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Short Communication

Capillary electrophoretic separation of recombinant granulocyte-colony-stimulating factor glycoforms

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ABSTRACT

Free zone capillary electrophoresis separated recombinant human granulocyte-colony-stimulating factor, expressed in Chinese hamster ovary cells, into two well-resolved species. Following incubation with neuraminidase, these species comigrated, eluting earlier than either of the original two species. This indicated that the observed heterogeneity was caused by different amounts of sialic acid present on the carbohydrate portion of the protein. It was determined that optimum separation occurred in the buffer pH range 7-9. Evidence is also presented to show that these glycoforms migrate in order of increasing numbers of siahc acids present.

INTRODUCTION

Determination of glycoprotein heterogeneity has become increasingly important in analytical biotechnology since many of the recombinant proteins with commercial value are highly glycostylated. Traditionally, the separation has been performed using gel isoelectric focusing (IEF). Glycoforms can contain different numbers of sialic acid residues and as a results show differences in their isoelectric points. Separation by IEF is based on the establishment of a stable pH gradient. Under the influence of an electric field the differently charged glycoforms migrate to the point where their p*I* values are equivalent to the pH and, hence, no further migration takes place. While the separations obtained with this technique are excellent, it is slow, labor intensive and the results are semi-quantitative. To overcome some of these problems, Pharmacia has introduced their PhastGel system that permits automation of staining but still requires a separate step for quantitation. Recently, the separations obtained by Phast IEF Gel and IEF in coated capillary columns have been compared for monoclonal antibody Her-2 4D5. Capillary IEF showed a superior degree of species resolution [1].

High-performance capillary zone electrophoresis (CZE) has shown great success in separating peptides and proteins. While these separations can typically be carried out with no more than pH and ionic strength manipulations, the application of CZE for the separation of proteins based on their isoelectric points or p*I* values is not such a simple procedure. Several publications have described the separation of glycoforms by isoelectric focusing using capillary electrophoresis instrumentation [2–5]. In all all of these instances separations were achieved using coated capillary columns. When uncoated columns were used, electroendoosmotic flow

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was a limiting factor that precluded the possibility of attaining stable focused zones necessary for IEF to occur. Very recently, automated IEF has been reported on two commercial capillary electrophoresis systems, one using chemical mobilization [6] and the other using vacuum mobilization [7].

We have evaluated the utility of free zone electrophoresis as an alternative technique for the separation of glycoforms. Recombinant human granulocyte-colony-stimulating factor (rhGCSF) produced in CHO cells was selected because it is a well-characterized protein present in a highly purified state, containing two O-linked carbohydrate moieties that differ only in having one and two sialic acid residues. This glycoprotein represents one of the simplest, yet well defined carbohydrate structures for the evaluation of experimental conditions that can control the separations of proteins differing in sialic acid content.

METHODS

hGCSF produced in mammalian cell lines was manufactured at Amgen, Thousand Oaks, CA, USA. All capillary zone electrophoresis was performed using a Beckman PACE 2000 high-performance capillary electrophoresis system. Capillaries of 50 cm × 75 μm were supplied by Beckman, those of 100 cm × 75 μm were assembled in our laboratory and were obtained from Polymicro Technologies (Phoenix, AZ, USA).

Separations were carried out with cathode placed at the detector end of the capillary. Before analysis was carried out, capillary electrophoresis system was set to maintain a constant temperature at 25°C. For each run, the capillary was rinsed with running buffer for 3 min. Sample was then introduced by low pressure for 5 s. After sample loading, voltages of 5-20 kV were applied and maintained constant throughout the run. Separated species were then monitored with UV detection set at 214 nm and detector signal was transmitted to a Chromjet integrator (Spectra-Physics, San Jose, CA, USA). Post-separation rinses with 0.1 A4 NaOH, followed by water were carried out for 3 min each to ensure removal of residual protein from the previous run. Experimental conditions are as described in the figure captions.

Sialic acid residues were removed from rhGCSF by digestion at 37°C with neuraminidase (Vibrio Cholerae Type II) in sodium acetate, pH 5.0.

RESULTS AND DISCUSSION

There are several major advantages in using recombinant derived proteins for developing new analytical separation methods. Since these proteins are intended for human therapeutic use, they are extremely well-characterized and highly purified. Carbohydrate structures on rhGCSF have been identified as NeuAcα2→3Galβ1→3(± NeuAcα2→6)GalNAcol [NeuAc = N-acetylneuraminic

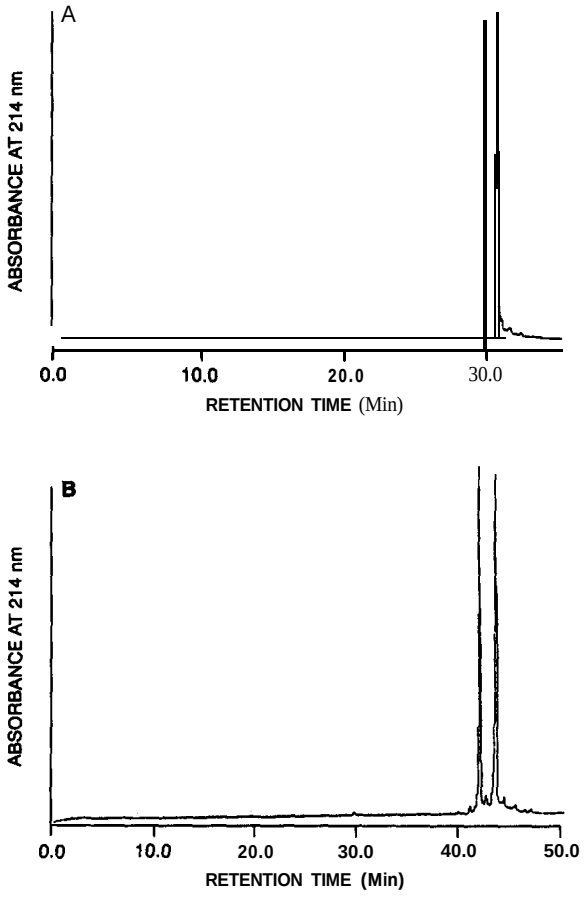

Fig. 1. Capillary electrophoretic separations of rhGCSF at 1 mg/ml in (A) pH 8.0, 50 mM phosphate-50 mM borate buffer and (B) pH 8.0, 50 mM phosphate-50 mM borate buffer with 2.5 mM diaminobutane. Separations were obtained under 30 kV using a 100 cm × 75 μm capillary.

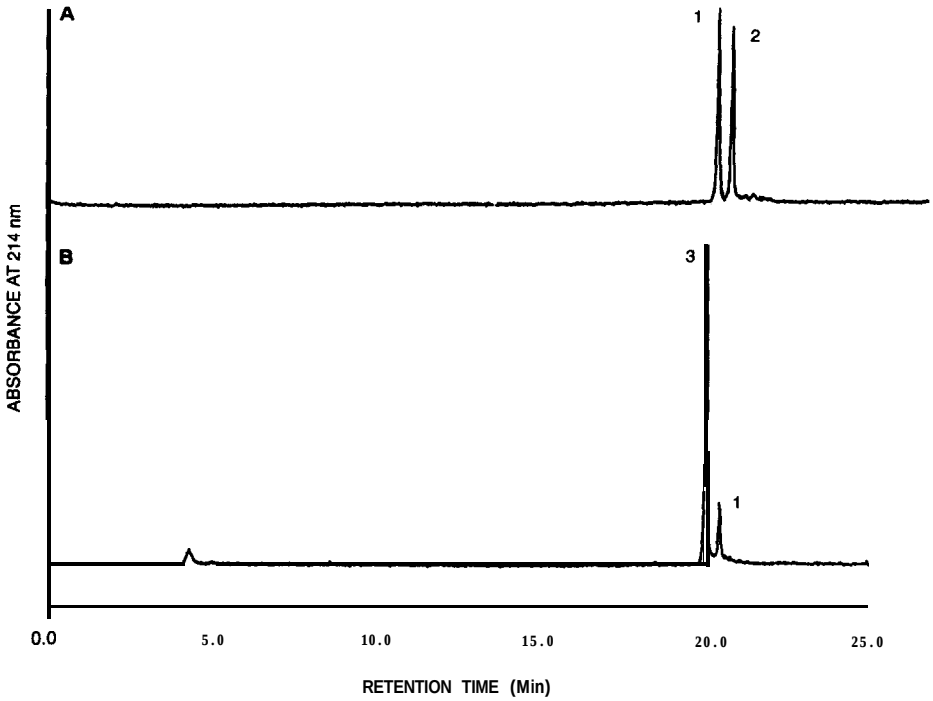

Fig. 2. Capillary electrophoretic separations in pH 9.0, 100 m*M* borate buffer. (A) rhGCSF and (B) rhGCSF after 10 h of incubation with neuraminidase. Separations were obtained under 20 kV using a 100 cm × 75 μm capillary. See text for peak identification.

acid, Gal = galactose, GalNA col = N-acetylgalactosamine (reduced)] and are present in equal amounts differing only in the number and linkage of the sialic acid residues present [8].

The separation of rhGCSF glycoforms was evaluated in buffers with pH values ranging from 2.5-9. At pH 5 or less, no rhGCSF migrated past the detector. rhGCSF may have precipitated on the column or its mobility was so slow that it did not migrate sufficiently in the time allotted for analysis (< 60 min). When pH 6 was used, rhGCSF migrated as two overlapping peaks. At pH values of 7-9, these separated into two equally sized peaks showing baseline resolution with little quantitative difference among the separations obtained at each pH.

The experimental conditions that can affect separations and resolution have been the subject of many studies [9–12]. From these reports it has been shown that column length, buffer type, ionic strength, pH, voltage and a variety of additives can all have a major impact. These were systematically evaluated on an individual basis for their effects on analysis time and peak resolution. Fig. 1A shows the optimum electrophoretic separation obtained for the two glycoforms present in rhGCSF. Enhancement of the resolution was next evaluated by incorporating organic additives such as methanol, ethanol, tetrahydrofuran, urea and 1 ,4-diaminobutane. All of these are known to reduce electroendoosmotic flow to different extents. Of these, 1,4-diaminobutane showed the most effectiveness in improving resolution at a final concentration of 2.5 m*M*. The results is shown in Fig. 1B.

At pH values of 6-9, sialic acids present on rhGCSF are negatively charged and migrate in the opposite direction from the detector. The electroendoosmotic flow dominates the overall migration carrying the negatively charged rhGCSF species toward the detector. Since the migration velocity of a charged species is the vector sum of the electroendoosmotic flow and that of the species, the presence of negatively charged sialic acid causes the glycoforms to migrate at a slower rate than the electroendoosmotic flow, with a net flow which is differ-

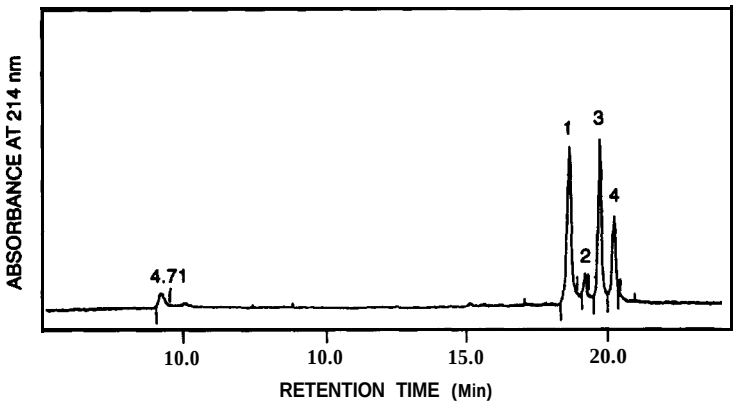

Fig. 3. Capillary elecrophoretic separations in pH 9.0, 100 m*M* borate buffer. Sample of rhGCSF was incubated with neuraminidase for 30 min and spiked with non-glycosylated rhG-CSF. Peaks: 1 = non-glycosylated rhGCSF; 2 = desialylated rhG-CSF; 3, 4 = glycosylated rhG-CSF.

ent for charged glycoforms containing one and two sialic acids. Glycoforms containing one sialic acid will migrate faster than those with two sialic acids. As a result, it can be expected that these glycoforms will migrate in order of increasing numbers of sialic acids. Peak 1 is assigned to the glycoform with one sialic acid and peak 2 to that containing two sialic acids (Fig. 2a).

To verify peak assignments, rhGCSF was incubated with neuraminidase which selectively removes sialic acid from the carbohydrate moiety. These incubation studies were conducted over 24 h with samples removed at various time points for CZE analysis. The results showed the peak 2 decreased more rapidly than peak 1. A new peak, identified as peak 3, emerged migrating faster than peak 1 and gradually increased in response as peak 1 and peak 2 decreased. Fig. 2b shows the rhGCSF separation profile after 10 h of incubation with neuraminidase.

It was of interest to compare the electrophoretic behavior of non-glycosylated rhGCSF produced in *Escherichiu coli* cells and desialylated rhGCSF. These could be baseline resolved with *E. coli* produced rhGCSF migrating faster than desialylated rhGCSF (Fig. 3). These results indicate that the carbohydrate structures remaining on rhGCSF at pH 9 affect to a significant extent the mobility of the protein under the experimental conditions used.

In addition to developing a capillary electrophoretic separation of isoforms, we investigated the feasibility of obtaining quantitative separation data. A series of concentrations from 0.10, 0.25, 0.50, 0.75 and 1.0 mg/ml were used and the areas of the separated isoforms determined. The results indicated that the amounts determined are consistent over the concentration range with a relative standard deviation of $< 2\%$. Retention time reproducibility was evaluated by injecting rhGCSF six times and determining consistency of retention times. The relative standard deviation was determined to be $\pm 0.1\%$.

CONCLUSIONS

The use of free zone capillary electrophoresis is shown to be a highly efficient and fast technique capable of separating glycoforms based on the number of sialic acids present. The glycoforms are eluted in order of increasing number of sialic acids and with this information the results can be directly compared to gel IEF. rhGCSF has a p*I* value of 4.5 and is negatively charged under the pH conditions in the range 7-9 used to separate the glycoforms. As a consequence, protein adsorption onto the capillary walls which are negatively charged at these pH values is minimized by charge repulsion. The effect of various additives was evaluated to further enhance separation but results indicated that pH control in itself was sufficient to obtain satisfactory baseline resolution. In contrast to gel IEF electro-

phoresis, the separation of the glycoforms is fast with total analysis times of < 30 min, simple to perform, provides for automation and the results are quantitative in terms of reproducibility and linearity.

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