Glycoprotein sizing on the Agilent 2100 bioanalyzer

Application

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Abstract

Glycoproteins analyzed either by traditional SDS-PAGE or on the Agilent 2100 bioanalzyer frequently migrate at a different size than anticipated from the known molecular weight. Because the glycan attachment can keep the protein from assuming a rod shape, and will also change the charge-to-mass ratio from the bound detergent, migration is often slowed down, making the glycoprotein appear too large. After enzymatic deglycosylation, the resulting proteins now migrate at the expected sizes.

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Introduction

In eukaryotic cells, glycosylation of proteins is a common post-translational modification. Many proteins appear as glycoforms with different isoelectric points (pI) and/or molecular mass. Glycan attachments to proteins, which may comprise multiple sites and contain highly branched moieties, make the molecule bulky and hydrophilic. When denaturing glycoproteins in a detergent solution for SDS-PAGE, the hydrophilic nature of the attachment will alter the stoichiometry of the detergent association, changing the charge density of the complex. Secondly, the bulky glycan portion will not permit the formation of a rod shaped protein-detergent complex. Because the shapes and the charge-to-mass ratios are not comparable to the proteins used in the ladder, migration times and hence molecular weight estimations of glycoproteins from electrophoretic techniques can be very different from the predicted size based on the molecular composition or mass spectrometry measurements. Analysis of such samples, performed on the Agilent 2100 bioanalyzer or with SDS-PAGE, is susceptible to these sizing anomalies. In order to accurately size the proteins, the glycan portion must be removed. Either hydrazinolysis or enzymatic methods can be used to cleave all common classes of oligosaccharides from glycoproteins. After the complete removal of the glycans, the Agilent 2100 bioanalyzer will provide accurate measurement of the molecular weight and concentration of the sample.

Experimental

An N-Glycosidase F Deglycosylation Kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All proteins and 2-mercaptoethanol were purchased from Sigma Aldrich, (St. Louis, MO, USA). PBS and purified water were purchased from Amresco, (Solon OH, USA). Novex Pre-Cast gels and Tris-Glycine SDS 2X sample buffer were purchased from Invitrogen (Carlsbad, CA, USA). GelCode® Blue Staining Reagent was purchased from Pierce Biotechnology (Rockford, IL, USA). The Agilent 2100 bioanalyzer and the Protein 200 Plus LabChip kit were obtained from Agilent Technologies Deutschland GmbH (Waldbronn, Germany).

Deglycosylation reactions

Proteins were dissolved to 10 mg/mL in deionized water; 5 µL aliquots were reduced and denatured with the kit denaturation buffer for 3 minutes at 98 °C. Then enzymatic deglycosylation was performed in the kit reaction buffer for 1 hour at 37 °C as directed in the Working Procedure for Complete Deglycosylation included in the Roche kit. Control protein (non-deglycosylated reactions) had additional reaction buffer added in lieu of enzyme solution. The control glycoprotein mixture included in the kit was reacted in a similar fashion.

SDS-PAGE

Samples were prepared in Tris–Glycine SDS 2X sample buffer, 2-mercaptoethanol was added to the sample. Samples were run on a Novex 12 % Tris–Glycine Pre-Cast gel at 120 V. Following electrophoresis, the gel was washed with water and stained with GelCode® Blue Staining Reagent for one hour. The gel was then destained by washing with water, and scanned using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA)

Protein 200 Plus Assay

The chip-based protein analysis was performed on the Agilent 2100 bioanalyzer using the Protein 200 Plus LabChip kit and dedicated Protein 200 Plus software assay. Samples containing 0.5 – 1.67 mg/mL of each protein were denatured as specified in the Reagent Kit Guide using the Protein 200 Plus Sample Buffer with added 2-mercaptoethanol. All chips were prepared according to the Reagent Kit Guide. The Protein 200 Plus LabChip kit includes 25 chips, a syringe, 4 spin filters and all required reagents except for reducing agent.

Results and discussion

The 2100 bioanalyzer separates samples through a viscous linear polymer in the microfluidic chip through the careful control of currents and voltages. In order for the sample to move in an electrical field, a charge must be associated with the proteins. According to the Protein 200 Plus protocol, samples are first heat denatured in a lithium dodecylsulfate (LDS) solution to give the proteins a uniform charge to mass ratio. Denatured proteins are assumed to bind dodecylsulfate with a fairly constant stoichiometry and to assume a rod shape. A noncovalently bound fluorophore, present in the gel/dye mix, becomes more

intensely fluorescent upon association with the detergent-protein complex. Samples are separated through the sieving gel matrix and detected by laser-induced fluorescence. Comparison of migration times of the sample proteins relative to the Protein 200 Plus ladder and the alignment to internal standards allows for the accurate sizing of the unknown samples. The implicit assumptions are that all detergent-protein complexes assume a completely unfolded cigar-like shape and that the charge-to-mass ratio of this complex is constant. This holds true for most reduced proteins. The shapes and the charge-to-mass ratios of glycoproteins are not comparable to the proteins used in the ladder and are therefore effecting the size determination. To determine the effect of glycosylation, several proteins were analyzed on the Agilent 2100 bioanalyzer before and after deglycosylation. Glycans can either be removed by hydrazinolysis or enzymatic methods. However, hydrazinolysis destroys the protein chain, so to analyze the intact protein, enzymatic procedures must be used. A number of endoglycosidases have been characterized that can remove various oligosaccharide chains, but many of them are fairly specific and thus of limited utility. One class of enzymes reacts with asparagine-linked glycan chains. N-glycosidase F is able to release all common classes of N-glycans from the protein backbone. A glycoprotein test mixture was provided with the N-Glycosidase F Deglycosylation kit. The test mixture, containing a1-acid glycoprotein, transferrin and ribonuclease B, was used as a positive control. After treatment with the glycosidase, all three proteins migrated faster at a smaller molecular weight (figure 1 and

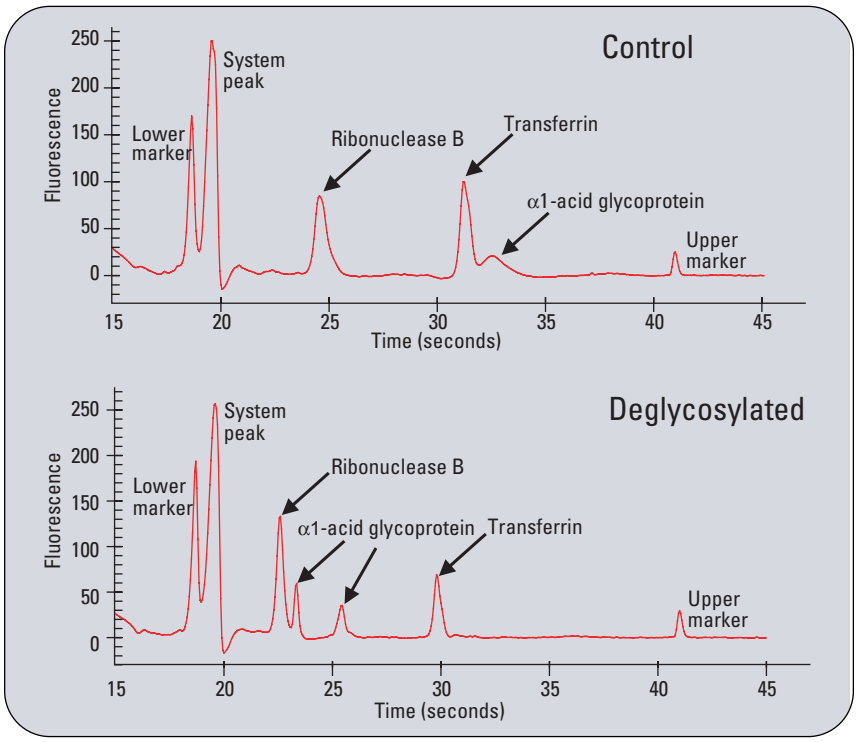

Figure 1
The glycoprotein test mixture provided with the N-Glycosidase F Deglycosylation kit showing a1-acid glycoprotein, transferrin and ribonuclease B was run as a positive control on a Protein 200 Plus kit. Also visible are the lower marker, system peak, and upper marker that are characteristic of Agilent 2100 bioanalyzer runs. The analysis of the proteins before and after reaction with N-glycosidase F is shown.

	Protein 200 Plus		SDS-PAGE		
Glycoprotein	**Mw (+)**	**Mw (-)**	**Mw (+)**	**Mw (-)**	**Theoretical Mw**
Ovalbumin	43,3	37.2 & 39.6	45,0	44,0	41,9
Ovomucoid	86,0	28,4	26,0	18,0	20,5
IgG Heavy Chain	57,9	51,3	50,0	49,0	50,0
IgG Light Chain	27,9	27,9	22,0	22,0	27,0
Transferrin	92,1	72,0	70,0	69,0	60,0
α 1-acid glycoprotein	110,0	22.7 & 36.5	40,0	26,0	22,0
Ribonuclease B	30,4	18,4	17,0	15,0	14,7

Table 1
Sizing comparison between the Agilent 2100 bioanalyzer and SDS-PAGE, before (+) and after deglycosylation (-), all sizes are shown in kDa.

table 1). Initially a1 -acid glycoprotein was a very broad peak (2 seconds wide). After treatment with N-glycosidase F, 2 peaks separated, the larger peak at 36.5 kDa probably represents incomplete reacted isoform(s). Unreacted ribonuclease showed 2 peaks at 18.8 and 30.4 kDa. After deglycosylation the larger peak moved to 18.8 kDa. Two glycoslyated forms of ribonuclease exist in addition to ribonuclease A, the nonglycoslyated form which was observed at 18.8 in both runs. Transferrin initially ran at 92.0 kDa and shifted to 72.0 kDa after treatment.

The proteins selected for analysis were observed to run at a larger molecular weight relative to the size anticipated from sequence information. The protein set contained several kinds of oligosaccharides. Before glycosidase treatment the ovalbumin peak ran at 43.3 kDa on the bioanalyzer. After treatment ovalbumin (figure 2a) exhibited a main peak with a leading shoulder. Both peaks (37.2 kDa and 39.6 kDa) migrated faster than the untreated protein. Ovalbumin from hen egg whites contains a mono-N-glycosylated form with a glycan chain on Asn-292. It is recognized to be microheterogeneous with respect to the glycan. This heterogeneity manifested as an asymmetrical peak with a leading edge in the starting material. The ovalbumin species did not react at the same rate with the enzyme during the 1-hour incubation, resulting in a large peak with a definitive shoulder.

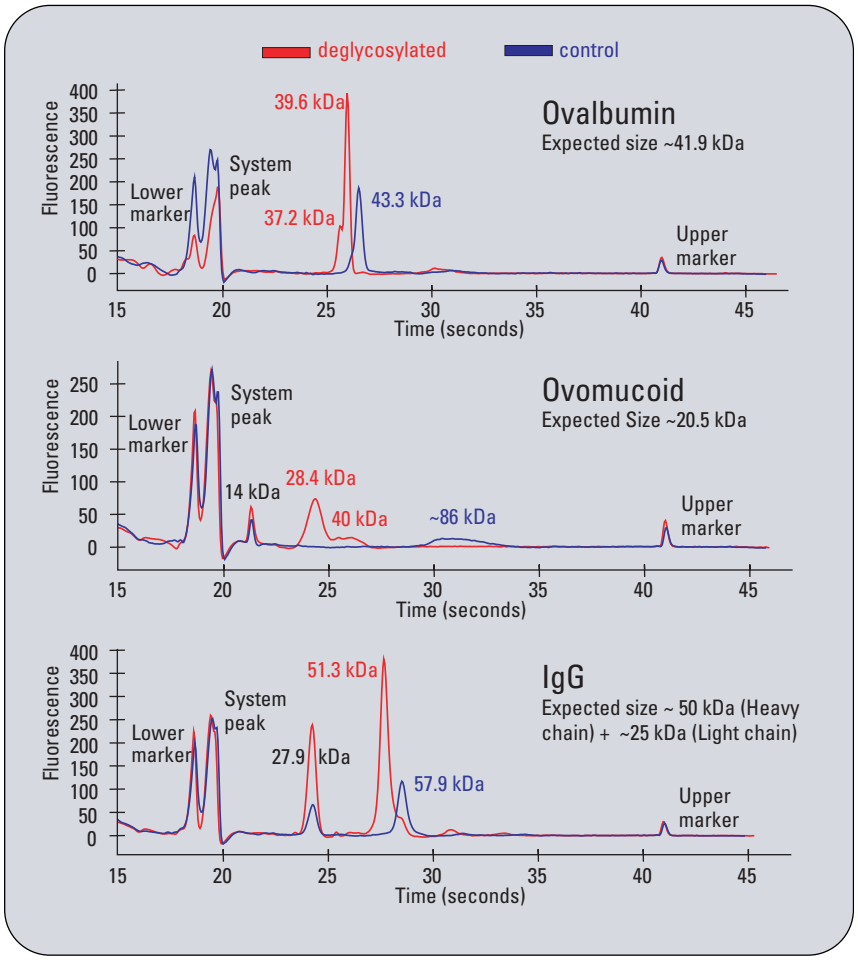

Figure 2
Ovalbumin, Ovomucoid and IgG were analyzed with the 200 Plus LabChip assay before (blue) and after (red) reaction with N-glycosidase F. After removal of the glycan attachments, proteins clearly shift to smaller sizes.

Ovomucoid was also a very broad peak at approximately 86 kDA before exposure to N-glycosidase F. After deglycosylation the peak migrated twice as fast at 28.4 kDa, with a trailing shoulder of incompletely deglycoslyated material at approximately 40 kDa. Ovomucoid contains both sulfated oligosaccharides and sialyloligo-saccharides and is frequently problematic on SDS-PAGE gels and the Agilent 2100 bioanalyzer. An impurity of approximately 14 kDa was also found to be present in the sample. This impurity, however, did not change size after treatment indicating it had no glycan attachments. The immuno γ-globulin is separated into heavy and light chains after

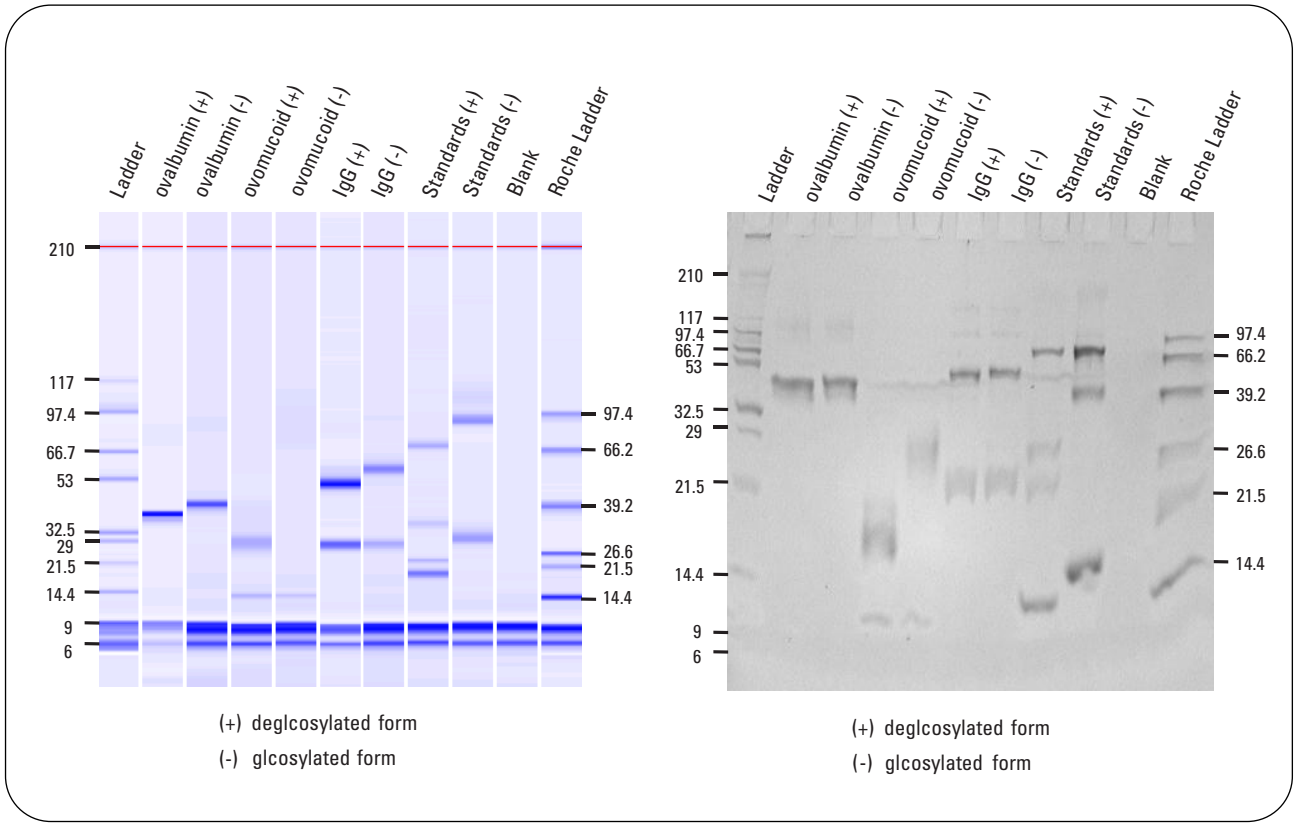

Figure 3
Comparison of the gel-like image generated by the Agilent 2100 bioanalyzer software and a SDS-PAGE slab gel. Both techniques show similar banding patterns. For some proteins, such as ovalbumin and IgG heavy chain, the shift in size after deglycosylation is more evident on the Agilent 2100 bioanalyzer.

reduction. When deglycosylated, the light chain migrated in the same position as before, whereas the heavy chain now moved faster. A small shoulder of heavy chain migrated at the original time, indicating the incomplete removal of the carbohydrate, similar to the observations of ovalbumin and ovomucoid. Even monoclonal antibodies are recognized to have isoforms that can be separated by capillary isoelectric focusing.

All proteins were also run on an SDS-PAGE slab gel. Comparison between the slab gel and the Agilent 2100 bioanalyzer is shown in figure 3. Clear and definitive sizing was determined by the Agilent 2100 bioanalyzer, where results are displayed in a tabular format, an electropherogram and a gel-like image. All samples contain an upper and lower marker. The upper and lower markers are used to align the samples with the ladder. This helps to reduce the "smiling" effect that is commonly seen on slab gels.

Conclusion

The Agilent 2100 bioanalyzer is designed to size and quantitate a wide range of proteins. Occasionally, as with SDS-PAGE, sizing anomalies will occur. Proteins that are heavily glycosylated can be

problematic because of the large carbohydrate attachments. Removal of the glycan components of these glycoproteins allow for the resulting protein to migrate more rapidly on the protein LabChips. Because most glycoproteins have heterogeneous carbohydrate attachments, it is reasonable that the reaction rates with the N-glycosidase F may vary. One major peak for each deglycosylated protein was present. Other peaks probably representing unreacted glycoprotein isoforms were also found. All of the different species, glycosylated, deglycosylated and various isoforms were clearly visualized using the Agilent 2100 bioanalyzer.

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