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Use of high-performance capillary electrophoresis to monitor charge heterogeneity in recombinant-DNA derived proteins

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ABSTRACT

The separation of charge variants of recombinant DNA-derived (rDNA) proteins by high-performance capillary electrophoresis (HPCE) has been explored with the following examples: human growth hormone (rhGH), a soluble form of a T4 receptor protein (rCD4) and tissue plasminogen activator (rt-PA). The separation of rhGH and deamidated variants were examined over the range of low pH (<2.5) to high pH (8.0) on a coated silica capillary. No resolution was observed at pH 2.5 while at pH values of 6.5 or greater the deamidated species were separated. At pH 3.5 the variant was partially resolved but no detector signal was observed at pH 4.5 and 5.0. HPCE was also used to monitor a glycoprotein (rt-PA) with charge heterogeneity presumably due to variable sialic acid content. At a pH value of 4.5, the charge heterogeneity was only observed as peak broadening. For rCD4 multiple peaks were observed at pH 5.5 but no signal was observed at pH 6.5 or above (the pI of rCD4 is 8). These results suggest that HPCE will prove to be a valuable technique for the analysis of charged variants present in rDNA products either as a consequence of natural microheterogeneity or due to degradative processes such as deamidation.

INTRODUCTION

Recently high-performance capillary zone electrophoresis (HPCE) has been applied to the analysis of polypeptide samples[1–6]. While most of the applications have been directed at peptide samples several examples of protein separations have been reported such as adrenocorticotropin (ACTH)[2], ribonuclease A[6], insulin[6], growth hormone[4–6], transferrin[2] and albumin[4]. With the advent of protein pharmaceuticals produced by recombinant-DNA (rDNA) technology there has been a resurgence in analytical protein chemistry. The early potential of HPCE in this field has been demonstrated with the successful analysis of biosynthetic human growth hormone (rhGH)[5–7]. HPCE has considerable potential to monitor charged variants of proteins as shown by the separation of deamidated rhGH[5,6], deamidated insulin[6] and human transferrin isoforms[3]. Since HPCE has significant potential in drug development with applications as diverse as fermentation control to stability monitoring,

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we decided to further investigate the separation of charged variants at different pH values. Deamidation has been chosen as an example of charge heterogeneity caused by degradative reactions, while sialic acid isoforms was used as illustrative of carbohydrate microheterogeneity. The rDNA-derived proteins included in this study range from a non-glycosylated protein (rhGH, mol.wt. 22 000 dalton) to a glycoprotein of moderate complexity (soluble human CD4, mol.wt. 40 000 dalton) to a glycoprotein of considerable microheterogeneity tissue plasminogen activator (rt-PA, mol.wt. 66 000 dalton). The separations were carried out on a coated capillary that is commercially available and the importance of pH on both the recovery and resolution of the charged variants was investigated.

METHODS

Capillary zone electrophoresis

All capillary electrophoresis was performed using the HPE 100 high-performance capillary electrophoresis system from Bio-Rad Labs. The capillaries used were enclosed in cartridges and coated on their internal surfaces with a covalently bonded linear polymer, significantly reducing both adsorption and electroendoosmosis[4]. The cartridges used were 20 cm × 25 μm I.D.

Free zone capillary electrophoresis was carried out with the polarity of the internal power supply of the instrument set such that the sample components would migrate toward the detector, *i.e.*, the cathode was at the detector end of the capillary for the lower pH runs (*e.g.*, pH 2.5 for rhGH), and at the inlet end for the higher pH runs (*e.g.*, pH 6.5 for rhGH). At the start of an analysis the capillary and the electrode reservoir at the detector end of the capillary were filled with phosphate buffer, and the inlet-side electrode reservoir was flushed with distilled water. A 10-μl volume of the sample solution (0.5 to 1.0 mg/ml) with an ionic strength approximately one-tenth that of the electrophoresis buffer was then placed by syringe into the reservoir just ahead of the inlet of the capillary. The power supply was turned on, and the sample electrophoresed into the capillary for 5 s at 8 kV. The inlet electrode reservoir was then flushed with the electrophoresis buffer, and the power supply turned back on at a constant voltage of 8 or 12 kV. Electropherograms were made by monitoring absorbance at 200 nm. Following a run, the capillary was flushed with buffer to remove uneluted components. Samples isolated from ion exchange chromatography were diluted 1:5 or 1:10 with distilled water depending on the ionic strength of the sample. Each analysis was performed a minimum of five times to demonstrate a reproducible electropherogram.

RESULTS

Fig. 1 shows the effect of pH on the separation of deamidated variants of rhGH. The sample used in this study contained approximately 10% deamidated material resulting from an incubation of rhGH in 10 m*M* sodium phosphate, pH 7.4 for 2 weeks at 4°C. The identification of the species observed in the HPCE separation was achieved by injection of standards isolated from ion-exchange chromatography (IEX)[5]. At pH 2.5 no separation was observed (A), while at pH 3.5 (B) the deamidated species was partially resolved as a shoulder (at approximately 4.5 min). No peaks

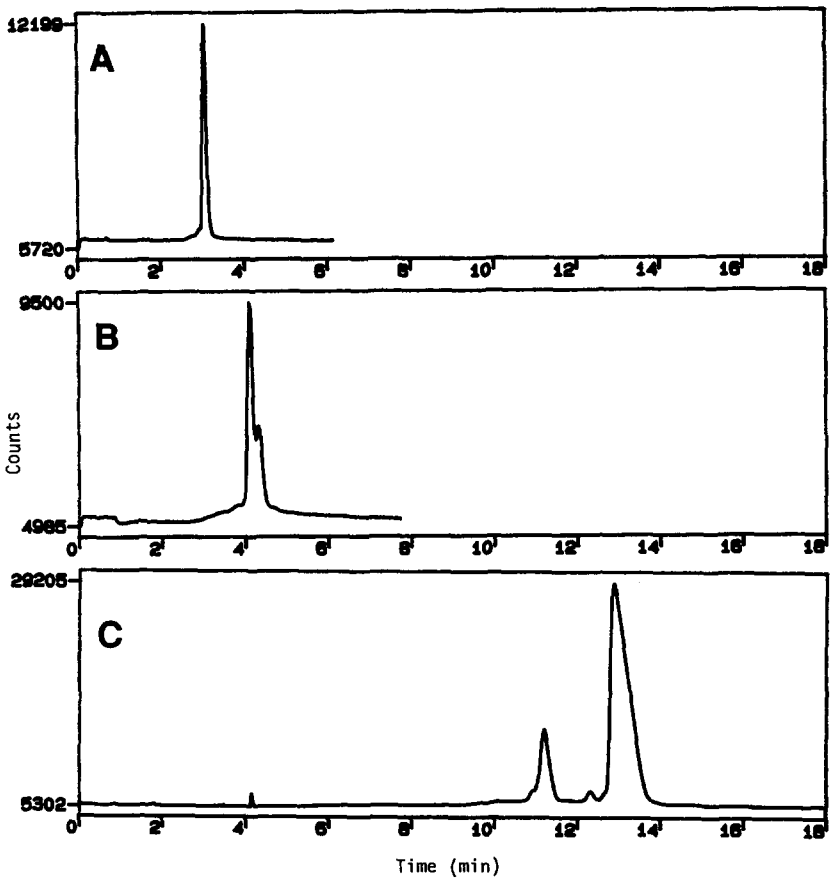

Fig. 1. Effect of pH on the separation of charged variants in degraded rhGH. The separation was carried out on a Bio-Rad coated silica capillary column (20 cm × 25 μm) at 8 kV using the conditions described in the Methods section. The earlier eluted fraction at pH 6.5 or later eluted fraction at pH 3.5 was identified as deamidated material. The pH conditions were 2.5 (A), 3.5 (B), and 6.5 (C). rhGH concentration 0.5 μg/μl.

were observed when the separation was run at pH4.5 or 5.5 (data not shown). In Fig. 1C at pH 6.5 the deamidated species was well resolved from native material (11.3 *vs.* 13.5 min). The reversal in migration order was caused by reversal in polarity of the capillary system to compensate for change in charge of the protein from a cationic to an anionic species at pH values above the p*I* of the protein.

Fig. 2 shows the effect of pH on the separation of charged variants of a highly purified sample of rCD4 (ref. 8). At pH 2.5 (A) only a single peak was observed, while at pH 3.5 (B) partial resolution was observed. The charged variants were well resolved at pH 4.5 (C) and almost baseline resolved at pH 5.5 (D). No signals were observed for separations carried out at pH 6.5 and above. Fig. 3 shows the results of analysis of the charge heterogeneity of rCD4 by HPCE after removal of sialic acid with neuraminidase[9].

Fig. 4 shows the analysis of a sample of rt-PA by HPCE (A) and of a sample

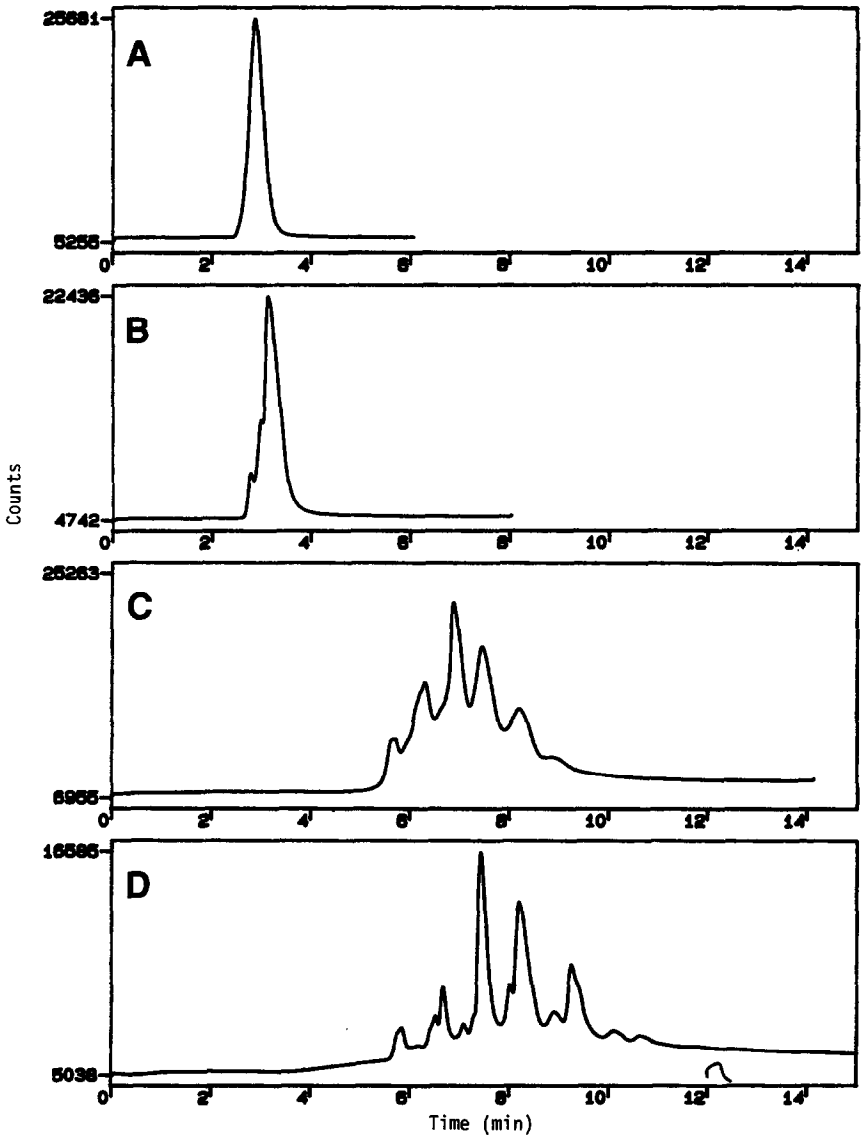

Fig. 2. Effect of pH on the separation of charged variants of rCD4. The separation was carried out on a Bio-Rad coated silica capillary column (20 cm × 25 μm) at 12 kV using the conditions described in the Methods section. The pH conditions were 2.5 (A), 3.5 (B), 4.5 (C) and 5.5 (D). rCD4 concentration was 0.5 μg/μl.

after removal of sialic acid residues with the enzyme neuraminidase[9] (B). The early eluting peak (2.5 min) was shown to be excipient related by a blank run (data not shown). Fig. 4B shows a much sharper peak profile which can be attributed to removal of the extensive charge heterogeneity due to the sialic acids.

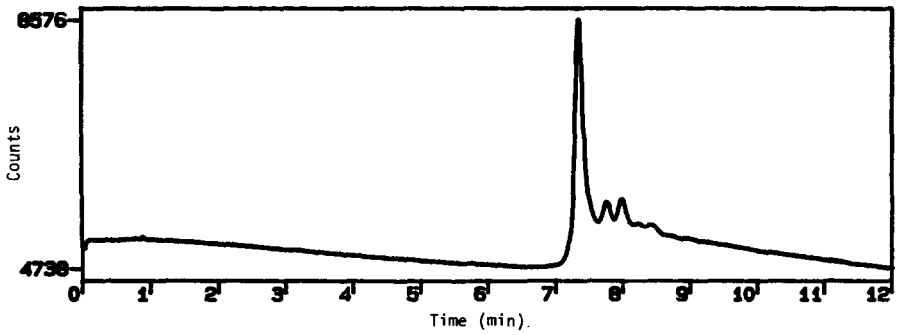

Fig. 3. The effect of sialic acid removal (neuraminidase treatment) on the electrophoretic pattern observed for rCD4. The analysis of rCD4 on the Bio-Rad coated capillary column (20 cm × 25 μm) was at 12 kV and at pH 4.5.

DISCUSSION

A major degradative pathway of recombinant DNA-derived human growth hormone (rhGH) has been identified as deamidation[10]. The site of deamidation was identified as asparagine (residue 149) which is converted to an aspartic acid (Asp). Since Asp has a pK_a of approximately 4.0 when incorporated into a polypeptide chain, we would expect that use of a buffer with a pH above this value would allow a

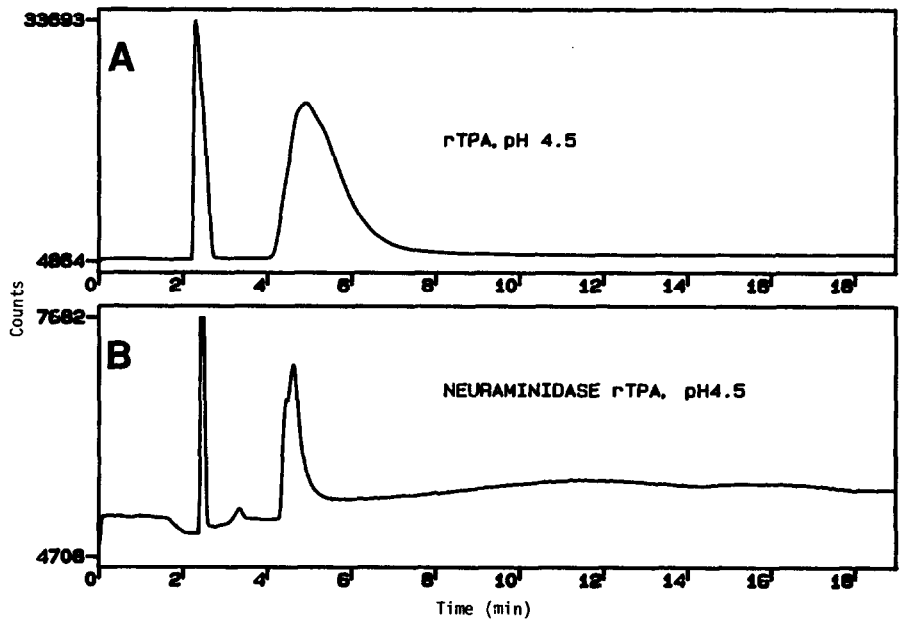

Fig. 4. The analysis of rt-PA by a Bio-Rad coated silica capillary column (20 cm × 2.5 μm) at 12 kV and pH 4.5 (A). (B) Analysis of a sample of rt-PA that has been treated with neuraminidase. rt-PA concentration was 1 μg/μl.

TABLE I

MOLECULAR WEIGHT HETEROGENEITY OF rt-PA

These molecular weight values were calculated from a recent study (see ref. 9).

		Molecular weight
Amino acid residues		59 008
Carbohydrates		
Amino acid residue[a]		
117	(Type of glycan: high mannose)	1200–1600
184	(Type of glycan: complex)	2000–3600
448	(Type of glycan: complex)	2000–3600
Type I (three carbohydrate chains)	Total mol.wt. of rt-PA variants:	64 000–68 000
Type II (two carbohydrate chains)	Total mol.wt. of rt-PA variants:	62 000–64 000

[a] Site of attachment of carbohydrate chain.

charge-based separation. However, the p*I* of rhGH and the presence of any free silanols on the walls of the capillary are important considerations in developing the HPCE separation. Previously the effect of pH on the analysis of rhGH by IEX was studied and the separation of charged variants was shown to be dependent on subtle changes in pH[11]. In the HPCE separation the use of pH values below the pK_a value of Asp resulted in little separation of the deamidated variant. No peaks were detected at pH 4.5 and pH 5.5 and since the p*I* of rhGH is 5.0, this negative result could be attributed to lack of migration or loss of sample due to adsorption on the walls of the capillary. This separation was carried out on a coated capillary, however, such a coating does not completely inactivate reactive silanols and protein adsorption is possible, particularly under conditions where sample aggregation is promoted by an isoionic species[12]. At pH values substantially above the p*I* of the sample and pK_a of the silanol groups, for example pH 6.5, both aggregation of the sample and protein/wall interactions are minimized due to charge repulsion and excellent separations were achieved (Fig. 1C). The rapid electrophoretic separation of variants produced by a major degradative pathway of rhGH illustrate the potential of HPCE in monitoring of the stability of protein pharmaceuticals. Such monitoring can have significant applications in areas of biotechnology such as quality control testing, formulation development and optimization of the fermentation process.

The recent introduction of large scale mammalian cell culture has allowed the production of rDNA derived glycoproteins as potential therapeutic agents[13]. However, the presence of sialic acid on the complex-type oligosaccharides introduces considerable charge as well as mass heterogeneity into the product. Table I lists the sites of N-linked glycosylation of rt-PA as residues 117, 184 and 448 with the two latter sites consisting of bi-, tri- and tetraantennary structures that contain sialic acid. Residue 184 is an optional glycosylation site and approximately 50% of the molecules contain two instead of three carbohydrate chains. This heterogeneity in addition to the microheterogeneity of each of the carbohydrate chains results in rt-PA containing a range of isoforms ranging in molecular weight from approximately 64 000 to 68 000 dalton. In Fig. 4A it can be seen that HPCE in unable to separate these isoforms and

a broad peak is observed. The type I variant (see Table I) was found to migrate at the front part of the broad peak, while the type II variant was concentrated at the back of the peak (data not shown). A 1:1 mixture of the two variants gave same profile as seen in Fig. 4A. However, removal of sialic acids by treatment of rt-PA with neuraminidase resulted in significant sharpening of the peak (Fig. 4B). This result might suggest that much of the peak broadening was caused by charge heterogeneity in the N-linked oligosaccharides. However an empirical relationship between electrophoretic mobility and molecular weight was reported[4] and extended to the tryptic peptides of rhGH[5]. In a separate study Grossman *et al.*[6] related mobility to molecular size of proteins. Thus it is likely that the molecular weight heterogeneity of rt-PA also contributes to the broad peak observed in Fig. 4A.

Despite a relatively high isoelectric point of rt-PA (in the range of 7.0 to 8.0) it was not possible to analyze samples above pH 4.5. This behavior parallels previous experience with reserved-phase HPLC on both silica and polymer based supports where we found that rt-PA could not be eluted from the column at pH values above 4.0[14]. In this study we used a coated capillary in an effort to reduce interactions between the sample and the capillary wall. Other authors have investigated a variety of approaches to minimize this problem such as the use of alkali metal salts[15] zwitterionic reagents[16], protein denaturants[4], putrescine[17] and the use of other coatings[18] in an attempt to improve both peak shapes and recoveries. Currently we are investigating the use of zwitterionic reagents and detergents in an effort to improve this separation. It has been reported that temperature gradients can severely reduce separation efficiencies[19]. In this study we used unthermostatted capillaries although the use of small diameters (25 μm) and low voltages minimized this potential problem[4].

CD4 is a glycoprotein expressed on the surface of a variety of cells of the immune system and binding of gp 120 to CD4 mediates the infectivity of the HIV virus[20]. Recombinant soluble CD4 (rCD4) is a truncated form of human CD4 that is secreted from transfected Chinese hamster ovary cells. This 368-amino acid glycoprotein contains two potential sites of N-linked glycosylation (Asn-271 and Asn-300[8,21]. The first site contains complex-type oligosaccharides, while Asn-300 has attached high-mannose or hybrid structures in addition to complex-type oligosaccharides. It was decided to investigate the potential of HPCE to monitor the charge variants of rCD4 due to variable sialic acid content. Since the sialic acid group has a pK_a value of 3.5 to 4.5 the effect of a range of pH values on the separation was investigated. From the results shown in Fig. 2A–D it can be seen that the optimal separation of rCD4 is around pH 5.5. The improved resolution observed for the charged variants of rCD4 relative to that of rt-PA may be related to the lower degree of sialyation with rCD4[8] as well as less molecular weight heterogeneity as rCD4 unlike rt-PA does not contain an optional glycosylation site. No signals were detected at pH 6.5 or above. Since the p*I* of CD4 is approximately 8.0, the loss of sample may be associated with protein adsorption on the capillary wall. As was observed in the analysis of rt-PA, sample loss can occur at pH values well below the isoelectric point. Fig. 3 shows the analysis of a sample of rCD4 that has been treated with neuraminidase to remove sialic acid. As in the case of rt-PA, the electropherogram of rCD4 is significantly sharpened (compare Fig. 3 with Fig. 2C) and indicates that much of the charge heterogeneity is due to sialic acid microheterogeneity.

In conclusion, HPCE has been shown to be a valuable new analytical method

for the monitoring of charged variants that can occur during the production of a biosynthetic protein pharmaceutical. However the pH studies indicate that a successful electrophoretic analysis requires careful optimization of the separation conditions.

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