Application of the Experion™ Automated Electrophoresis System to Glycoprotein Visualization and Analysis

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Introduction

The most common posttranslational modification encountered in proteins is glycosylation, in which carbohydrate moieties ranging from a few simple structures to large complexes are added to nascent proteins. Glycoproteins are broadly distributed, existing not only in eukaryotes, but also in eubacteria and archaea (Lechner and Wieland 1989, Messner 1997).

Because of the implications of glycoprotein biochemistry in disease and the fact that many proteins of biotechnological significance are glycosylated, studies of the structure, distribution, synthesis, and biological roles of glycoproteins are of great importance. Glycoproteins play crucial roles in numerous cellular processes, including structural roles, participation in signaling, ligand-receptor interactions, pathogen recognition, immune recognition, and mediation of protein sorting (Varki 1993, Spiro 2002). Misglycosylation and modification of steps in glycosylation pathways can disrupt these functions and have been linked to clinical pathologies, such as mental retardation and gastrointestinal abnormalities (Freeze 2001, Spiro 2002, Martin and Freeze 2003).

One of the conventional methods for studying glycosylated proteins is SDS-PAGE, which is used to monitor glycoprotein size, purity, and stability, and to track changes to glycoproteins following enzymatic and/or chemical treatment. The Experion automated electrophoresis system, a microfluidics-based system that integrates protein electrophoretic separation, staining, destaining, detection, and analysis, can be used as an alternative to SDS-PAGE. The Experion system delivers reduced time-to-results, possesses intuitive sample comparison tools, and reports additional sample information such as percent purity. Moreover, the sensitivity, resolution, and sizing and quantitation performance of Experion Pro260 protein analysis are comparable to or better than similar measurements determined using standard SDS-PAGE methodologies (Zhu et al. 2005). In this report, we show that the Experion system is also an effective alternative to SDS-PAGE in routine experiments where separation, visualization, and monitoring of glycoprotein status are required.

Methods

The proteins used in this study (Table 1) were obtained from Sigma-Aldrich, with the exception of bovine IgG (Bio-Rad Laboratories catalog #500-0208). Deglycosylation was performed using the GlycoPro enzymatic deglycosylation kit and the GlycoPro prO-LINK extender kit (ProZyme). Denaturing conditions were used to maximize the exposure of substrates to enzymes.

Identical amounts of each protein were analyzed by SDS-PAGE and with the Experion system (Bio-Rad Laboratories). For SDS-PAGE, samples were prepared by mixing equal volumes of protein sample and 2x Laemmli sample buffer with or without 5% β-mercaptoethanol. Following separation on Criterion™ Tris-HCl 4–20% precast gels, gels were stained with Bio-Safe™ colloidal Coomassie Blue stain and imaged using a GS-800™ densitometer and Quantity One® software (Bio-Rad Laboratories). For Experion analyses, samples were combined with reducing or nonreducing sample buffer and separated using the Experion Pro260 analysis kit according to the protocol described in the instruction manual.

Table 1. Proteins used in this study. Theoretical molecular weight (MW) calculated using http://ca.expasy.org/tools/pi_tool.html. Sequence data were searched using http://ca.expasy.org/cgi-bin/sprot-search-de (UniProt Knowledgebase, Swiss-Prot, and TrEMBL). %CHO = % carbohydrate.

Protein	%CHO	Theoretical MW (kD)	Source
β-Casein	0	25.1	Bovine milk
Myelin basic protein	0	14.2*	Mouse
Immunoglobulin G (IgG)	3	150*	Bovine
Egg white albumin (ovalbumin)	4	42.9	Chicken
Apotransferrin	6	77.8	Bovine
Ribonuclease B	8	13.7	Bovine pancreas
α-1-Antitrypsin	13	46.7	Human plasma
Peroxidase	18	35.1	Horseradish
Glucose oxidase	18	65.2	*Aspergillus niger*
Lectin	18	26.1	*Ulex europaeus* I
Fetuin	25	38.4	Fetal calf serum
Kininogen, high MW	40	72.0	Human plasma
α-1-Acid glycoprotein	45	23.5	Human

* Multiple isoforms of varying MW.

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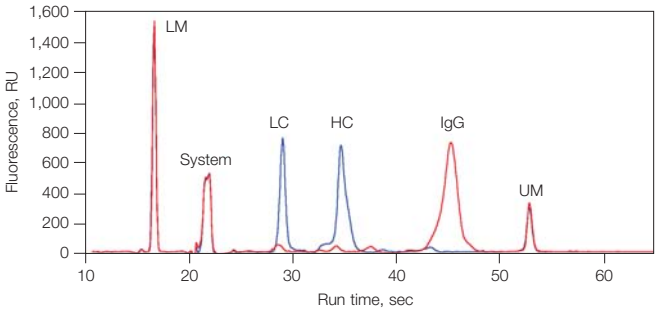

Fig. 1. Experion Pro260 analysis of bovine IgG. Bovine IgG was separated under nonreducing conditions (—) and reducing conditions (—) using the Experion system and the Experion Pro260 analysis kit. LC, light chain; HC, heavy chain; LM, lower marker; UM, upper marker; System, system peaks.

Results and Discussion

Separation of Immunoglobulin G and Kininogen

Immunoglobulin G (IgG) is a multimeric, ~150 kD glycoprotein composed of two heavy and two light polypeptide chains held together by disulfide bonds. To visualize both intact IgG and its constituent subunits, we analyzed bovine IgG under both nonreducing and reducing conditions using the Experion system and SDS-PAGE. Shown in Figure 1 is an overlay of Experion electropherograms generated under both conditions. As expected, a single peak was detected at 162 kD under nonreducing conditions, while two peaks (representing the light and heavy chains) were detected at 30 kD and 62 kD under reducing conditions.

Not all glycoproteins, however, behaved as expected when separated using the Experion system. This was especially true for highly glycosylated proteins, which tended to migrate aberrantly compared to separations performed by SDS-PAGE. As an extreme example of this, Figure 2 shows the separation of high molecular weight human plasma kininogen, a ~110 kD highly glycosylated (~40% carbohydrate) protein cofactor involved in blood coagulation. When separated under nonreducing conditions by SDS-PAGE, kininogen appeared as a doublet with a major band at 98 kD and a fainter band at ~109 kD; under reducing conditions, the products appeared as two bands of approximately 45 kD and 62 kD (Figure 2C). This latter band pattern is expected, as the larger ~109 kD polypeptide is composed of two smaller peptides linked by a disulfide bond (Zhang et al. 2000). However, kininogen displayed different migration characteristics when separated with the Experion system: Under nonreducing conditions, it was also a doublet, but appeared as larger peaks (a primary species at approximately 147 kD and a minor species at 180 kD), and under reducing conditions, the two kininogen cleavage products migrated as a single broad peak at ~82 kD. The observation of a single peak under reducing conditions (rather than the two sharp bands seen on SDS-PAGE gels) indicates that the glycosylated nature of kininogen influenced the migration of the two polypeptides so that they were not completely separated from one another. Deglycosylation of kininogen and separation under reducing conditions resulted in more similar separation patterns and subunit sizes for the Experion and gel separation methods (37 kD and 48 kD, compared to 31 kD and 44 kD, respectively; data not shown).

Glycoproteins often migrate with decreased mobility by SDS-PAGE because the carbohydrate does not bind SDS. This reduces the net charge-to-mass ratio of the protein-SDS complex, resulting in an increased apparent molecular weight (Hames and Rickwood 1990, Westermeier 1997). Additionally, the shapes of denatured glycoproteins, especially those with large oligosaccharide structures, may not be comparable to those of unglycosylated sizing standards, leading to aberrant molecular weight estimates. However, both SDS binding and glycoprotein shape are expected to apply equally to separations by the Experion system and SDS-PAGE, as are additional effects of net charge due to carbohydrate composition (such as the presence of sialic acid).

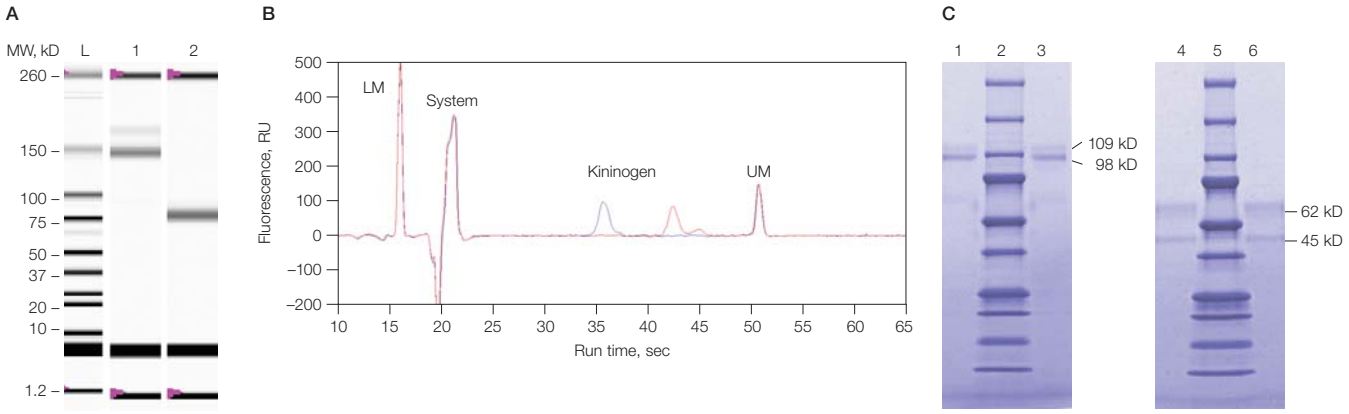

Fig. 2. Comparison of separation of human kininogen by the Experion system and by SDS-PAGE. A, simulated gel image generated by the Experion system showing separation of the Pro260 ladder in lane L, kininogen under nonreducing conditions in lane 1, kininogen under reducing conditions in lane 2; **B**, electropherogram overlay of kininogen under reducing (—) and nonreducing (—) conditions; **C**, SDS-PAGE analysis using Tris-HCl 4–20% gels and showing separation of Precision Plus Protein™ standards in lanes 2 and 5, kininogen under nonreducing conditions in lanes 1 and 3, and kininogen under reducing conditions in lanes 4 and 6. LM, lower marker; System, system peaks; UM, upper marker.

Thus, other dissimilarities between SDS-PAGE and the microfluidics-based Experion system likely contribute to the sizing differences observed. For example, the Experion system uses a separation medium composed of a viscous linear polymer, while the SDS-PAGE gel medium is composed of crosslinked polyacrylamide; these differences result in different pore structures and sieving properties. Moreover, whereas the SDS-PAGE method applies protein staining after separation (thereby excluding any effects of the dye on electrophoretic migration), the Experion system couples protein detection to the separation step in a dynamic process in which dye molecules interact with the SDS-coated proteins throughout separation; the added dye molecules presumably alter sizing results by unevenly affecting the charge-to-mass ratios of both the standard proteins and sample glycoproteins.

Since we generally observe a variance in sizing accuracy of <10% from the expected size when analyzing proteins on the Experion system (Nguyen and Strong 2005, Zhu et al. 2005), the data presented here suggest either that there is considerably more interaction between the kininogen glycoprotein and the Experion matrix or that the dye bound to the glycosylated protein retards migration more through the Experion matrix (decreased charge-to-mass ratio) than through a crosslinked polyacrylamide matrix. Differences in the amounts of bound SDS and dye seem to explain these migration differences, as the apparent molecular weights of the proteins detected by each technique appear to be proportional to the degree of glycosylation; highly glycosylated kininogen (~40% carbohydrate) exhibits more anomalous migration behavior when analyzed using the two methods than the considerably less glycosylated IgG (~3% carbohydrate).

Separation of Proteins Before and After Deglycosylation

To confirm the correlation between protein carbohydrate content and electrophoretic mobility observed using these two techniques, various glycoproteins with carbohydrate contents ranging from approximately 3% to 45% were analyzed. The native and deglycosylated forms of the samples were run in adjacent wells of Experion Pro260 chips and Criterion gels. Figure 3 shows the separation of each protein sample before and after deglycosylation. β-Casein and myelin were included as negative controls for the deglycosylation reactions (these proteins are not glycosylated and so are not expected to exhibit any migration shift after treatment).

In each case, the overall banding patterns obtained by both means of separation were similar, and the proteins and any contaminants were readily identified. Table 2 summarizes the sizes of the major protein species identified by the Experion system and Quantity One software before and after deglycosylation. The failure of peroxidase to exhibit a change in migration following deglycosylation is reportedly due to the fucose (α1 → 3)GlcNAc core glycan structure in the glycoprotein, which is not recognized by the peptide-N-glycosidase F used in the reaction (Tams and Welinder 1995).

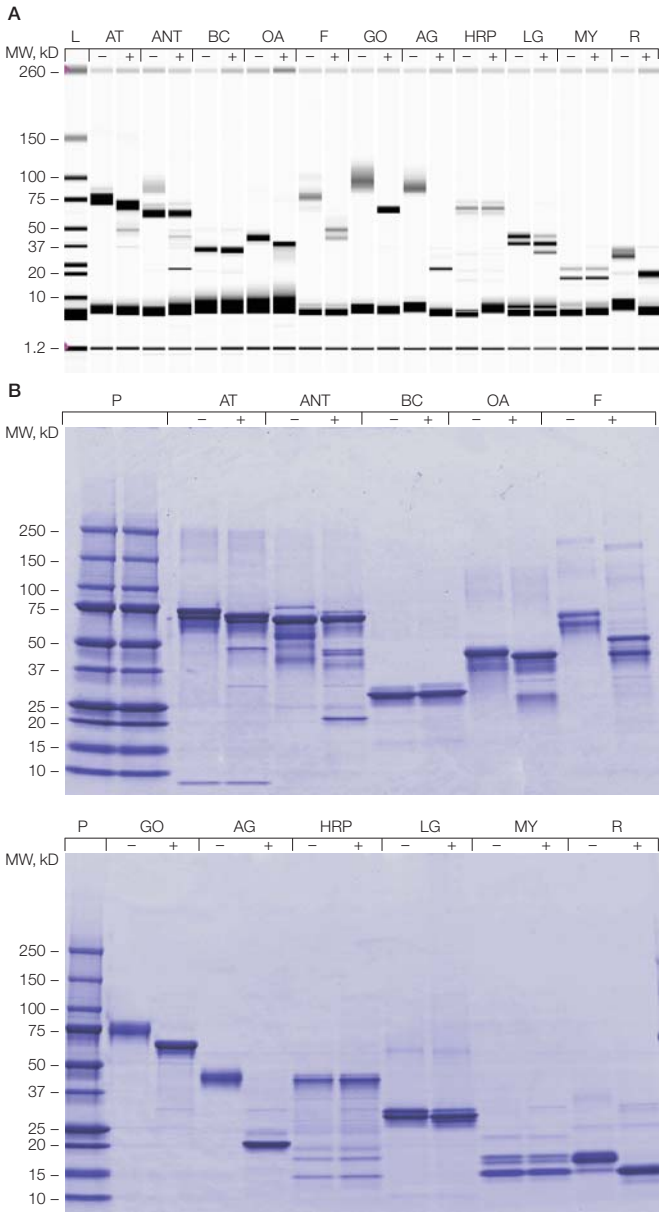

Fig. 3. Separation of proteins before (–) and after deglycosylation (+). **A**, separation with the Experion system; **B**, separation by SDS-PAGE. L, Pro260 ladder; P, Precision Plus Protein standards; AT, apotransferrin; ANT, α-1-antitrypsin; BC, β-casein (unglycosylated control); OA, ovalbumin; F, fetuin; GO, glucose oxidase; AG, α-1-acid glycoprotein; HRP, peroxidase; LG, lectin; MY, myelin basic protein (unglycosylated control); R, ribonuclease B.

A plot of the % difference between the gel- and the Experion-determined molecular weights suggests that sizing results vary more between the two systems for proteins in their glycosylated forms (Figure 4). A linear fit of this data set indicates a relatively strong correlation ($r^2 = 0.71$) between carbohydrate content and variation in sizing using the Experion system compared to SDS-PAGE. An increase in apparent molecular weight of as great as 2% for each 1% increase in carbohydrate was predicted. After removal of the carbohydrate groups, protein migration became more similar between the two systems, differing by less than 20% for most proteins.

Table 2. Apparent size of proteins before and after deglycosylation.

Protein	%CHO		Major Peaks or Bands (kD) Glycosylated Experion System	Glycosylated SDS-PAGE	Deglycosylated Experion System	Deglycosylated SDS-PAGE
β-Casein	0		34.5	29.0	33.8	29.5
Myelin basic protein	0		24.5 22.9 21.7 18.1 15.0	23.3 17.9 17.0 15.2	24.7 23.1 21.9 18.1 15.1	23.3 18.1 17.3 15.4
IgG	3	Nonreducing	162			
		Reducing	62.0 30.0	53.0 26.0	55.0 30.0	51.0 26.0
Egg white albumin (ovalbumin)	4		42.6	45.8	38.4	44.4
Apotransferrin	6		77.9 73.1	73.4 68.5	72.1 68.0 49.2	70.1 66.0 48.5
Ribonuclease B	8		33.1 29.6	18.5 17.5	19.8	15.6
α-1-Antitrypsin	13		87.2 70.5 62.8	78.0 65.7 55.7	72.0 63.0 48.3 44.1 23.0	73.8 67.0 61.6 47.6 45.8 22.2
Peroxidase	18		67.1	42.8	67*	42.8*
Glucose oxidase	18		96.2	75.0	64.8	61.9
Lectin	18		43.8 38.6	30.5 28.8	38.6 33.1	29.0 27.9
Fetuin	25		100 77.6	72.1 65.0	48.9 43.0	54.8 46.4
Kininogen, high MW	40	Nonreducing	180 147	109 98.0		
		Reducing	82 (broad peak)	62.0 45.0	48.0 37.0	44.0 31.0
α-1-Acid glycoprotein	45		88.0	43.3	22.7	20.4

* Horseradish peroxidase is refractive to the glycosidase treatment used in this study (Tams and Welinder 1995).

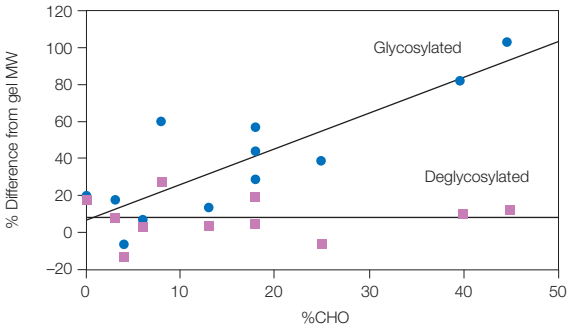

Fig. 4. Effect of carbohydrate content (%CHO) on protein sizing by the Experion system and by SDS-PAGE. The % difference between the molecular weight determined using the Experion Pro260 analysis kit and that determined by SDS-PAGE is plotted against %CHO. For the glycosylated curve, $r^2 = 0.7107$.

Using the Experion System to Monitor the Efficiency of Deglycosylation Reactions

The progress of deglycosylation and other enzymatic or chemical reactions can be monitored using the Experion system (He and Strong 2005). The overlay features and alignment capabilities of Experion software facilitate such analysis by allowing direct sample comparisons. Figure 5 shows Experion separations before and after deglycosylation for several of the glycoproteins examined in this study and illustrates the ease with which the software can be used to detect differences between samples. Moreover, using the overlay function of the software permits straightforward monitoring of reaction progress to assist in optimization.

To demonstrate this approach, the deglycosylation of fetuin and α-1-acid glycoprotein were monitored using both the Experion system and SDS-PAGE gels. Figure 6 shows overlays of Experion electropherograms captured at different time points

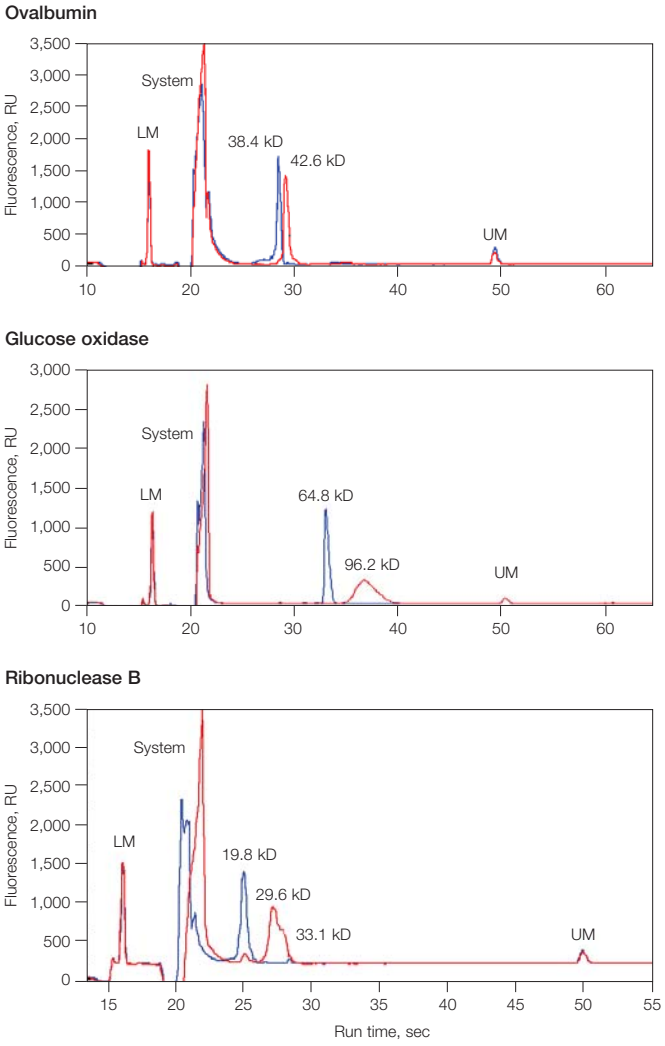

Fig. 5. Experion Pro260 analysis of deglycosylation. Shown are electropherogram overlays of various glycoproteins before (—) and after (—) deglycosylation. LM, lower marker; System, system peaks; UM, upper marker.

Table 3. Reproducibility of protein sizing and quantitation. Samples were analyzed using the Experion Pro260 analysis kit. SE = standard error of the mean.

				Sizing			Quantitation		
Sample Name	Deglycosylation	%CHO	# of Replicates	Mean MW (kD)	SE	%CV	Mean Concentration (ng/µl)	SE	%CV
Ovalbumin	+		7	39.9	0.02	0.2	411.8	22.2	14.3
	–	4	7	44.8	0.07	0.4	375.6	11.2	7.9
Glucose oxidase	+		14	68.4	0.12	0.7	549.8	8.6	5.9
	–	18	14	100.9	0.12	0.4	546.8	8.6	5.9
α-1-Acid glycoprotein	+		14	25.0	0.07	1.1	530.9	14.5	10.2
	–	45	14	98.3	0.45	1.7	569.6	13.7	9.0

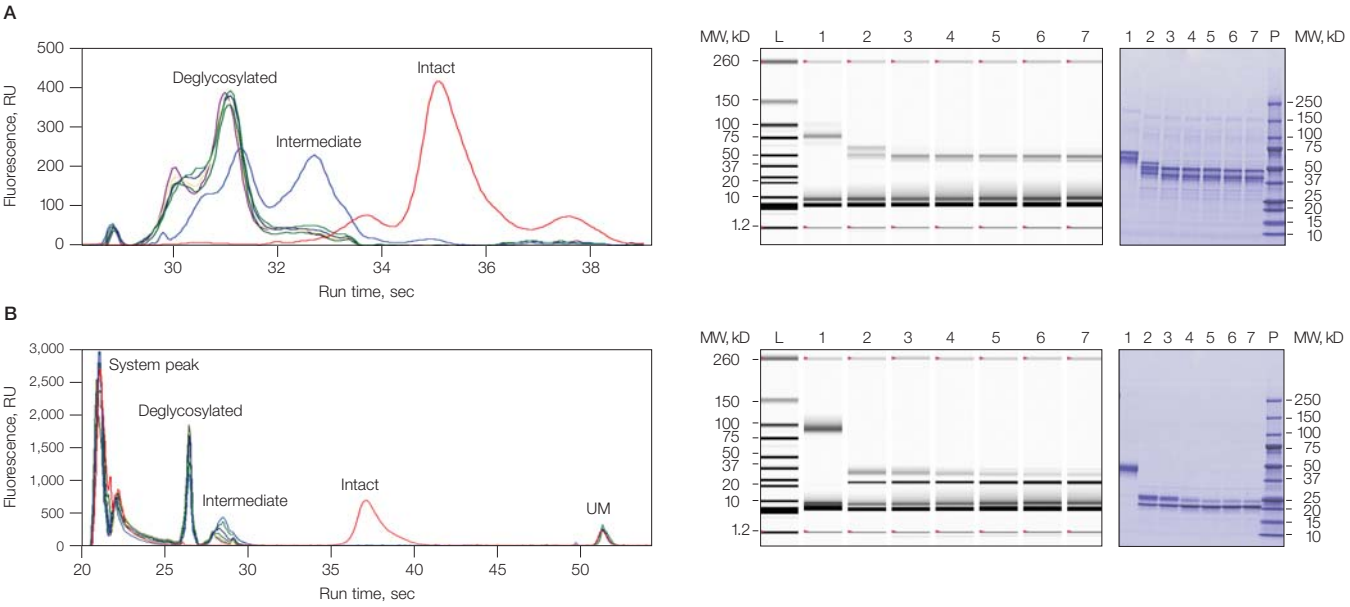

Fig. 6. Time dependence of deglycosylation. Shown are Experion electropherogram overlays (left), Experion simulated gel views (center), and SDS-PAGE analyses (right) of samples taken at various time points during enzymatic deglycosylation. **A**, fetuin samples deglycosylated for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), 1 hr (lane 4), 1.5 hr (lane 5), 2 hr (lane 6), and 3.5 hr (lane 7); **B**, α-1-acid glycoprotein samples deglycosylated for 0 min (lane 1), 5 min (lane 2), 15 min (lane 3), 1.5 hr (lane 4), 4 hr (lane 5), 5.5 hr (lane 6), and overnight (lane 7). L, Pro260 ladder; P, Precision Plus Protein standards.

during the deglycosylation process for each glycoprotein. These overlays allow visualization of the differences in the protein population between time points and clearly indicate the endpoint of the reaction. Figure 6 also presents the same data in an Experion simulated gel view as well as the results from the SDS-PAGE gels. After 10 min of incubation with the mix of glycosidases, the partially deglycosylated form of fetuin was still present, but by 30 min, fetuin had been completely converted to the final, presumably fully deglycosylated 43–49 kD form, as no further molecular weight changes were evident (Figure 6A). The deglycosylation of α-1-acid glycoprotein also appeared to pass through an intermediate stage, but one that was more persistent, as the partially deglycosylated form was present even after overnight incubation (Figure 6B). A deglycosylation reaction performed several months earlier found the reaction complete after 3 hr of incubation (data not shown). The discrepancy between these results suggests the enzymatic activity of some or all of the glycosidases used in the reaction had decreased, indicating that a check of the activity of the glycosidases and further optimization would be needed if complete deglycosylation were desired. A thorough confirmation of complete deglycosylation may be determined by other methods, such as mass spectrometry or periodate oxidation of available carbohydrate groups coupled with fluorescence detection, but the Experion system allows a much quicker, more streamlined approach to monitoring the progress of deglycosylation reactions.

Reproducibility of the Experion System for Glycoprotein Analysis

Since earlier results indicated that sizing accuracy of the Experion system was affected by the degree of protein glycosylation, it was important to determine whether the reproducibility of assay measurements was also adversely affected. To test the reproducibility of the results obtained by the Experion Pro260 analysis kit, seven Experion Pro260 chips were run with three glycoproteins of widely different carbohydrate content: ovalbumin, glucose oxidase, and α-1-acid glycoprotein. The Experion system sized all three proteins within a 2% coefficient of variation (CV) and quantitated all three proteins within a 15% CV (Table 3), indicating that assay reproducibility is independent of protein carbohydrate content.

Conclusions

The data presented here demonstrate the application of the Experion system to the visualization and analysis of glycoproteins. Although proteins with varying degrees of glycosylation can be quickly separated on the Experion system, sizing results may vary from those obtained using conventional SDS-PAGE, especially for highly glycosylated proteins. SDS-PAGE provides only apparent molecular weight information, and several factors may influence the sizing data obtained using the Experion system and SDS-PAGE, such as sieving matrix composition and structure, the percentage, structure, and charge of carbohydrate groups, and the staining process. The Experion system is particularly effective at monitoring and tracking changes to a protein during deglycosylation reactions, facilitating optimization of these reactions, and enabling easy monitoring of the stability or purity of their protein products. The Experion system is a powerful tool that provides reproducible sizing and quantitation data, quick visualization, overlay capability, and digitized results — readily saved and distributable — on glycosylated and unglycosylated proteins in a fraction of the time required to perform similar analyses by SDS-PAGE.

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