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Rapid quantitative capillary zone electrophoresis method for monitoring the micro-heterogeneity of an intact recombinant glycoprotein

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Abstract

A simple high-resolution capillary zone electrophoresis (CZE) method capable of rapidly assessing the micro-heterogeneity of a 24 kDa molecular weight glycoprotein, has been developed. Separation is carried out using a bare silica capillary at a pH of 2.5 in a commercially available electrophoresis buffer system composed of triethanolamine and phosphoric acid. Over 30 peaks were detected within a run time of 15 min using a 27 cm capillary and approximately 60 peaks were detected using a 77 cm capillary. Although most of the peaks arise from differences in the oligosaccharide structures present on the one glycosylation site on this molecule, other forms of micro-heterogeneity due to the presence of the nonglycosylated form of this glycoprotein and various types of chemical degradation, e.g., deamidation, are also responsible for the multitude of peaks observed. Although the exact chemical identity of each peak in the resulting electropherogram of this glycoprotein is not known, useful information can be obtained for assessing comparability, stability, and batch consistency. Factors impacting the resolution, precision, accuracy, and robustness of the assay are also discussed along with inherent advantages and limitations associated with measuring the micro-heterogeneity of intact glycoproteins.

Keywords: Capillary electrophoresis; Glycoproteins; Protein micro-heterogeneity

1. Introduction

A majority of the therapeutic recombinant proteins developed in biopharmaceutical companies are glycoproteins. This class of proteins contains complex carbohydrate structures, referred to as oligosaccharides, which are chemically linked to specific amino acid residues along the polypeptide chain. Since the carbohydrate portions of glycoproteins play an important role in their therapeutic activity [1–9], extensive characterization of the carbohydrates attached to these proteins is often performed during the development of a biopharmaceutical [10]. Although significant strides have been made in developing methods for analyzing carbohydrates [11–23], these methodologies are complex and time-consuming. The key steps used in these methods include the following:

(1) Enzymatic cleavage of the oligosaccharides from the intact glycoprotein.
(2) Purification of the released oligosaccharides.
(3) Chemical labeling of the oligosaccharides.[1]
(4) Additional purification of the oligosaccharides to remove labeling reagents.[1]
(5) Electrophoretic or HPLC separation.

An alternative approach that would simplify and speed-up the characterization of glycoprotein micro-heterogeneity involves direct analysis on the intact molecule. Mass spectrometry (MS) offers some capability in this area [24], however, the resolution of complex high molecular mass species with different ionization efficiency present limitations to this

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[1] Step is not required for oligosaccharide analysis when using high pH ion exchange chromatography with pulsed amperometric detection (PAD) or mass spectrometry.

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approach. In the past, several CE methods for detecting glycoprotein micro-heterogeneity, using the intact glycoprotein, have been reported [25–44]. However, all these methods are applicable to certain types of proteins and are often not quantitative. In this work, we describe a new CZE method to detect and quantify differences in the various glycoforms and charge heterogeneity of a glycoprotein.

2. Materials and methods

2.1. Chemicals and reagents

The CZE buffer used throughout this work was 100 mM triethanolamine–phosphoric acid, pH 2.5 (catalog no. 05100-SB) (MicroSolv, Long Branch, NJ, USA), Neuraminidase (from *Arthrobacter ureafaciens*) and PNGase F (from *Flavobaceterium meningosepticum*) were obtained from Boehringer Mannheim (Indianapolis, IN, USA), Leucine was obtained from Aldrich (Milwaukee, WI, USA), sucrose and dynorphin A (fragments 1–13) were obtained from Sigma (St. Louis, MO, USA). The uncoated bare silica CElect-FS or TSP050375 capillaries, 50 μm I.D. and 375 μm O.D., were obtained from Supelco (Supelco Park, Bellefonte, PA, USA) and Polymicro Technologies (Bloomfield, NJ, USA), respectively. The 0.5 mL, 10 000 molecular weight (MW) cutoff membrane Ultrafree centrifugation filter units (catalog no. UFV5BGC00) were obtained from Millipore (Milford, MA, USA).

2.2. Capillary electrophoresis

All studies were conducted on a P/ACE Model 5500, automated HPCE instruments equipped with a variable-wavelength UV–vis detector (Beckman-Coulter Instruments, Fullerton, CA, USA). The Beckman-Coulter HPCE Windows version software was utilized for all data acquisition, manipulation, calculations, and display.

2.2.1. Experimental conditions for conducting CZE

All CZE experiments were performed using the triethanolamine–phosphoric acid buffer mentioned in Section 2.1. Unless stated otherwise, the CE running conditions for a 27 cm capillary were as follows: separation voltage 10 kV, temperature of 25 °C, and UV detection at 214 nm with a 5 nm bandwidth. Sample loading was performed hydrodynamically with a typical time duration of 10 s. The CE running conditions for a 77 cm capillary were the same as for a 27 cm with the exception of an increase in running voltage to 30 kV and an increase in injection time to 30 s. For all CE runs, samples were held in the autosampler at ambient temperature. Typical glycoprotein sample concentration was between 1.0 and 1.5 mg/mL. The buffer matrix of the samples contained 50 mM leucine, at a pH of 4.5 (pH was achieved by titrating the buffer solution with HCl). In some cases, the samples contained sucrose at a concentration of 3.0 mg/mL, however, the presence or absence of sucrose had no impact in the resulting electropherograms. All samples were stored at −70 °C and were run with an internal standard, dynorphin A (fragments 1–13) peptide, which was dissolved in water at a concentration of 1 mg/mL and frozen in 30 μL aliquots at −70 °C. In certain occasions, sample pre-concentration and/or buffer exchange were required. To achieve this, 0.5 mL, 10 000 MW cutoff ultrafree centrifugation spin units were used at 2–8 °C in an Eppendorf model 5417R centrifuge (Hamburg, Germany).

2.2.2. Conditioning of capillary

Capillary conditioning involved the following washing sequence: deionized water, 0.1 M NaOH, deionized water, and running buffer (100 mM triethanolamine–phosphoric acid, pH 2.5). Each wash was run for 5 min. This same sequence of washing steps was also used between injections. For long-term storage, the capillary was flushed with deionized water and stored at room temperature with the capillary inlet and outlet immersed in vials containing deionized water.

3. Results and discussion

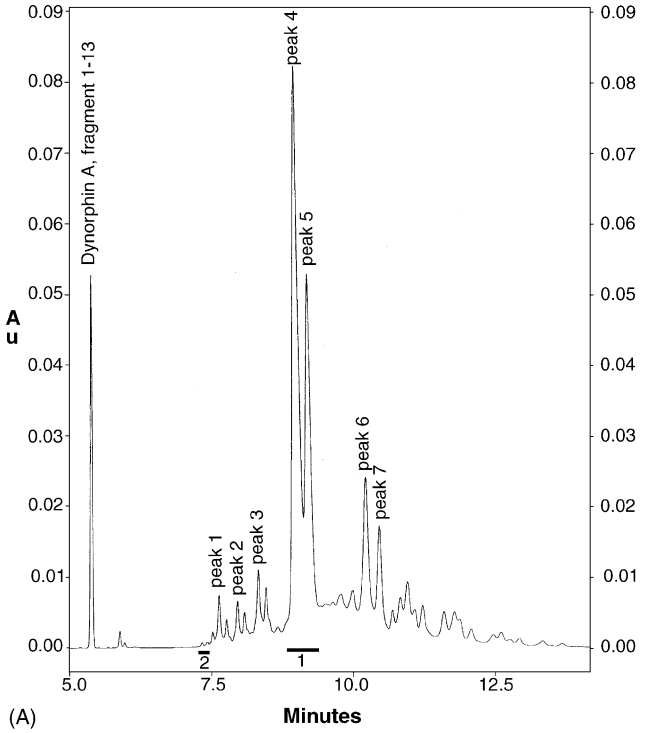
3.1. CZE of glycoprotein in 100 mM triethanolamine/phosphoric acid pH 2.5 buffer

An electropherogram obtained for the glycoprotein used throughout this study on a 27 cm capillary in a 100 mM triethanolamine/phosphate pH 2.5 electropherosis buffer, is shown in Fig. 1A. Results shown in Fig. 1B demonstrate the reproducibility of this method. Further improvement in resolution can be achieved using a 77 cm capillary (see Fig. 2A). When neuramindase (an enzyme that removes the terminal sialic acids residues from oligosaccarides) was added to the glycoprotein, a much simpler electrophoregram was obtained, see Fig. 2. Upon removal of the N-linked oligosaccharide, with PNGase-F, an even simpler CZE pattern was observed (Fig. 3). Hence, the complex electropherogram obtained for this glycoprotein, results from the glycosylation and the variation in sialylation of the different oligosaccharide structures attached to the single glycosylation site on this protein. Additional evidence presented in Section 3.7 indicates that some of the micro-heterogeneity of this glycoprotein, detected by this CZE method, is also due to factors other than differences in carbohydrate structures.

3.2. Day-to-day reproducibility (intermediate precision) of peak areas and migration times

The day-to-day reproducibility of the migration times and peak areas of the seven major peaks indicated in Fig. 1A using Dynorphin A fragments 1–13 as an internal standard[2]

[2] Even with appropriate capillary conditioning the capillary-to-capillary and buffer-to-buffer variations caused variations in peak migration time and peak area to be detected. Application of an internal standard, however, significantly helps to reduce these variations.



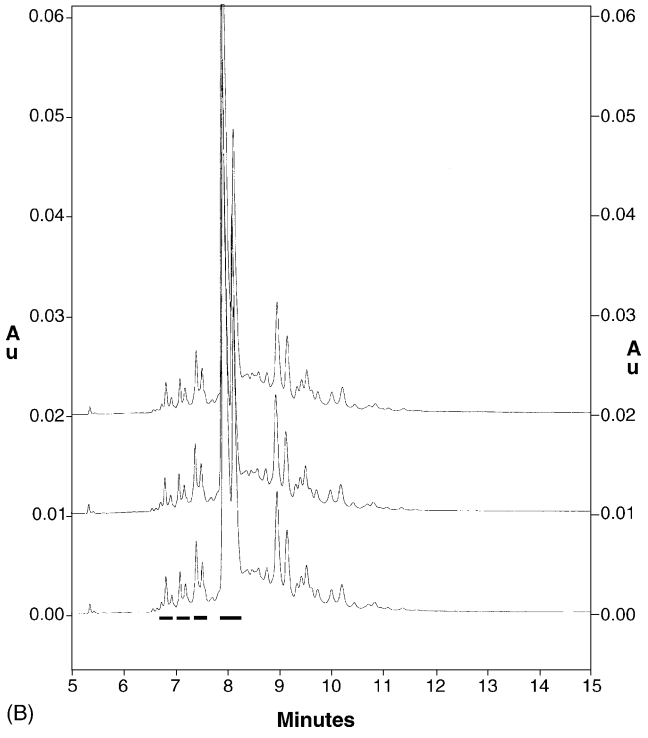


Fig. 1. (A) CZE electropherogram of the glycoprotein used in this study on a 27 cm bare silica capillary. (B) The reproducibility of the CZE method is demonstrated by the qualitatively reproducibility of the electropherograms from three repetitive injections of the same glycoprotein sample. Electrophoretic conditions used to conduct these runs are indicated in Section 2.2.1. Although different glycoprotein samples were used in (A) and (B), both glycoprotein samples were in a 50 mM leucine, pH 4.5 buffer at a concentration of 1.5 mg/mL. Regions in the electropherogram labeled by bar 1 and 2, at the bottom of (A), and as bars at the bottom of (B) are discussed in Sections 3.5 and 3.7.

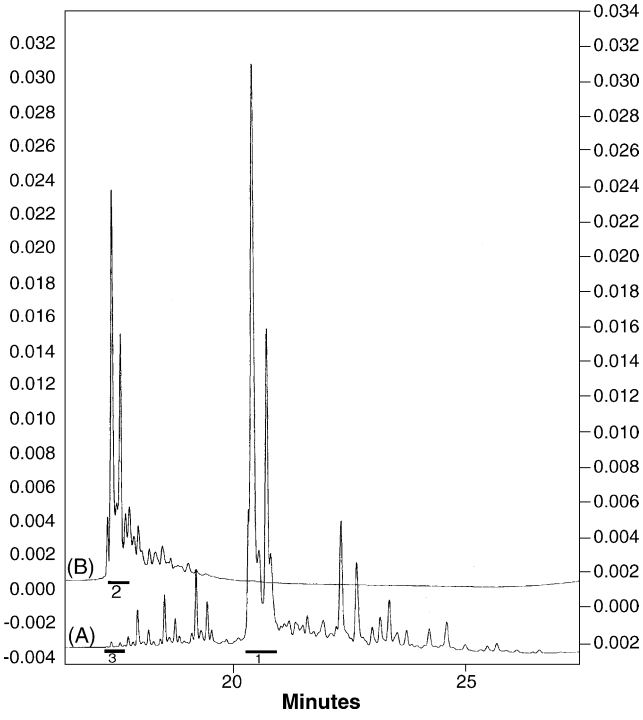


Fig. 2. CZE electropherogram of the glycoprotein used in this study on a bare silica capillary 77 cm long (A) without neuraminidase and (B) with neuraminidase. CZE electropherogram of the glycoprotein treated with neuromindase was conducted for 18 h at 25 °C. Electrophoresis conditions used to conduct this run are indicated in Section 2.2.1. Regions in the electropherogram labeled with bars at the bottom of each figure are discussed in Section 3.5.

was less than 0.7% and 7% (expressed as RSD), respectively. Additional reproducibility data are also shown in Section 3.8.

3.3. *Effect of sample matrix*

Results shown in Fig. 4A–C, for samples of the glycoprotein in three different buffers systems, indicates that the resolution between various peaks was dependent on the nature of the sample matrix [45–47]. This data indicates improved resolution obtained for samples in low ionic strength and low pH buffer. The enhanced resolution achievable by injecting a sample at a much lower ionic strength relative to the electrophoretic buffer is well known and is due to stacking effects [48–50]. The effect of sample pH may be related to the isoelectric point (p*I*) of this glycoprotein, which spans a range from 6.5 to 8.0. Hence the initial movement of the glycoprotein is actually in the direction of the anode instead of cathode, reducing the resolution enhancing effect of electrophoretic stacking. From this data, we have concluded that in order to achieve high electrophoretic resolution, the samples matrix should low conductivity and pH. A buffer system composed of leucine or leucine/sucrose (see Section 2.2.1) was found to satisfy the above mentioned buffer matrix requirements. Since most of our work was conducted in this buffer, the glycoprotein samples could be run as is, without any sample pretreatment. However, when needed, glycoprotein samples

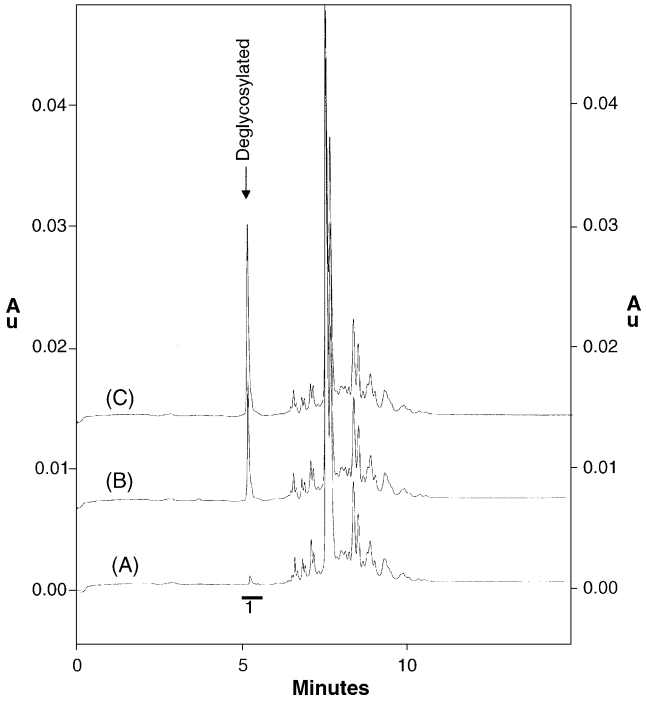


Fig. 3. CZE electropherograms obtained on a sample of the glycoprotein used in this study after treating it with PNGase F at 37 °C (which removes the N-linked oligosaccharide from this molecule) for different lengths of time. (A) Control, untreated sample; (B) after 3 h; and (C) after 8 h. Bar 1 indicated at the bottom of (A) corresponds to where the nonglycosylated form of this protein migrates (see Section 3.5.2).

were buffer exchanged into the leucine or leucine/sucrose buffer by either overnight dialysis, at 2–8 °C, or in 1 h using centrifugation filter units (see Section 2.2.1).

3.4. Stability of glycoprotein to the acidic pH of the electrophoresis buffer

Since sialic acid residues are known to be labile under acidic conditions [51], the impact of the low pH used during electrophoresis was explored. To achieve this, the glycoprotein was exposed to pH 2.5 for increasing amounts of time by decreasing the running voltage potential. Results obtained from this study are shown in Fig. 5. The data indicates no significant change in the electrophoresis pattern other than some peak broadening, which is a characteristic phenomenon of running samples under reduced voltage [52].

3.5. Identification of the protein glycoforms

Prior characterization work on the glycoprotein used this study, by a number of analytical techniques, showed that about 60% of the glycoforms on this molecule have a disialylated biantennary structure, BiNA2. Correlating this information with the electropherogram obtained for this glycoprotein has led us to speculate that the peak region indicated by bar 1 in Fig. 1A, which includes the two largest peaks in the electropherogram, peaks 4 and 5, represents

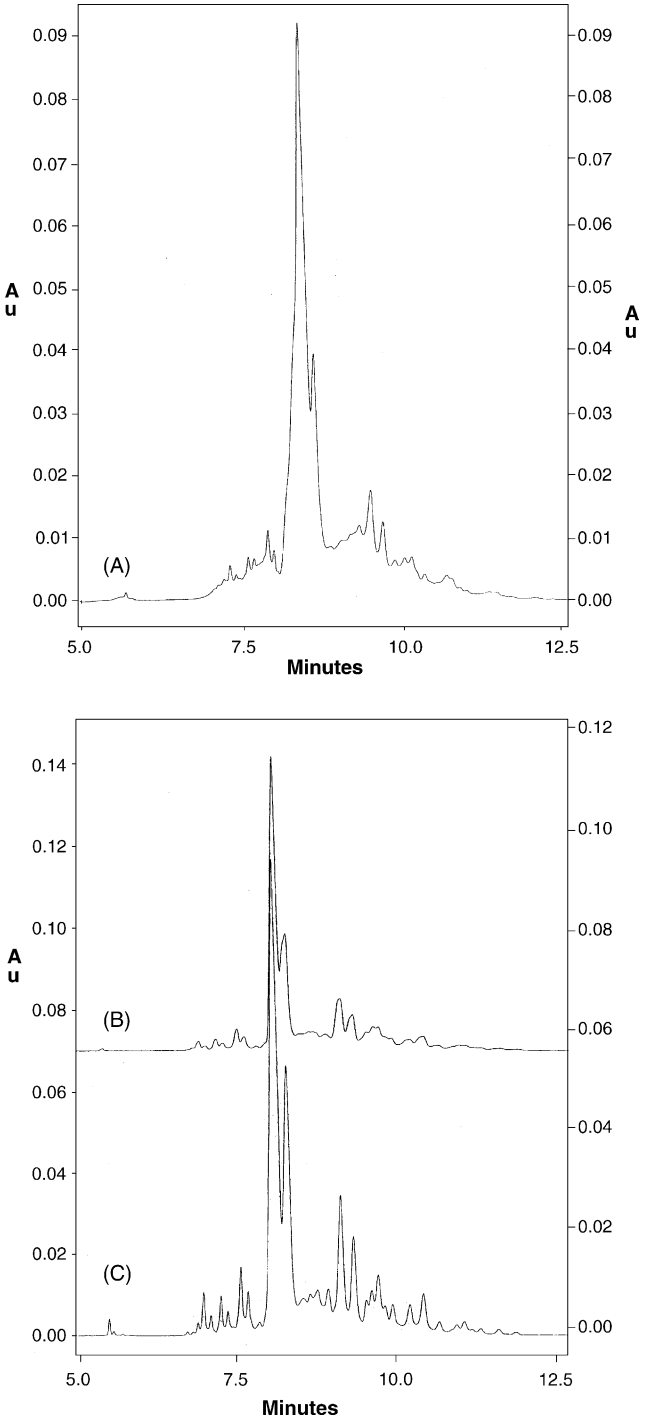


Fig. 4. The effect of the sample matrix buffer on the CZE electropherogram of the glycoprotein used in this study: (A) sample buffer, 200 mM NaCl, 100 mM sodium phosphate, pH 7.2, (B) sample buffer, 50 mM NaCl, 25 mM sodium phosphate, pH 7.2, and (C) sample buffer, 50 mM leucine pH 4.5.

the disialylated biantennary glycoforms. In addition, data shown in Fig. 2 reveals that digesting the glycoprotein with neuramindase results in the loss of peaks 4 and 5 and the appearance of two new major peaks in the region indicated by bar 2 in Fig. 2B. These two new major peaks are believed to correspond to the nonsialylated biantennary glycoforms.

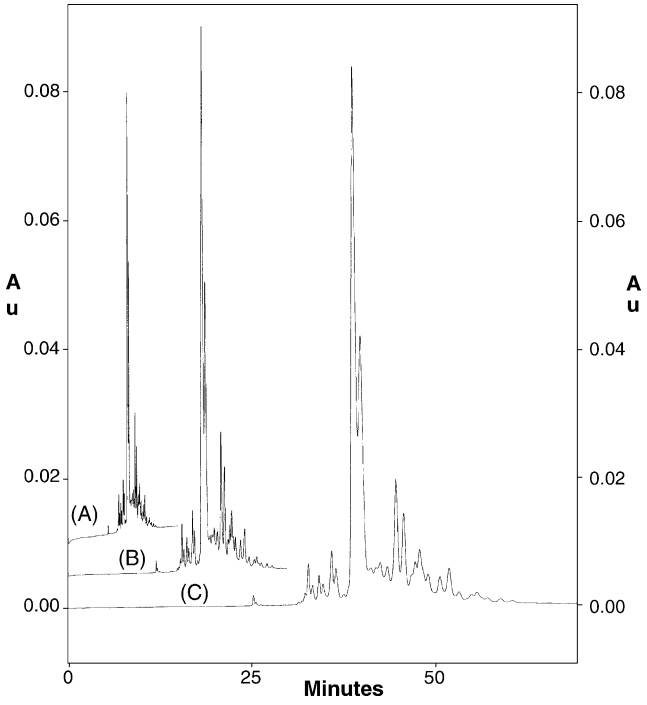


Fig. 5. Stability of the glycoprotein at pH 2.5 in CZE electrophoresis buffer assessed by increasing the exposure time of the glycoprotein to pH 2.5 by reducing the running voltage: (A) 10 kV, (B) 5 kV, and (C) 2.5 kV.

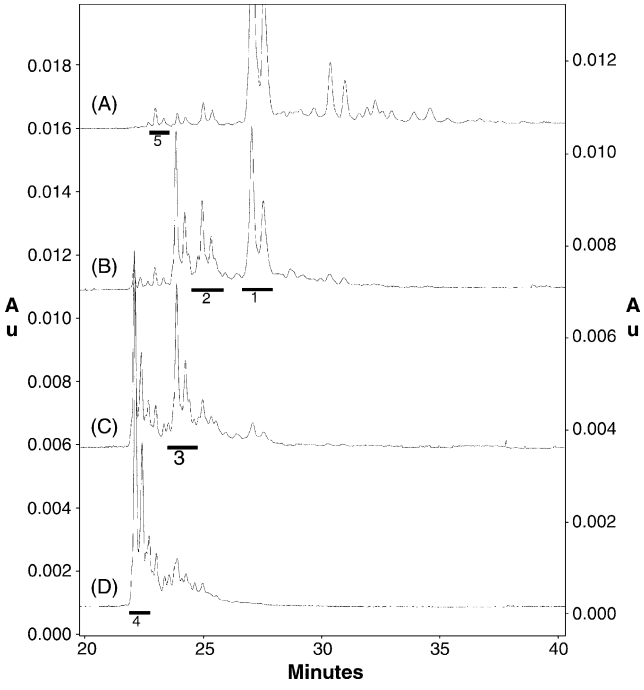


Fig. 6. A kinetic study on the enzymatic removal of the terminal sialic acid residues on the glycoprotein studied in this work by spiking a sample of this glycoprotein with neuramindase and holding it at 25 °C in the auto-injector. Aliquots from this sample were then taken at different times to conduct CZE: (A) control, untreated sample; (B) after 1 h; (C) after 2 h; and (D) after 7 h. Regions in the electropherograms labeled by bars 1–5, at the bottom of each electropherogram, are discussed in Section 3.5.1.

To further identify other peaks in the electropherogram, enzymatic hydrolysis studies as a function of time were conducted using neuramindase and PNGase F. Results from these studies are discussed in Sections 3.5.1 and 3.5.2.

3.5.1. Neuramindase treatment of the glycoprotein as a function of time

A series of electropherograms obtained for a glycoprotein sample treated with neuraminidase as a function of time is shown in Fig. 6. The two predominant peaks initially present in the electropherogram, see bar 1 in Fig. 6B, rapidly decreases with time. As time progresses, three sets of peaks, labeled bar 2 in Fig. 6B, bar 3 in Fig. 6C, and bar 4 in Fig. 6D, emerge. With increasing time the size of the peaks in the two areas, labeled as bar 2 and bar 3, decrease. The peaks indicated by bar 2, however, decrease faster than those indicated by bar 3. At the end of the experiment, only the peak set indicated by bar 4, which increased with time, remained.

These results indicate a stepwise cleavage reaction of sialic acid residues from the intact glycoprotein, which for each class of antennary glycoforms should proceed as indicated below:

Biantennary (2)

Disialylated (2) ⇒ monosialylated (2) ⇒ nonsialylated (2)

Triantennary (3)

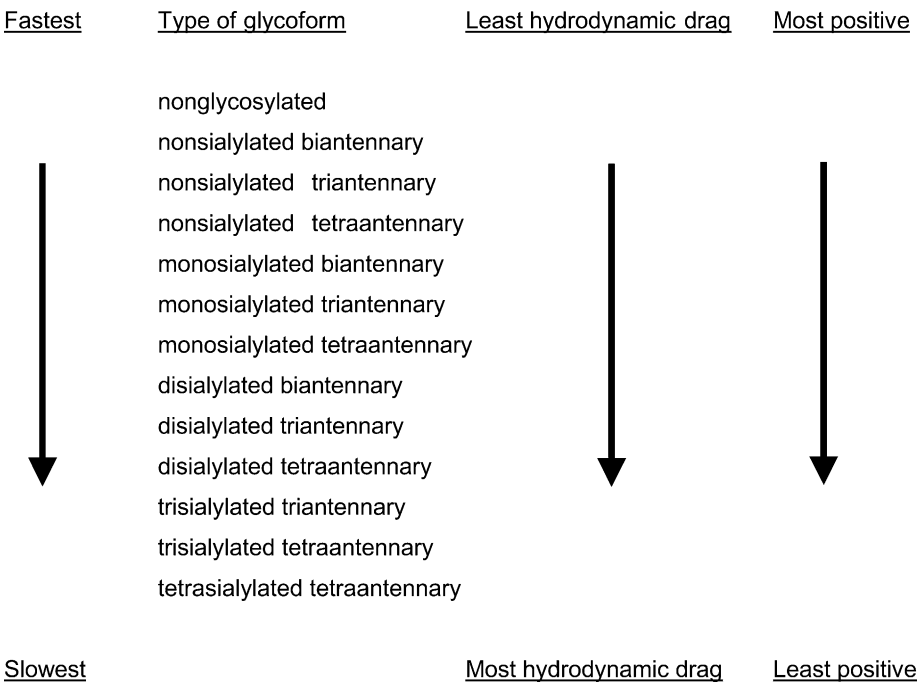
Trisialylated (3) ⇒ disialylated (3) ⇒ monosialylated (3) ⇒ nonsialylated (3)

Tetraantennary (4)

Tetrasialylated (4) ⇒ trisialylated (4) ⇒ disialylated (4) ⇒ monosialylated (4) ⇒ nonsialylated (4)

Note: ( ) indicates the glycoform's antennary structure.

Although all of the above glycoforms are positively charged at pH 2.5, the strength of the net positive charge decreases as the antennary structure of the oligosaccharide on the glycoform increases. This decrease in positive charge is due to the associated increasing level of sialylation, and therefore, negative charge (the p*K* values for most carboxyl groups on sialic acids are in the pH 2.5 range [53]) with higher antennary structures. Hence glycoforms having higher levels of sialylation have a lower electrophoretic mobility towards the cathode. In addition, the increasing hydrodynamic volume (and, therefore, frictional drag) of glycoforms containing higher antennary structures leads to a further reduction to electrophoretic mobility. Since the electroosmotic flow at pH 2.5 is effectively zero, due the reduced ionization of the silanol groups on the silica capillary wall at this pH [54], movement of all glycoforms past the UV detector is totally dependent on their inherent electrophoretic mobility. As a result, the combined effect of net charge and hydrodynamic drag yields the following predicted order of elution for the major glycoforms:



As a result of the above information the peaks labeled by bar 3 in Fig. 6 most likely to correspond to the monosialylated biantennary glycoforms. This is based on the dominance of these peaks during the early phase of the reaction and the subsequent loss of these peaks due to the eventual cleavage of the one remaining sialic acid. Hence, at the end of the hydrolysis reaction, the two major peaks labeled by bar 4 in Fig. 6D should correspond to the nonsialylated biantennary glycoforms. The progressive increasing level of these peaks during the course of the reaction further supports this assignment. Peaks migrating in the area labeled by bar 2 in Fig. 6B most likely correspond to monosialylated oligosaccharide with higher antennary structure. This is based in the initial increase level of these peaks and their eventual disappearance, much like that observed for the monosialylated biantennary glycoforms, during the reaction. However, the level that these peaks reach is significantly reduced in comparison to those peaks assigned as monosialylated biantennary glycoforms. This reduced transient level of the monosialylated higher antennary glycoforms is consistent with the overall lower amounts of tri and tetrasialylated glycoforms initially present in the glycoprotein preparation. Finally, the peaks in the region between the non and monosialylated biantennary glycoforms, labeled by bar 5 in Fig. 6A, did not appear to change significantly during the course of the experiment. Hence its identity is unknown.

3.5.2. PNGase F treatment of the glycoprotein as a function of time

A series of electropherograms obtained for a glycoprotein sample treated with PNGase F as a function of time is shown in Fig. 3. With increasing time the size of the initial minor peaks, indicated by bar 1 in Fig. 3A, progressively increase and most likely correspondsto the nonglycosylated form of the glycoprotein. The initial amount of these peaks observed in the electropherograms (Fig. 3A) most likely represents the amount of nonglycosylated form of the glycoprotein original present.[3] As the size of this small cluster of peaks increases with time, the relative ratios of the later eluting peaks appear to remain constant. This observation supports the conclusion that the rate of the cleavage of the different types of oligosaccharides on the protein backbone is not dependent on the chemical structure of the oligosaccharides present.

3.6. Anion-exchange fractionation of the protein

Additional evidence supporting the assignments of the various CZE peak regions made in Section 3.5 was obtained by fractionating the glycoprotein via preparative anion-exchange chromatography (AEC) (data not shown). The fractions collected across the AEC chromatogram were analyzed by both CZE and HPLC [15]. In the latter case, fractions were deglycosylated and the released carbohydrates were analyzed. HPLC chromatograms of the oligosaccharide profile for the AEC fractions (P1, P3, and P4) and the non-fractionated glycoprotein (control) sample are shown in Fig. 7A. CZE electropherograms of the glycoform profile (intact glycoprotein) for the corresponding AEC fractions and non-fractionated glycoprotein (control) sample are shown in Fig. 7B. Prior detail characterization work has shown that

[3] In conducting this experiment, the enzymatic removal of the oligosaccharide, from the asparagine residue, with PNGase F results in the conversion of this asparagine to an aspartic acid. Such a modification increases the negative charge on the modified glycoprotein. However, at the running pH of 2.5, the free carboxyl group on the aspartic acid is assumed to be fully protonated. Hence, the migration of the PNGase F treated glycoprotein should compare to the true nonglycosylated protein.

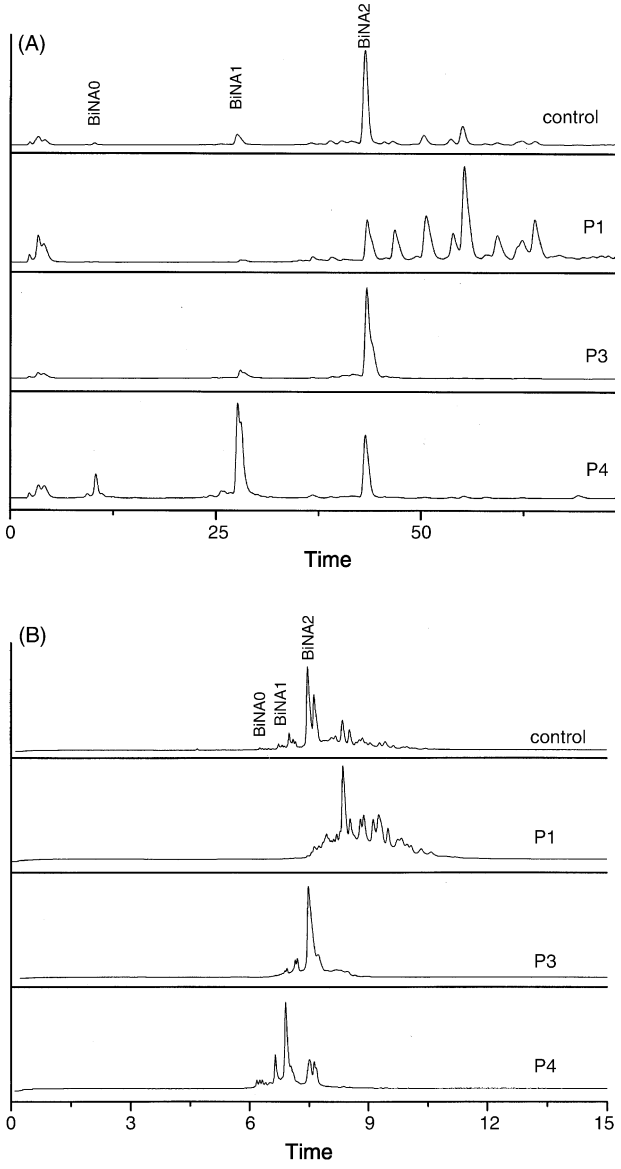


Fig. 7. Characterization of the same AEC fractions (labeled P4, P2, and P1 that correspond to fractions obtained at increasing elution times, respectively) obtained across the glycoprotein AEC preparative elution profile by (A) HPLC analysis [15], which looks at the released oligosaccharides, and by (B) CZE analysis, which looks at the intact glycoforms. In each case, a control sample is shown at the top of each figure that corresponds to the oligosaccharide, in the case of HPLC, and glycoform profiles, in the case of CZE, for the unfractionated glycoprotein sample.

the region to the right of the peak area labeled BiNA2 in the control HPLC chromatogram (see Fig. 7A) contains tri and tetrantennary oligosaccharides that were sialylated with three or more sialic acid residues. Similarly, the region in the chromatogram labeled BiNA2 corresponds to the area where the diasialylated biantennary oligosaccharides migrate. Finally, the regions labeled BiNA1 and BiNA0 correspond to where mono-sialylated biantennary oligosaccharides and nonsialylated biantennary oligosaccharides migrated, respectively. In the case of CZE, information generated in the present work (see Section 3.5) has lead us to identify the region in the CZE electropherogram to the right of the peak region labeled BiNA2 in the control CZE electropherogram (see Fig. 7B) as the region where glycoforms containing tri and tetrantennary oligosaccharides sialylated with three or more sialic acid residues migrate. Similarly, the region in the electropherogram labeled BiNA2 has been assigned to the area where glycoforms containing disialylated biantennary oligosaccharides could be found. Finally, the regions labeled BiNA1 and BiNA0 correspond to where glycoforms containing mono-sialylated biantennary oligosaccharides and nonsialylated biantennary oligosaccharides migrate, respectively. A comparison of the known key peak assignments in the various HPLC profiles with the CZE results for the same sample clearly provide additional experimental support to the tentative CZE peak region assignments made in Section 3.5.

3.7. Detection of other forms of micro-heterogeneity (deamidation)

An interesting pattern in the electropherogram (see highlighted areas indicated by a series of small bars at the bottom of Fig. 1B) is the appearance of multiple doublet peaks. A possible explanation for this pattern is the occurrence of an additional modification in the protein that impacts its electropheoretic mobility. Other measurements have shown

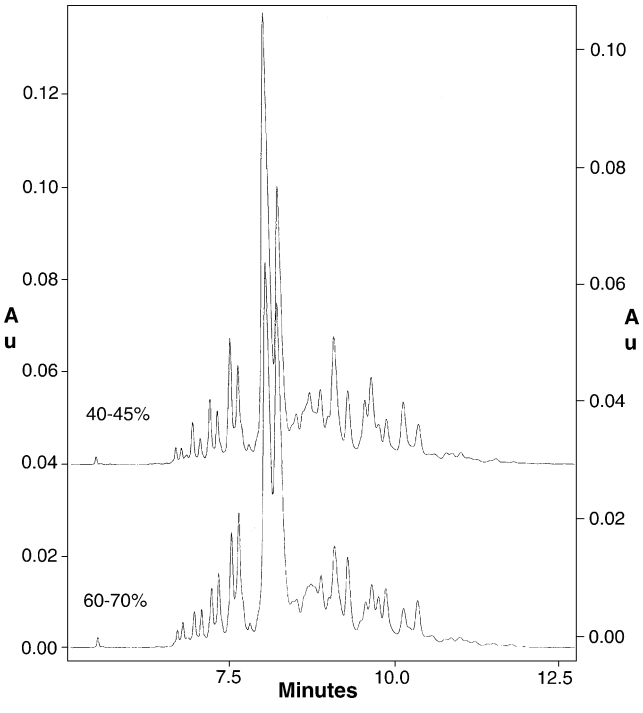


Fig. 8. The effect of deamidation on the resulting CZE electropherogram of the glycoprotein studied in this work is shown by running two samples of this glycoprotein having known different amounts of deamidation (levels of deamidation are indicated in the figure). Deamidation levels were determined by a separate HPLC peptide map assay specifically developed to monitor deamidation on this glycoprotein.

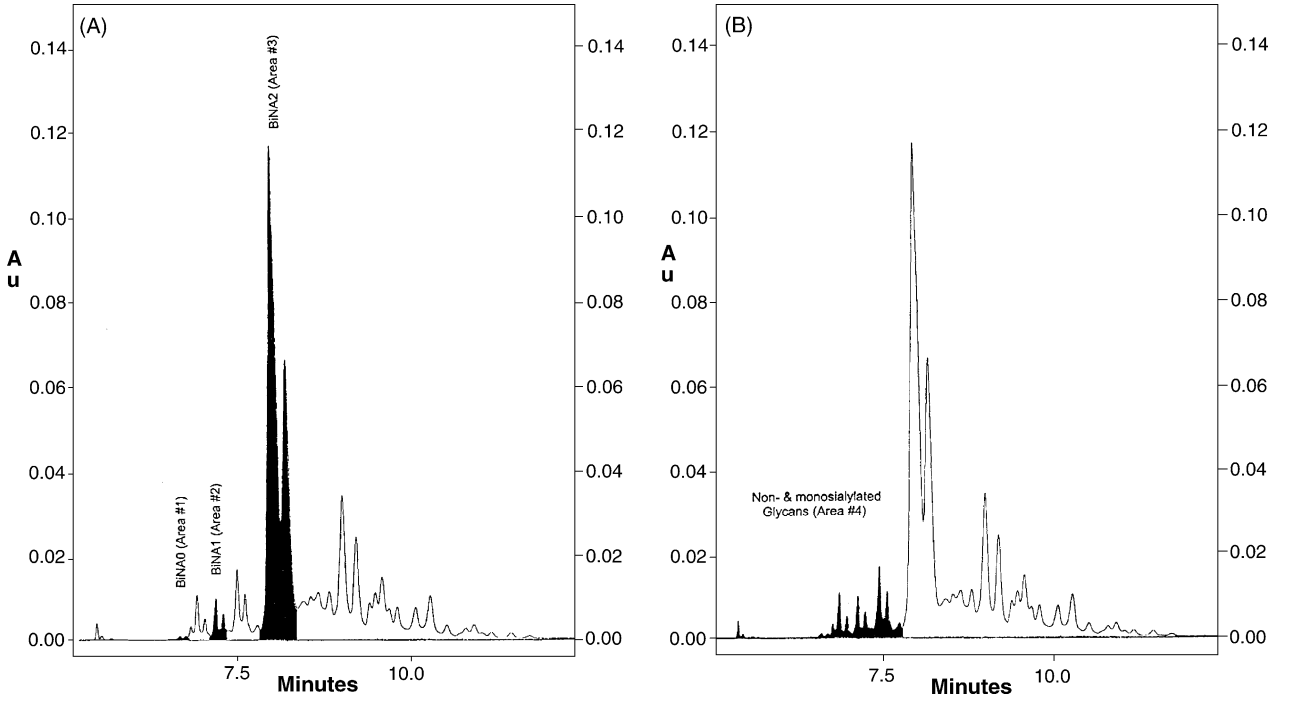


Fig. 9. Peak region assignments in the glycoprotein electropherogram used to provide quantitative information via Eqs. (1)–(3): (A) peaks assigned in this figure correspond to the following: BiNA0 (area #1), biantennary oligosaccharides with no terminal sialic acid residues; BiNA1 (area #2), biantennary oligosaccharides with one sialic acid terminal residue; and BiNA2 (area #3), biantennary oligosaccharides with two terminal sialic acid residues and (B) nonglycosylated protein and monosialylated glycoforms (area #4).

that the glycoprotein is deamidated[4] to a significant level, typically 40–50% at a single asgaragine site. Two samples of this glycoprotein with significantly different known levels of deamidation are shown in Fig. 8. In the sample with a higher level of deamidation, the height of the slower migrating peak in each doublet is typically higher in comparison to the corresponding peak in each doublet for the sample with a lower level of deamidation. Hence, the peak region containing the largest doublet (see bar 1 in Fig. 1A), that we believe corresponds to the major disialylated biantennary glycoforms (see Section 3.5), has been used as a relative indicator of the amount of deamidation present in this glycoprotein, see Eq. (4) in Section 3.8. Additional data supporting the use of these peaks to assess deamidation in this glycoprotein is provided in Section 3.9.2.

3.8. Quantitation of sialylation, glycosylation, and deamidation

Based on peak assignments and peak information obtained in Sections 3.5–3.7, Eqs. (1)–(4) were used to calculate the following parameters:

$$\text{Percent non and monosialylated glycoforms} = \frac{100\,(\text{area } 4)}{\text{total area}} \quad (1)$$

$$\text{Percent biantennary glycoforms} = \frac{100\,(\text{area } 1 + \text{area } 2 + \text{area } 3)}{\text{total area}} \quad (2)$$

$$\text{Percent sialylation of the biantennary glycoforms} = \frac{100\,(\text{area } 1 + 0.5\,[\text{area } 2])}{\text{area } 1 + \text{area } 2 + \text{area } 3} \quad (3)$$

$$\text{Percent deamidation} = \frac{100\,(\text{area } 6)}{\text{area } 5 + \text{area } 6} \quad (4)$$

Areas 1–4 used in Eqs. (1)–(3) correspond to peak areas shown in Fig. 9, while areas 5 and 6 in Eq. (4) correspond to peak areas obtained from peaks 4 and 5, respectively, in Fig. 1A.

These parameters provide quantitative information on the level of sialylation [55,56], glycosylation and deamidation of the glycoprotein. Since all calculations were based on the ratio of peak areas obtained within the same electropherogram, the use of an internal standard to improve reproducibility was not necessary.[5]

[4] Although the additional negative charge resulting from deamidation should lead to a reduction in this peaks migration time, the low pH of the electrophoresis buffer should neutralize it (as discussed in footnote 3). Hence, it is not clear how deamidation causes the reduction in peak migration time. Possible interaction of the deamidation residue with other appropriate groups on the glycoprotein could create a microenvironment allowing the negative charge to persist.

[5] The linear behavior of these peak areas as a function of sample load was assessed by varying the time of injection by a factor of 4. Resulting plots of peak area (nomalized with internal standard) versus injection time gave linear plots (data not shown), which when analysed by linear regression analysis gave correlation coefficients greater than 0.997.

Table 1
Summary of quantitative data collected on the reference standard of the glycoprotein studied in this work over a period of 10 months

Data set	%Mono and nonsialylated	%Biantennary	%Sialylation of biantennary	%Deamidation
1	12.6	53.6	96.6	35.5
2	12.3	54.3	96.8	35.2
3	12.2	54.5	96.9	36.5
4	13.1	53.4	96.6	35.4
5	12.9	53.2	96.3	35.7
6	12.3	54.2	97.0	35.0
7	13.6	52.5	96.4	36.7
8	13.5	53.5	96.2	36.1
9	13.6	53.1	96.5	37.7
10	13.0	53.3	96.4	36.2
11	12.2	54.5	97.0	35.6
12	13.0	53.4	96.4	35.7
13	13.2	53.7	96.7	37.2
14	14.3	51.5	96.2	37.4
15	12.5	53.1	96.8	35.8
16	13.0	53.2	96.6	36.0
17	13.8	51.9	96.6	35.6
18	13.5	51.5	96.2	36.4
19	14.3	51.4	96.4	38.8
20	14.3	51.8	96.5	38.7
21	14.4	52.3	96.4	37.6
22	13.3	53.3	96.6	37.1
Average	13.2	53.1	96.6	36.4
SD	0.7	1.0	0.2	1.1
%RSD	5.4	1.8	0.3	3.0

In calculating the specific parameters, using Eqs. (1)–(4), it must be emphasized that the numerical values generated are not absolute. This is due to the tentative peak identification. Nevertheless, the ability to measure these parameters reproducibly was assessed by using the same control sample in all CZE runs. Results obtained for this control sample, over time, are shown in Table 1. The data indicates good reproducibility of the method over a period of 10 months.

3.9. Applications of the CZE method

3.9.1. Changes in protein micro-heterogeneity due changes in host cells and growth media conditions

The impact of using a different strain of CHO cells and different growth media condition on the micro-heterogeneity of the glycoprotein was assessed by CZE. Results from this method are shown qualitatively in Fig. 10A and B and quantitatively in Tables 2 and 3. The data show unique qualitative and quantitative differences.

3.9.2. Assessment of protein stability

The ability of CZE to detect and quantify changes in the amounts of deamidation and desialylation in the glycoprotein used in this study as a function temperature and pH over time is shown in Fig. 11. In this figure, CZE data is compared to data obtained via two separate HPLC methods capable of monitoring deamidation and desialylation on the glycoprotein. In the case of deamidation, a reversed-phase HPLC peptide map was used that could separate the intact and deamidated peptide allowing the %deamindation to be calculated via UV detection and appropriate peak integration. In the case of desialylation, a separate HPLC method was used

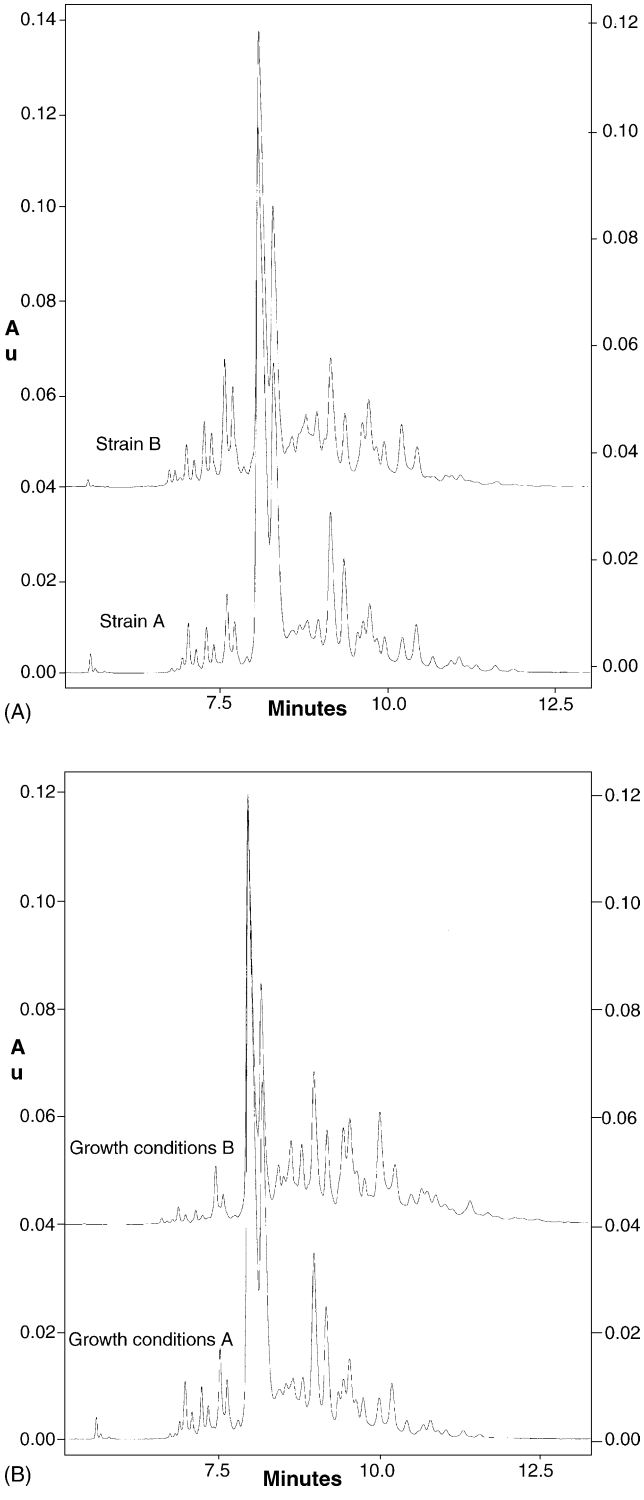


Fig. 10. (A) CZE electropherograms showing the effect of growing the same glycoprotein in two different CHO cell strains. (B) The resulting CZE electropherograms showing the effect of growing the same glycoprotein in same CHO cell strain, but in two different growth media.

Table 2
Comparison of quantitative CZE data (obtained from Fig. 10A) for the glycoprotein made using two different CHO strains (established cell lines)

Parameter	%Mono and nonsialylated		%Biantennary		%Sialylation of biantennary		%Deamidation	
Strain ID	A	B	A	B	A	B	A	B
Sample 1	11.7	17.4	55.1	47.8	96.5	93.7	33.6	34.8
Sample 2	11.5	17.5	55.6	47.9	97.0	93.6	33.6	35.0
Sample 3	11.6	17.7	55.7	47.9	96.9	93.5	33.5	34.8
Average	11.6	17.5	55.5	47.9	96.8	93.6	33.6	34.9
SD	0.1	0.2	0.3	0.1	0.3	0.1	0.1	0.1
%RSD	0.9	0.9	0.6	0.1	0.3	0.1	0.2	0.3

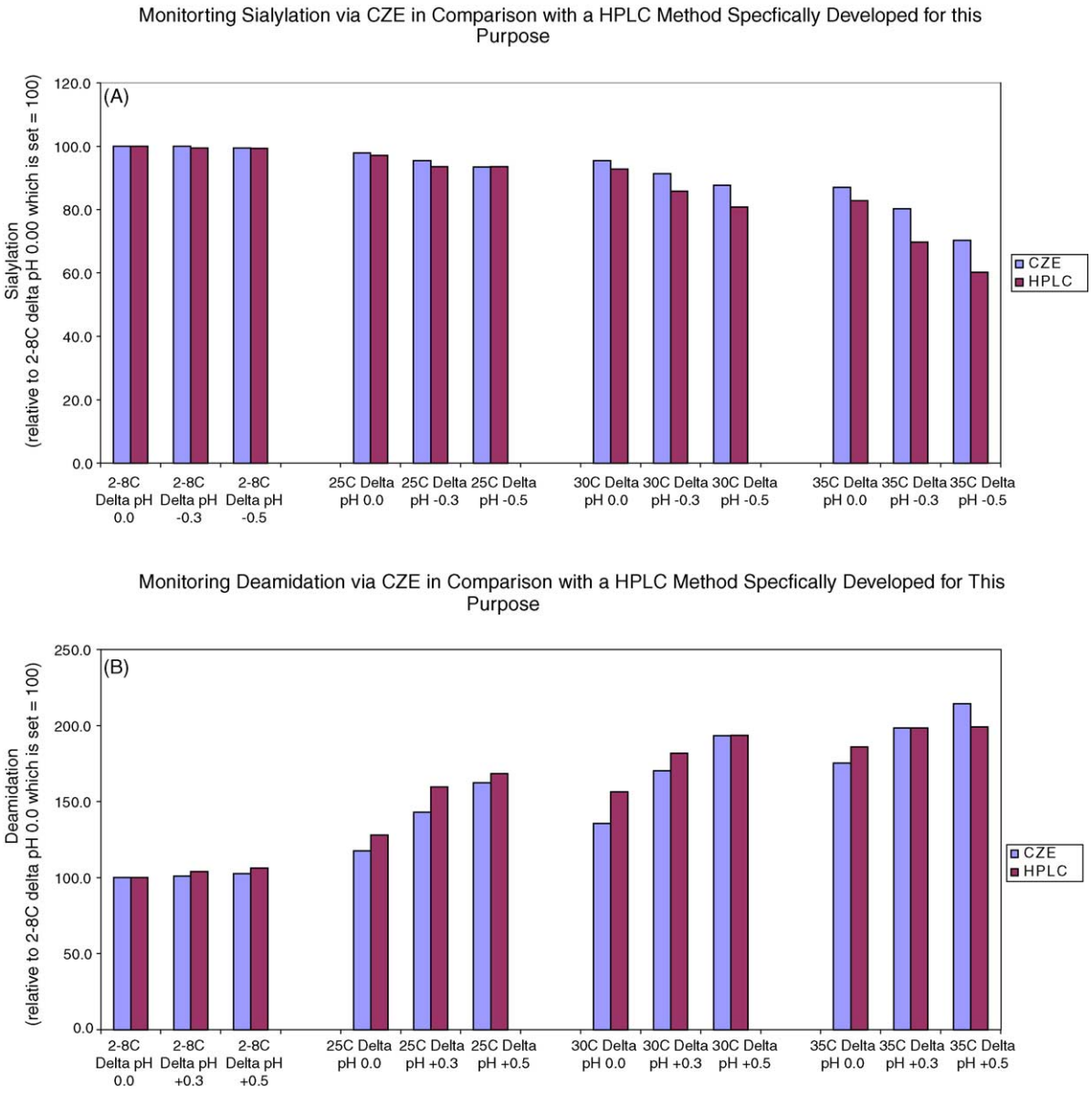


Fig. 11. Results showing the stability indicating capability of the CZE method developed for the glycoprotein used in this study is demonstrated by its ability to monitor changes in the amount of desialylation (using data obtained via Eq. (3)) and deamidation (using data obtained via Eq. (4)) when this molecule is stored for 3 months at 2–8, 25, 30, 35 °C at target pH value and at pH values ± 0.3 and ±0.5 from target pH. In each case, data obtained by the CZE method (indicated by the blue color bars) is compared with data obtained using a separate, but specific HPLC method (indicated by the red colored bars) for measuring (A) deamidation and (B) desialylation (see discussion in Section 3.9.2 for information about both HPLC methods). Values reported in both plots are percent values relative to their initial value measured at 2–8 °C at target pH (which was assigned an initial value of 100%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

Table 3
Comparison of quantitative CZE data (obtained from Fig. 10B) for the glycoprotein made using two different growth conditions

Parameter	%Mono and nonsialylated		%Biantennary		%Sialylation of biantennary		%Deamidation	
Growth condition	A	B	A	B	A	B	A	B
Sample 1	11.7	6.5	55.1	38.2	96.5	98.0	33.6	31.5
Sample 2	11.5	6.4	55.6	37.8	97.0	98.0	33.6	31.2
Sample 3	11.6	6.5	55.7	37.8	96.9	97.9	33.5	31.3
Average	11.6	6.5	55.5	37.9	96.8	98.0	33.6	31.3
SD	0.1	0.1	0.3	0.2	0.3	0.1	0.1	0.2
%RSD	0.9	0.9	0.6	0.6	0.3	0.1	0.2	0.5

based on the procedures outlined in [55,56], which involves the separation and quantification of the enzymatic released and fluorescently tagged oligosaccharides [15]. Although there are some differences in the actual levels of deamidation and desialylation determined by CZE and HPLC, the trends in the level of deamidation and desialylation are consistent.

4. Conclusions

The micro-heterogeneity of the glycoprotein detected by the CZE method developed in this paper can be attributed to absence or presence of various forms of the oligosaccharides and to the amount of deamidation on the glycoprotein. Although the glycoprotein studied in this paper has only one site for carbohydrate attachment, the resulting CZE method has shown the level of micro-heterogeneity to be extensive (similar level of complexity have been reported for other glycoproteins [57,58]). Using a long capillary, we have been able to detect over 60 peaks. Because sialic acid residues contain carboxyl groups that have pK_a values in the pH range of the electrophoresis buffer used for the CZE separation [53], an increase in the number of sialic residues per glycoprotein molecule increases the net negative charge on the glycoprotein. Variations in the amount of net negative charge along with differences hydrodynamic frictional drag between various glycoforms allow for their electrophoretic separation.

The CZE method developed in this paper provides a high-resolution fingerprint of the micro-heterogeneity of the glycoprotein studied in this work. In most cases, samples could be injected without any sample preparation. In other cases, only simple buffer exchange and concentrating of the sample was required. The end result is a very rapid and robust assay that could be used for characterization and stability and studies.

Although a tentative identification of some peaks or peak regions observed in the electrophoregram has been made, more definitive identification is required to confirm these assignments. Furthermore, identification of the many other unknown peaks is also desirable. Although peak identification by CE-MS work would be attractive, the incompatibility of the CZE buffer with MS prohibits the use of this powerful hyphenated technique.

Results obtained in using this CZE method on other glycoproteins, at present, have been variable. In particular, for glycoproteins having a much higher molecular weight, such as antibodies, results have been disappointing. Opportunities for achieving similar successful separations, as demonstrated in this paper, with other glycoproteins appear to be limited to glycoproteins of similar or lower molecular weight. These observations point out the general difficulty in applying CZE methods developed in the literature from one protein to another. This difficulty is most likely due to the wide variability in physico-chemical properties of proteins. However, when suitable methods are found, the high efficiency and resolution of CZE can be realized. Such situations are illustrated by results reported in this paper and the use of CZE for the analysis of recombinant human erythropoietin [59] and carbohydrate-deficient transferrin [60].

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References

[1] D.A. Cumming, Glycobiology 1 (1991) 115.
[2] D.T.-Y. Lui, Trends Biotechnol. 10 (1992) 114.
[3] A. Varki, Glycobiology 3 (1993) 97.
[4] N. Jenkins, M.A. Curling, Enzyme Microb. Technol. 16 (1994) 354.
[5] R.A. Dwek, in: F. Brown, A. Lubiniecki, G. Murano (Eds.), Characterization of Biotechnology Pharmaceutical Products, vol. 96, Dev. Biol. Stand., Karger, Basel, 1988, p. 43.
[6] K.J. Morrow Jr., Genet. Eng. News 22 (2002) 54, 59.
[7] J. Alper, Science 301 (2003) 159.
[8] A. Wright, S.L. Morrison, J. Immunol. 160 (1998) 3393.
[9] P.M. Rudd, T. Elliot, P. Cresswell, I.A. Wilson, R.A. Dwek, Science 291 (2001) 2370.
[10] A. Gervais, Y.-A. Hammel, S. Pelloux, P. Lepage, G. Baer, N. Carte, O. Sorokine, J.-M. Strub, R. Koerner, E. Leize, A.V. Dorsselaer, Glycobiology 13 (2003) 179.
[11] S. Honda, A. Makino, S. Suzuki, K. Kahehi, Anal. Biochem. 191 (1990) 228.
[12] P. Jackson, Anal. Biochem. 196 (1991) 238.

[13] R.R. Townsend, in: Z. El Rassi (Ed.), Carbohydrate Analysis: High-Performance Liquid Chromatography and Capillary Electrophoresis, Elsevier, Amsterdam, New York, 1995, pp. 181–209.
[14] Y. Zhao, S.B.H. Kent, B.T. Chait, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1629.
[15] K.R. Anunmula, S.T. Dhume, Glycobiology 8 (1998) 685.
[16] J. Charlwood, H. Birrell, P. Camilleri, J. Chromatogr. B 734 (1999) 169.
[17] K.R. Anumula, Anal. Biochem. 283 (2000) 17.
[18] A.D. Hooker, D.C. James, Mol. Biotechnol. 14 (2000) 241.
[19] N. Viseux, X. Hronowski, J. Delaney, B. Domon, Anal. Chem. 73 (2001) 4755.
[20] G.-H. Zhou, G.-A. Luo, Y. Zhou, K.-Y. Zhou, X.-D. Zhang, L.-Q. Huang, Electrophoresis 19 (1998) 2348.
[21] N. Callewaert, S. Geysens, F. Molemans, R. Contreras, Glycobiology 11 (2001) 275.
[22] J.A. Saba, J.P. Kunkel, D.C.H. Jan, W.E. Ens, K.G. Standing, M. Bulter, J.C. Jamieson, H. Perreault, Anal. Biochem. 305 (2002) 16.
[23] H.J. An, T.R. Peavy, J.L. Hedrick, C.B. Lebrilla, Anal. Chem. 75 (2003) 5628.
[24] J. Siemiatkoski, Y. Lyubarskaya, D. Houde, S. Tep, R. Mhatre, Carbohydr. Res. (submitted for publication).
[25] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelsberg, Anal. Biochem. 205 (1992) 115.
[26] P.M. Rudd, I.G. Scragg, E. Coghill, R.A. Dwek, Glycoconj. J. 9 (1992) 86.
[27] E. Watson, F. Yao, J. Chromatogr. 630 (1993) 442.
[28] E. Watson, F. Yao, Anal. Biochem. 210 (1993) 389.
[29] D.C. James, R.B. Freedman, M. Hoare, N. Jenkins, Anal. Biochem. 222 (1994) 315.
[30] N.K. Klausen, T. Kornfelt, J. Chromatogr. A 718 (1995) 195.
[31] K. Yim, J. Abrams, A. Hsu, J. Chromatogr. A 716 (1995) 401.
[32] S. Hoffstetter-Kuhn, G. Alt, R. Kuhn, Electrophoresis 17 (1996) 418.
[33] J.F. Kelly, S.J. Locke, L. Ramaley, P. Thibault, J. Chromatogr. A 720 (1996) 409.
[34] K. Kopp, M. Schluter, R.G. Werner, Arzneim. -Forsch./Drug Res. 46 (1996) 1191.
[35] M.A. Strege, A.L. Lagu, J. Chromatogr. A 780 (1997) 285.
[36] M. Taverna, N.T. Tran, T. Merry, E. Horvath, D. Ferrier, Electrophoresis 19 (1998) 2572.
[37] A. Pantazaki, M. Taverna, C. Vidal-Madjar, Anal. Chim. Acta 383 (1999) 137.
[38] F.-Y. Che, J.-F. Song, X.-X. Shao, K.-Y. Wang, Q.-C. Xia, J. Chromatogr. A 846 (1999) 599.
[39] L.E. Somerville, A.J. Douglas, A.E. Irvine, J. Chromatogr. B 732 (1999) 81.
[40] M. Kinoshita, E. Murakami, Y. Oda, T. Funakubo, D. Kawakami, K. Kakehi, N. Kawasaki, K. Morimoto, T. Hayakawa, J. Chromatogr. A 866 (2000) 261.
[41] B.C. Giordano, M. Muza, A. Trout, J.P. Landers, J. Chromatogr. B 742 (2000) 79.
[42] J.S. Patrick, A.L. Lagu, Electrophoresis 22 (2001) 4179.
[43] V. Sanz-Nebot, P. Gonzalez, I. Toro, A. Ribes, J. Barbosa, J. Chromatogr. B 798 (2003) 1.
[44] V. Sanz-Nebot, F. Benavente, A. Vallverdu, N.A. Guzman, J. Barbosa, Anal. Chem. 75 (2003) 5220.
[45] F. Foret, K. Kleparnik, P. Gebauer, P. Bocek, J. Chromatogr. A 1053 (2004) 43.
[46] S. Hjerten, S. Mohabbati, D. Westerlund, J. Chromatogr. A 1053 (2004) 181.
[47] S. Mohabbati, S. Hjerten, D. Westerlund, J. Chromatogr. A 1053 (2004) 201.
[48] F.E.P. Mikkers, F.M. Everaerts, Th.P.E.M. Verheggen, J. Chromatogr. 169 (1979) 1.
[49] R.-L. Chien, D.S. Burgi, J. Chromatogr. 559 (1991) 141.
[50] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, New York, 1993, p. 205.
[51] R. Schauer, Adv. Carbohydr. Chem. Biochem. 40 (1982) 131.
[52] R.P. Oda, J.P. Landers, in: J.P. Landers (Ed.), Handbook of Capillary Electorphoresis, second ed., CRC Press, Boca Raton, FL, 1996, p. 24.
[53] R. Aubeck, C. Eppelsheim, C. Brauchle, N. Hammpp, Analyst 118 (1993) 1389.
[54] F.E. Regnier, D. Wu, in: N.A. Guzman (Ed.), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, p. 287.
[55] P. Hermentin, R. Witzel, E.-J. Kanzy, G. Diderrich, D. Hoffman, H. Metzner, J. Vorlop, H. Haupt, Glycobiology 6 (1996) 217.
[56] P. Hermentin, R. Witzel, Pharm. Pharmacol. Commun. 5 (1998) 33.
[57] P.M. Rudd, C. Colominas, L. Royle, N. Murphy, E. Hart, A.H. Merry, H.F. Hebestreit, R.A. Dwek, Proteomics 1 (2001) 285.
[58] P.M. Rudd, B.P. Morgan, M.R. Wormald, D.J. Harvey, C.W. van den Berg, S.J. Davis, M.S.J. Ferguson, R.A. Dwek, J. Biol. Chem. 272 (1997) 7229.
[59] European Pharmacopoeia, fourth ed., 1316, 2002, p. 1123.
[60] C. Lanz, U. Marti, W. Thormann, J. Chromatogr. A 1013 (2003) 131.