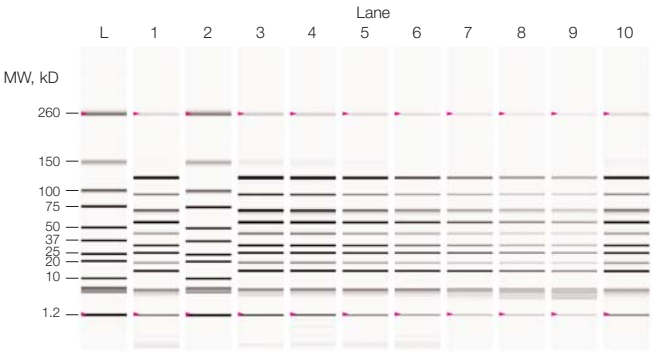

Fig. 3. Simulated gel view showing the influence of salt on detection sensitivity. Lanes L and 2, separation of Pro260 ladder; lanes 1 and 10, separation of a mixture of nine proteins in 0.15 M NaCl; lanes 3–9, separation of a mixture of nine proteins (each at 400 ng/µl) in 0.01, 0.05, 0.15, 0.30, 0.50, 0.75, and 1.0 M NaCl, respectively. Note the decreasing signal intensity at higher salt concentrations. To observe the salt-dependent effect on signal intensity, Experion software scaling was set to "Global".

To better quantitate the degree to which high ionic strength solutions affect the peak heights of proteins, we plotted the percentage change to the peak heights for each of the nine proteins in the 800 ng/µl samples. Figure 4 illustrates the plot for each of the nine proteins and shows a reduction in peak height of 5–10% for every 0.1 M increase in salt concentration. As expected, similar decreases in signal were obtained when other commonly used salts, such as KCl and $(NH_4)_2SO_4$, were tested at concentrations up to 1.0 M (data not shown). Consequently, when assessing samples in buffers containing elevated salt levels, and in cases where high sensitivity is needed (for example, when evaluating sample purity), higher protein concentrations may be required to compensate for reduced peak heights.

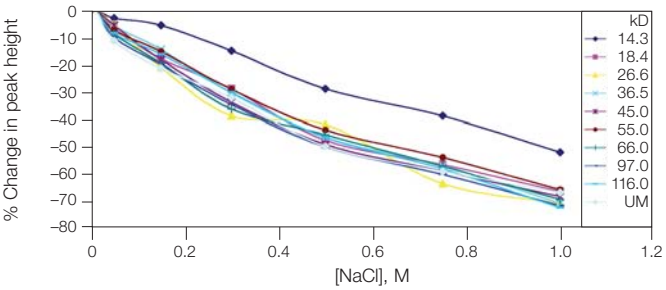

Fig. 4. Influence of salt concentration on peak height of proteins separated by the Experion system. Percentage change in peak height of each of nine proteins is plotted as a function of NaCl concentration to illustrate the dramatic decrease in peak height that occurred in buffers of increasing salt concentration. For each protein, the peak height in 0.01 M NaCl served as the reference. UM = upper marker.

Conclusions

When protein samples are analyzed using the Experion automated electrophoresis system and the Experion Pro260 analysis kit, the presence of high salt (up to 1.0 M NaCl) in the sample does not appear to significantly alter the accuracy and reproducibility of sizing and quantitation, even though there is a notable decline in signal strength as salt levels are increased. This result applied equally to every one of nine different proteins, where the amount of each protein spanned a concentration range of 50–400 ng/µl, for a total protein load of 0.45–3.6 mg/ml. However, these quantitation parameters may vary at lower protein concentrations (<50 ng/µl) and when the salt concentration or ionic strength of the sample solution is elevated (>1.0 M), because the peak heights for all proteins decrease under these conditions and lead to lower overall assay sensitivity. Additionally, a combination of high protein (>800 ng/µl) and high salt concentrations (>0.50 M NaCl) can lead to protein precipitation, which may influence the level of protein in a sample, thereby affecting quantitation accuracy.

References

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