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Use of acidic and basic pH and calcium ion addition in the capillary zone electrophoretic characterization of recombinant human deoxyribonuclease, a complex phosphoglycoprotein

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Abstract

This paper describes the analysis of recombinant human deoxyribonuclease (rhDNAse), an acidic and complex phosphoglycoprotein, by capillary zone electrophoresis (CZE). Separation performance was found to be dramatically improved by the addition of calcium ions to the CZE running buffer, due to the influence of calcium binding on the charge and the electrophoretic behavior of rhDNAse. The pH dependent calcium binding effects on the electrophoretic separation were demonstrated at both acidic and basic pH, resulting in a two-dimensional (pH 4.8 and 8.0) calcium aided analysis that achieved multipeak resolution of the complex, glycosylation based, charge microheterogeneity of rhDNAse. Two-dimensional investigation of neuraminidase- and alkaline phosphatase-digested protein further demonstrated that the acidic pH resolved acidic charge heterogeneity and that the basic pH discriminated neutral heterogeneity. This work demonstrates the resolving power of CZE for the analysis of a complex microheterogeneous glycoprotein, and emphasizes the importance of employing multiple separation conditions in accordance with known structural characteristics of the protein.

Keywords: Buffer composition; DNAse; Enzymes; Phosphoglycoproteins; Glycoproteins

1. Introduction

Recombinant human deoxyribonuclease I (rhDNAse I, Pulmozyme), an enzyme manufactured in Chinese hamster ovary cells, has been approved for the alleviation of the debilitating effects of cystic fibrosis [1–3]. rhDNAse is a glycoprotein (molecular mass is approximately 34 000) that possesses two fully occupied asparagine linked glycosylation sites that contribute approximately 12% to the average molecular mass of the protein [4,5]. Significant charge based heterogeneity occurs from the glycans of rhDNAse, which include approximately 0.6 mol of mannose-6-phosphate and 2.0 mol of sialic acid per mol of protein on a heterogeneous mixture of high mannose, hybrid, and complex glycosylation structures. The isoelectric point (p*I*) of the enzyme, predicted from the amino acid composition, is 4.6; however, after acidic post-translations modifications,

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the actual p*I* of the protein is visualized on isoelectric focusing (IEF) gel electrophoresis as a multitude of bands between p*I* 3.5 and p*I* 4.5 (Fig. 1). Characterization of such a heterogeneous protein represents a significant challenge. Separation conditions must be guided by the known structural features of the molecule.

The DNAse I molecule, 260 amino acids in length, contains two high-affinity calcium binding sites that are associated with structural stability (Fig. 2) [6]. Human and bovine DNAse I sequences share 77% homology and, while the structure of human DNAse I has not been published, the crystal structure of the human form is considered to be superimposable with the published bovine form [7]. Calcium ion binding protects the structural stability of bovine DNAse (bDNAse), preventing both inactivation by proteases [8] and reduction of a disulfide bridge (C173–C209) that is essential for activity [9]. Similarly, rhDNAse, in the presence of calcium, is resistant to protease digestion and denatures at a higher temperature in comparison to the protein in a calcium free environment [10]. Crystallographic data also indicates that the bovine molecule binds one Ca^{2+} ion near the non-essential C101–C104 disulfide bond [6], while the human molecule binds two Ca^{2+} ions within the same (D99–D107) exposed loop structure [7].

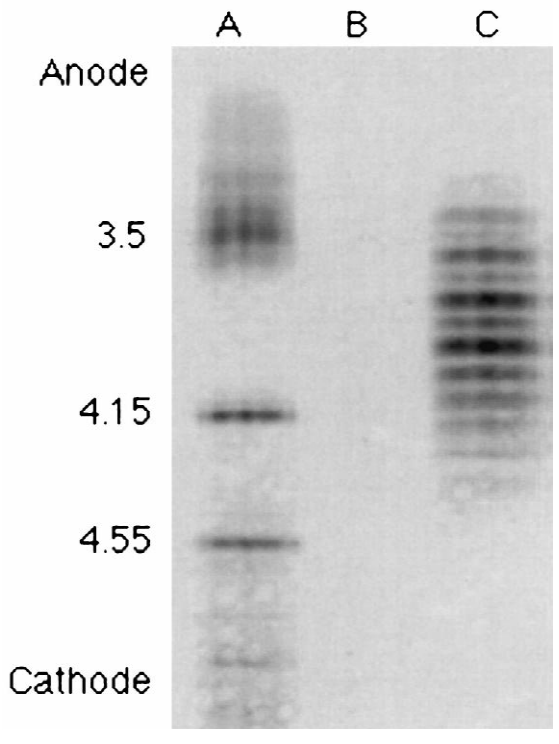

Fig. 1. Isoelectric focusing gel electrophoresis of rhDNAse. The predicted p*I* of the rhDNAse protein is 4.6; however, post-translational glycosylation, with both sialic acid and phosphate containing structures, generate considerable negatively charged heterogeneity, resulting in a ladder of approximately 14 bands (Lane C) between the p*I* markers of 3.5 and 4.5 (Lane A) on an isoelectric focusing gel. (B) blank.

The reversible interaction between the bDNAse molecule and calcium is highly pH dependent. At pH 9.0, there are four strongly bound ions, at pH 7.5, there are two strongly bound and three weakly bound calcium ions, and at pH 5.5, the protein binds strongly only one calcium ion while weakly binding four more [11]. The binding of calcium by bDNAse also causes changes in circular dichroism and in far UV and optical rotatory dispersion spectra; however, these changes do not correlate to any observable difference in the shape or volume of the protein, as determined by ultracentrifugation sedimentation [8,12]. The binding of calcium also appears to reverse pH induced conformational changes of DNAse, reducing the increased Stokes radius of the molecule that occurs at alkaline pH (9.5), indicating a role in the stabilization of the conformation of this protein by calcium ions [13].

In this paper, we present capillary zone electrophoresis (CZE) analysis of rhDNAse and demonstrate the importance of protein specific buffer additives, calcium in this case, upon the separation. The analysis of this therapeutic recombinant protein is challenging due to the complexity of a product that undergoes post-translational glycosylation, leading to both size- and charge-based heterogeneity. CZE, a high-resolution technique that separates analytes based on charge and size differences, is well suited for the analysis of such a complex protein [14–20]. The strongly pH dependent, calcium-aided separation was investigated at both basic and acidic pH, resulting in a two-dimensional analysis that demonstrated resolution of both neutral and negatively charged glycosylated forms of the protein. This paper further demonstrates that CZE is a reproducible and robust method for analysis of heterogeneous recombinant glycoproteins.

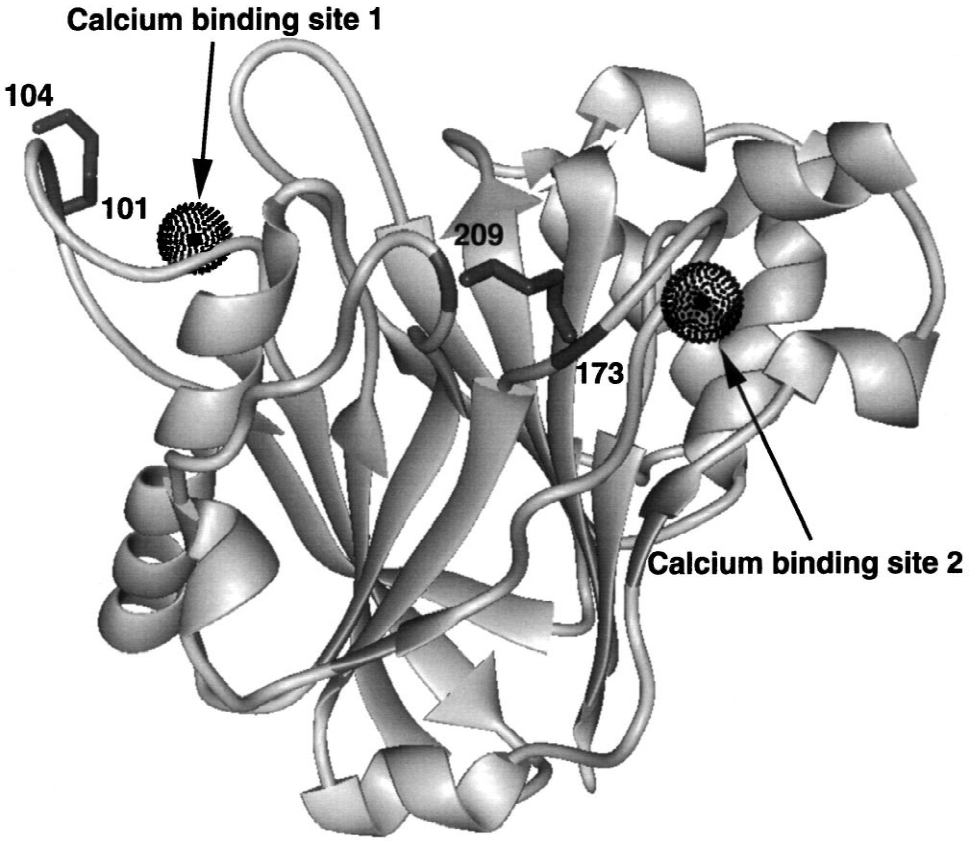

Fig. 2. Schematic three-dimensional representation of bovine DNAse. DNAse contains two high affinity calcium binding sites (shaded spheres) which are associated with structural stability of the molecule. The first site is located at an exposed loop segment that extends from D99 to D107 and contains the non-essential C101–C104 disulfide bond. The second high affinity calcium binding site is also located on an exposed loop segment, D201–T207, and appears to be responsible for the protection of the essential disulfide bridge C173–C209.

2. Experimental

2.1. Instrumentation

A P/ACE System 5500 (Beckman Coulter, Fullerton, CA, USA) with System Gold software version 8.10 was used for all electrophoretic experiments at pH 6.6 and 4.8. For all experiments at pH 8.0, a HP3DCE (Hewlett–Packard, Palo Alto, CA, USA) with HP ChemStation software version A.04.01 was employed.

2.2. Reagents

Analytical grade buffer [6-amino-*n*-caproic acid (EACA)] and the inorganic salts, calcium chloride, sodium chloride, manganese chloride and cobalt sulfate were obtained from Fluka (Ronkonkoma, NY, USA). Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis–Tris) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Poly(vinyl alcohol) (PVA) coated capillaries were purchased at Hewlett–Packard. Milli-Q water (Millipore, Bedford, MA, USA) was used to prepare all the buffers and sample solutions. Recombinant human deoxyribonuclease I (fully deamidated at the asparagine-74 site), as well as the neuraminidase-, alkaline phosphatase-,

and dual-digested forms of the protein, were prepared at Genentech.

2.3. Methods

2.3.1. Alkaline phosphatase digest

rhDNAse (10 mg) was dialyzed into 50 m*M* Tris, 2 m*M* calcium chloride, pH 8.4, and digested with 800 units of Boehringer Mannheim (Indianapolis, IN, USA) alkaline phosphatase, at a molar enzyme to protein ratio of approximately 1:60 for 6 h at 37°C. Phosphate content was analyzed by anion-exchange HPLC [4] and determined to be 0.6 mol of phosphate per mol of rhDNAse prior to digestion. Phosphorylated rhDNAse species were not detected after digestion with alkaline phosphatase.

2.3.2. Neuraminidase digest

rhDNAse (10 mg) was dialyzed into 10 m*M* sodium acetate, 2 m*M* calcium chloride, pH 5.5, and digested with 6 units of Boehringer Mannheim neuraminidase at a molar enzyme to protein ratio of approximately 1:333 for 24 h at 37°C. Sialic acid content was determined by derivatization with *o*-phenylenediamine and fluorescence analysis on HPLC [5] and was found to be 2 mols of sialic acid per mol of rhDNAse prior to digestion and 0.2 mol sialic acid per mol of rhDNAse after digestion.

2.3.3. Dual enzyme digest

Neuraminidase-digested rhDNAse (3.3 mg) was dialyzed into 50 m*M* Tris, 2 m*M* calcium chloride, pH 8.4 and digested with 264 units of alkaline phosphatase at a molar enzyme to protein ratio of approximately 1:60 for 6 h at 37°C.

2.3.4. Capillary zone electrophoresis

Laboratory-prepared PVA coated capillaries were used for all preliminary experiments and those performed at pH 4.8 [21]. Experiments at pH 8.0 used commercial (Hewlett–Packard PVA-coated capillaries. On the Beckman P/ACE sample injections at acidic and neutral pH were at 0.5 p.s.i (34.5 mbar). Injections at the basic pH on an HP3DCE were applied at 0.75 p.s.i (50 mbar). The voltage was applied in the reverse polarity mode and protein absorbancy was monitored at 214 nm. Detailed descriptions of specific capillary inner diameters and lengths, separation conditions and buffer concentrations are included in the figure captions.

2.3.5. Enzyme-linked immmunosorbent assay (ELISA) for rhDNAse recovery

Microtiter plates (96 well) were coated with antibody to rhDNAse (anti-rhDNAse) and blocked for 2 h with 200 μl assay diluent containing 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 m*M* calcium chloride, 4 m*M* magnesium chloride, 0.1% bovine serum albumin, 0.05% Polysorbate 20 and 0.01% Thimerisol at pH 7.5. The plates were washed with phosphate buffered saline, pH 7.4, containing 0.05% Polysorbate 20 (PBS–Tween) and 100 μl of standards, samples and controls were added to the wells and incubated for 2 h at ambient temperature. The plates were washed to remove unbound material, then 100 μl of horseradish peroxidase-labeled anti-rhDNAse was added to each well and incubation was continued for 2 h. After another wash step, 100 μl of substrate solution (5 mg tablet of *o*-phenylenediamine in 12.5 ml of 4 m*M* hydrogen peroxide in PBS) was added to each well and incubated in the dark for 15–25 min. The reaction was stopped with 100 μl of 2.25 *M* sulfuric acid to each well. An SLT 340 EAR AT plate reader (SLT Labinstruments, Research Triangle Park, NC, USA) was used to measure absorbancy at 490–492 nm, with 405 nm as the reference. A four parameter logistic curve-fitting program was used to determine sample concentrations and controls were monitored for consistency of assay performance.

2.3.6. Isoelectric focusing gel electrophoresis

A ServaLyte PreCotes 3-6 precast IEF gel (Serva, Heidelberg, Germany), 0.15 mm thick on polyester backing, with Serva Anode fluid pH 3 (L-aspartic acid+L-glutamic acid) and Serva glycine Cathode fluid pH 7, was prefocused on an LKB (Pharmacia, Sweden) Ultraphor IEF unit model 2217 at 4°C for 1 h. A 10 μg aliquot of rhDNAse was loaded 2 cm from the cathode and focused at 1250 v for 25 min, 1450 v for 25 min and 1650 v for 2 h. The focused gel was fixed in a 10% trichloroacetic acid, 5% (w/v) sulfosalicylic acid solution for 45 min, washed twice for 20 min each in a 20% ethanol–6.25% acetic acid solution, then stained in a 0.125% Coomassie R-250–0.05% copper sulfate–37.5%

ethanol (v/w/v) solution for 120 min. A 20 min intermediate destain with a solution of 0.5% copper sulfate– 7.5% acetic acid–40% methanol (w/v/v) was used to help clear the background. In addition, the gel was wiped with cotton swabs to remove stain precipitates. Finally, the gel was unstained in a 20% ethanol–6.25% acetic acid solution (v/v) until the desired background was obtained, rinsed with water and then air dried.

3. Results and discussion

The goal of this work was to develop a CZE method for the analysis of rhDNAse I protein microheterogeneity resulting from post-translational glycosylation. Preliminary experiments were performed with a buffer close to neutral pH, since the sialic acid and phosphate residues are expected to be negatively charged in this pH region. As shown in Fig. 3A, a broad peak with poor resolution of the expected charge microheterogeneity was observed. The stock solution of rhDNAse contains 1 m*M* calcium chloride in order to stabilize the structure of DNAse I. During the application of an electric field in a CZE separation, the negatively charged protein migrated in a direction opposite to the unbound calcium ions initially present in the sample injection plug. The use of a separation buffer lacking additional calcium ions most likely destabilized the protein, resulting in the poor peak shape observed at pH 6.6. With the addition of 5 m*M* calcium chloride to the pH 6.6 buffer, the mobility of the protein decreased, due to binding of the divalent calcium cation to negatively charged sites on the protein surface and an improved separation was observed (Fig. 3B).

As metal ion binding is highly pH dependent, experiments were performed in the pH range from 4.5 to 9.0 in order to determine the effect of pH, in the presence of calcium ions (5 m*M* calcium chloride), on the CZE separation of the various forms of rhDNAse. The resulting electropherograms (data not shown) indicated, as expected, reduced mobility of rhDNAse with decreasing pH. Sample migration is a result of the surface charge on the molecule, and the decrease in the net negative surface charge of the protein near the observed p*I* (ranging from 3.5 to

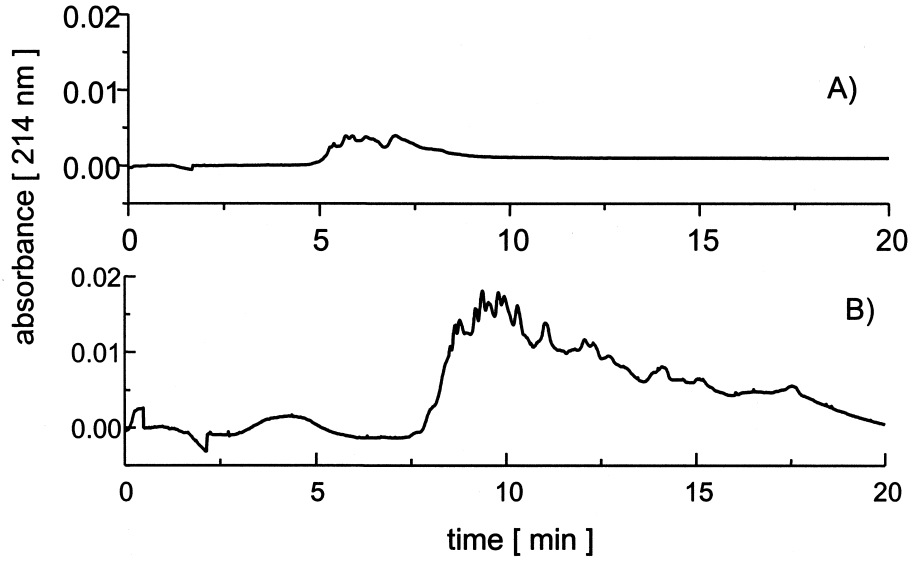

Fig. 3. Preliminary CZE experiments at pH 6.6. (A) 20 m*M* MES adjusted with Bis–Tris, pH 6.6 (no calcium); capillary=PVA coated, 37 cm (effective length 20 cm)×75 μm I.D., E=540 V/cm; injection=10 s by pressure; sample=1 mg/ml rhDNAse. (B) 5 m*M* calcium, 20 m*M* MES adjusted with Bis–Tris, pH 6.6; conditions as above; sample=1.5 mg/ml rhDNAse.

4.5) correlated to reduced mobility in CZE in the coated capillary.

3.1. Acidic pH

At acidic pH, calcium ion interactions with the protein are expected to occur at the high affinity structural site, near the essential disulfide bond. Under pH conditions close to the calculated p*I* of 4.6 (not considering post-translational modifications) [22], the amino acid residue portion of the protein molecule is expected to have little charge, while the exposed and fully charged glycosylation based sialic acid (2 mol/mol) and phosphate (0.6 mol/mol) moieties will significantly contribute to an overall net negative surface charge.

Fig. 4 shows the influence of calcium concentration on the electrophoretic separation of rhDNAse at pH 4.8. The complexity of charged glycoform heterogeneity was evident, and baseline separation was not achieved. As expected, increasing the calcium concentration reduced the electrophoretic mobility of the charged variants. In Fig. 4A, with no calcium added, the most predominant acidic peak (at approximately 18 min) had an electrophoretic mobility of 4.1×10^{-5} cm^2/Vs. In Fig. 4B–F, the

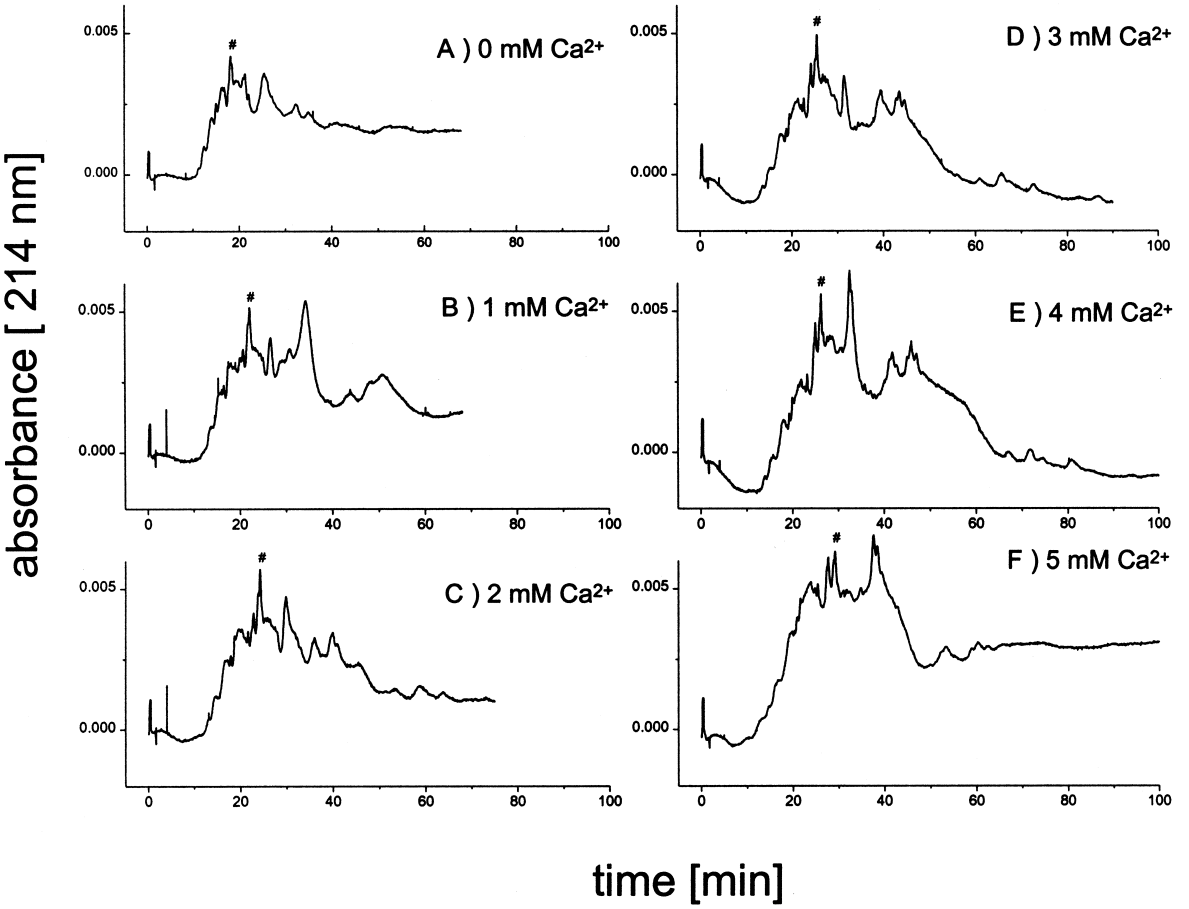

Fig. 4. Optimization of calcium concentration in the running buffer for CZE separation at pH 4.8. (A) $Ca^{2+}=0$ m*M*, 28 μA; (B) $Ca^{2+}=1$ m*M*, 29.6 μA; (C) $Ca^{2+}=2$ m*M*, 31 μA; (D) $Ca^{2+}=3$ m*M*, 33.5 μA; (E) $Ca^{2+}=4$ m*M*, 36 μA; (F) $Ca^{2+}=5$ m*M*, 39.5 μA. Separation conditions=200 m*M* EACA, pH 4.8, adjusted with acetic acid. Injection: 6 s by pressure (34.5 mbar). Sample=rhDNAse at 1.0 mg/ml in aqueous stock solution. Capillary=PVA coated, 27 cm (effective length 20 cm)×50 μm I.D., E=444 V/cm. Capillary flushed with buffer at 35 p.s.i. for 2 min between injections. #: peak for mobility calculation.

mobility of this peak decreased to 3.5, 3.2, 2.8, 2.7 and 2.6×10^{-5} cm^2/Vs with 1–5 m*M* calcium additions in 1 m*M* increments, respectively. The mobilities for the other less acidic peaks in the profile also decreased proportionally with increased calcium ion concentrations. Initially, the ability of calcium ion additions to reduce protein peak mobilities resulted in improved resolution of the multiple charged glycoforms; however, above 3 m*M* calcium, the effect on the mobilities of each major peak appeared to plateau, with little further decrease in peak mobility, suggesting that the protein was not interacting with additional calcium ions.

Reproducibility of the separation and good protein recovery are important characteristics of a quality control analysis [14]. Fig. 5 shows run-to-run repeatability and capillary-to-capillary reproducibility at pH 4.8 with 3 m*M* calcium. Reproducibility, determined from the relative standard deviation (RSD) for the migration time of the largest peak present in the electropherogram was found to be 0.5% ($N=6$) for consecutive runs on the same capillary and 9.5% ($N=3$) for different capillaries. Protein recovery from the capillary was determined by a collection method, wherein the protein was injected and separated under the same conditions as in the previous figures. After sample injection and application of voltage for 10 min, with the regular

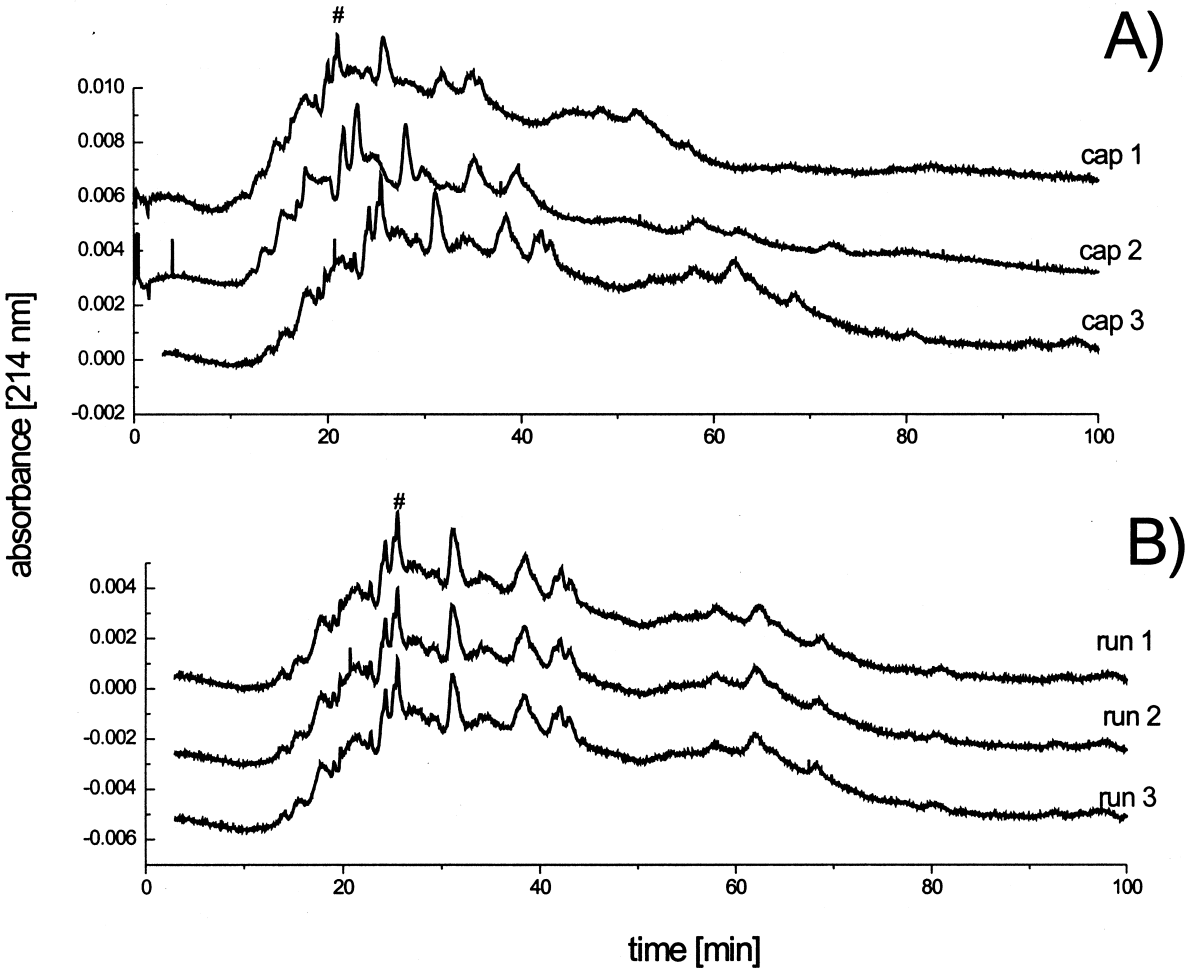

Fig. 5. Electrophoretic repeatability at pH 4.8: (A) Different batches of capillaries; (B) Run-to-run on a single capillary. For the separation conditions see Fig. 4 (3 m*M* Ca^{2+}). #: peak for RSD calculation.

buffer in the inlet and outlet vials, the capillary contents were collected in a replacement outlet vial containing ELISA buffer by applying pressure at the inlet side. Multiple injections were collected to ensure sufficient concentration for the ELISA measurement, which revealed a recovery of 85% ($N=2$) of the injected protein concentration, an acceptable recovery given the precision of the experiment.

3.2. Basic pH

As mentioned above, calcium binding capacity is strongly influenced by pH, and, therefore, a similar series of experiments investigated the influence of calcium concentration on the electrophoretic separation of rhDNAse at basic pH (pH 8.0). At this pH, the amino acid portion of the molecule will be highly negatively charged, and, while the glycosylation based sialic acid (2 mol/mol) and doubly charged phosphate (0.6 mol/mol) moieties will add further negative charges, the contribution of both moieties to the overall surface charge will not be as significant as at acidic pH. In a basic environment, both high affinity binding sites are expected to be occupied with calcium ions (3 in total), as well as increased interactions of calcium with lower affinity sites.

Fig. 6 shows that the overall influence of calcium concentration on the electrophoretic separation of rhDNAse at pH 8 was similar to that at acidic pH; protein mobility was reduced with increasing amounts of calcium, resulting in separation into four major peak areas and near baseline separation of the most basic peak. The effective electrophoretic mobility of the most predominant acidic peak (at approximately 17 min) was 1.2×10^{-4} cm^2/Vs. In Fig. 6B–F, the mobility of this peak decreased to 8.9, 7.4, 6.5, 5.4 and 4.6×10^{-5} cm^2/Vs with 1–5 m*M* calcium additions in increments of 1 m*M* calcium, respectively. In contrast to pH 4.8, the decreasing trend of protein electrophoretic mobility with increasing calcium concentration does not plateau at basic pH, indicating that more calcium–protein interactions may occur above the 5 m*M* calcium concentration level at pH 8.0.

Fig. 7 shows run-to-run repeatability and capillary-to-capillary reproducibility of the pH 8.0 separation at 3 m*M* calcium. Reproducibility for consecutive runs on the same capillary was determined to be 2.2% RSD ($N=6$) and 2.5% ($N=3$) for different capillaries, for the migration time of the largest peak present in the electropherogram. Protein recovery, determined in a similar method to that at acidic pH, revealed a recovery of 77% ($N=2$), an acceptable value given the precision of the experiment.

3.3. Other metal additions

rhDNAse would be expected to bind under basic pH conditions to other divalent metal ions, such as manganese, cobalt and magnesium [23]. To determine if enhanced separation power was calcium specific or could also be obtained through addition of other cations, two sets of experiments were performed. A monovalent cation, sodium, and a divalent cation, manganese, were chosen as additives at pH 8.0 and pH 4.8. Sodium had no effect on migration time or electropherogram profile; however, divalent manganese did affect the migration time of rhDNAse at both acidic and basic pH, indicating that Mn^{2+} bound to the negatively charged protein and decreased the electrophoretic mobility (data not shown). Although the manganese cation interacted with rhDNAse, there was less enhancement of the separation power when compared to the same concentration of calcium (data not shown), consistent with the lower overall affinity of bDNAse for manganese relative to calcium [12].

3.4. Enzymatic digestions

The extent of the protein charge microheterogeneity resulting from the sialic acid and phosphate residues was analyzed by selectively removing the contribution of each charge through specific enzymatic digestions. Fig. 8 shows the electrophoretic profiles of charge specific enzymatic digests of rhDNAse at pH 4.8 in the presence of 3 m*M* Ca^{2+}, in comparison to the native protein (Fig. 8A). Fig. 8B presents the electropherogram of the alkaline phosphatase digest (phosphate removal), with the charge based microheterogeneity resulting only from differences in sialic acid content. Comparison of Fig. 8A and B reveals a small shift in the pattern of peaks to longer time, with a more efficient, defined separation of the glycoform peaks. The profile was similar to that of the undigested protein, indicating

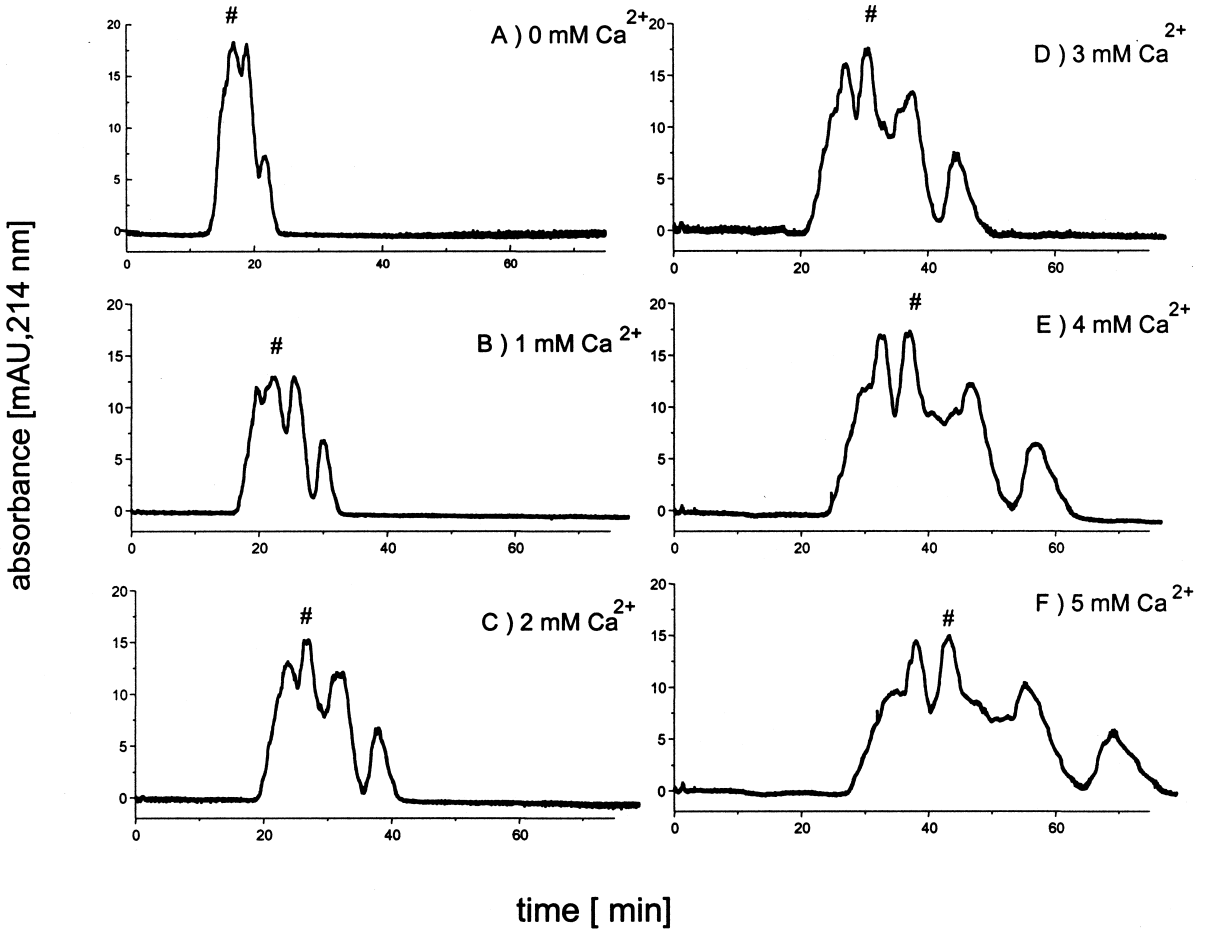

Fig. 6. Optimization of calcium concentration for CZE separation at pH 8: (A) Ca^{2+}=0 m*M*, 12 μA; (B) Ca^{2+}=1 m*M*, 13 μA; (C) Ca^{2+}=2 m*M*, 15 μA; (D) Ca^{2+}=3 m*M*, 17 μA; (E) Ca^{2+}=4 m*M*, 18 μA; (F) Ca^{2+}=5 m*M*, 19 μA. Separation conditions=pH 8.0, 20 m*M* Tris adjusted with HCl, 3 m*M* Ca^{2+}. Injection=10 s of water by pressure (50 mbar), 20 s sample, 10 s water. Sample=rhDNAse at 0.5 mg/ml in aqueous stock solution. Capillary=PVA coated, 64.5 cm (effective length 56 cm)×50 μm I.D., E=465 V/cm. Capillary flushed with buffer 5 bar for 10 min between injections. #: peak for mobility calculation.

that the separation pattern of the latter was associated with the sialic acid content of the glycans. Neuraminidase treatment (Fig. 8C) (removal of sialic acids) resulted in a separation pattern that represented the charge heterogeneity of the protein due to phosphorylation. The strong basic shift in the protein profile compared to the undigested protein indicated that the phosphate residues were not the main source of charge contributing to the CZE profile of the intact protein, a further indication that the sialic acid residues represented a large part of the microheterogeneity. Removal of both sialic acid and phosphate residues led to the large basic shift seen in Fig. 8D. The doubly digested protein had no glycosylation based charges and, at acidic pH, the profile of the nearly neutral rhDNAse glycoform variants did not return to the baseline, suggesting solubility issues that were also observed to a lesser degree in the neuraminidase digest profile (Fig. 8C).

Fig. 9 shows the electrophoretic profiles of the same charge specific enzymatic digests of rhDNAse at pH 8.0 in the presence of 3 m*M* calcium. The electropherogram of the alkaline phosphatase digest (Fig. 9B) revealed a profile of the sialic acid

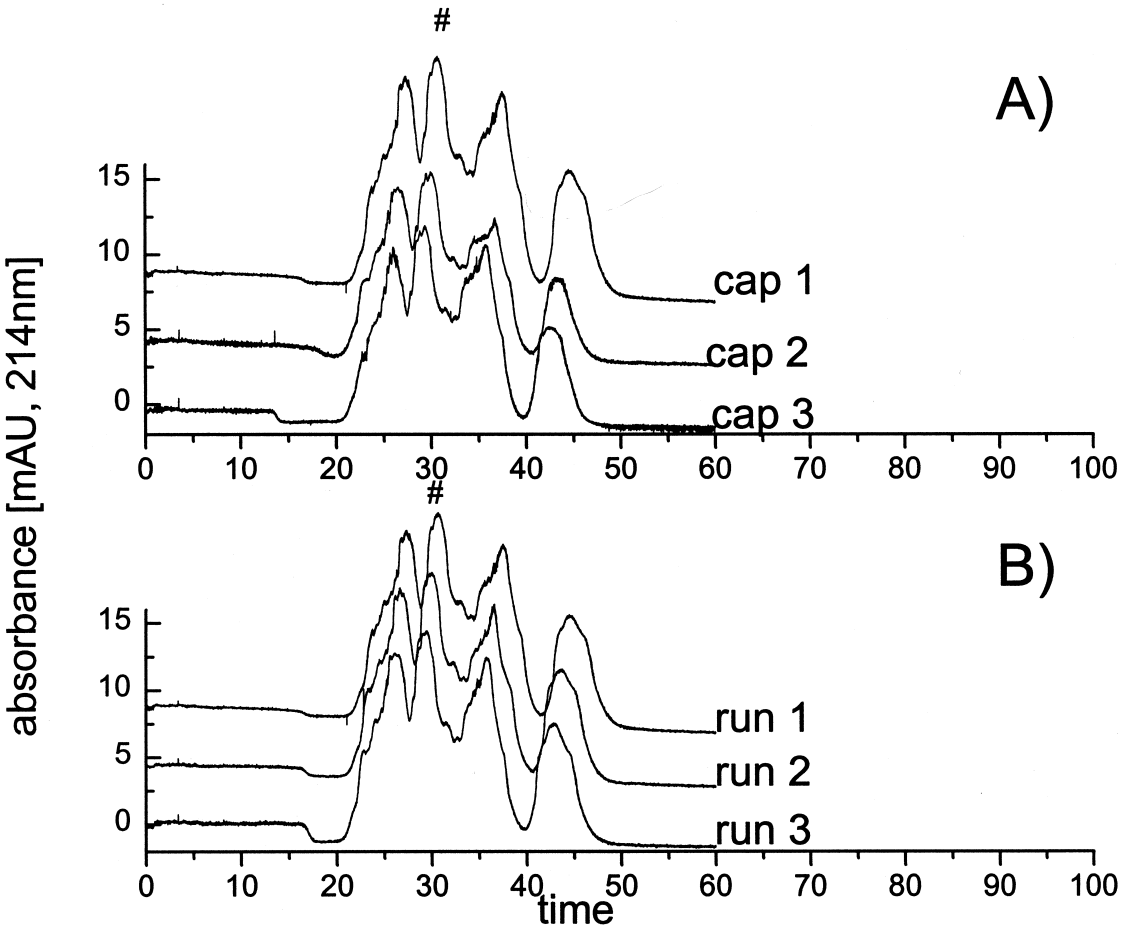

Fig. 7. Electrophoretic repeatability at pH 8: (A) Different batches of capillaries (B) Run-to-run on a single capillary. For separation conditions see Fig. 6 (3 m*M* Ca^{2+}). #: peak for RSD calculation.

microheterogeneity that was very similar to the undigested protein (Fig. 9A), providing further evidence that the native protein separation profile was reflective mainly of sialic acid content. Interestingly, the most basic peak region in this profile (Fig. 9B, at approximately 45 min) was most likely due to the alkaline phosphatase enzyme, which was observed in this same position in IEF gel electrophoresis, i.e. a higher p*I* than the most basic rhDNAse-related bands (data not shown). Neuraminidase cleavage of sialic acid residues (Fig. 9C) revealed a phosphorylation based profile that had a high degree of heterogeneity. The most basic region of the electropherogram (>35 min, peak region 3) contained glycoforms without phosphate or sialic acid (compare to Fig. 9D). There appeared to be separation of the overall phosphorylation-based charged state of the protein, in which the most basic non-phosphorylated peak region (peak 3 from ~35 to 65 min) corresponded to that measured by anion-exchange HPLC analysis [4], i.e. 55%. The more acidic region containing phosphorylated glycoforms also appeared to separate. Integration of the CZE profile for peak region 1, from approximately 20 to 24 min, and peak region 2, from roughly 24 to 35 min, were measured at 5 and 39% of the total peak area, respectively. This result was consistent with the amounts of di- and mono-phosphorylated glycoforms measured by anion-exchange HPLC, indicating that the CZE separation was based on different negatively charged zones.

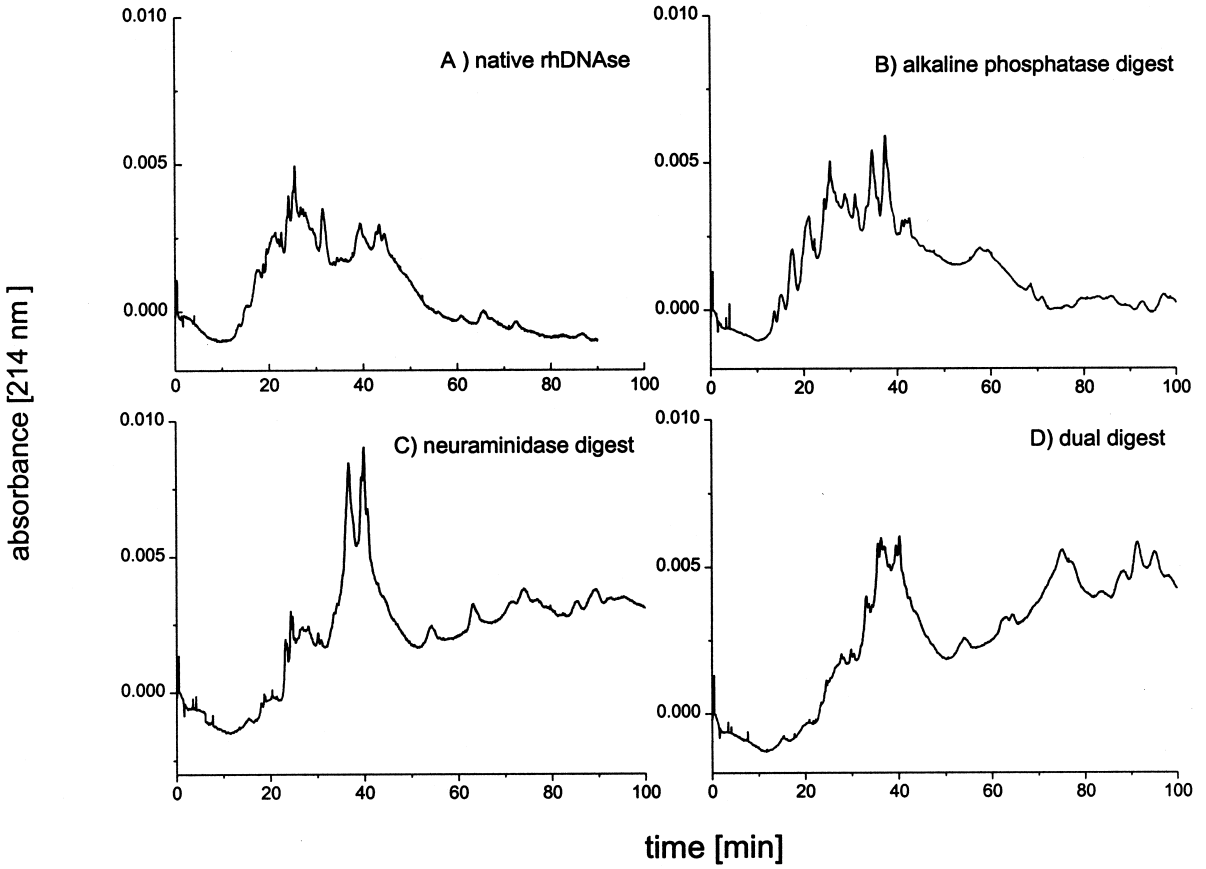

Fig. 8. Electropherograms of enzyme treatments at pH 4.8: (A) rhDNAse, undigested; (B) alkaline phosphatase digest; (C) neuraminidase digest; (D) dual digest=neuraminidase+alkaline phosphatase. For separation conditions see Fig. 4 (3 m*M* Ca^{2+}).

Analysis of the doubly digested protein at pH 8.0 (Fig. 9D) revealed a broad peak region that was partially resolved. One potential source of the remaining heterogeneity was the nearly 2300 range in molecular mass differences for the uncharged glycans characterized at the two glycosylation sites (high mannose vs. complex tetraantennary structures), accounting for approximately 7% of the overall protein size. Alternatively, the heterogeneity could be due to the charge based distribution of the number of calcium ions bound to the protein at lower affinity sites. The doubly digested protein peak pattern was also similar to the shape of the most basic (non-phosphorylated) peak already present in the neuraminidase digest profile. In contrast to the profile of this dual digest at acidic pH (Fig. 8D), the amino acid portion of the protein retained sufficient negative charge and demonstrated no solubility or electrophoretic problems.

A two pH profile of rhDNAse emerges from a comparison of electropherograms at pH 4.8 and pH 8.0 in the presence of 3 m*M* Ca^{2+} (Fig. 10A and B). The electrophoretic mobility of the protein measured faster at pH 8.0 than at pH 4.8, consistent with the increased amount of charge remaining on the protein at high pH. The degree of calcium interaction with the protein contributed contrasting effects to the overall charge of the molecule at pH 4.8 and pH 8.0. At pH 4.8, the single calcium ion, expected to bind to the high affinity site, reduced the small contribution of the negatively charged amino acids to the overall protein charge. Thus, sialylated (0–4 residues at each of the two sites) and phosphorylated (0–2 residues at each of the two sites) glycan hetero-

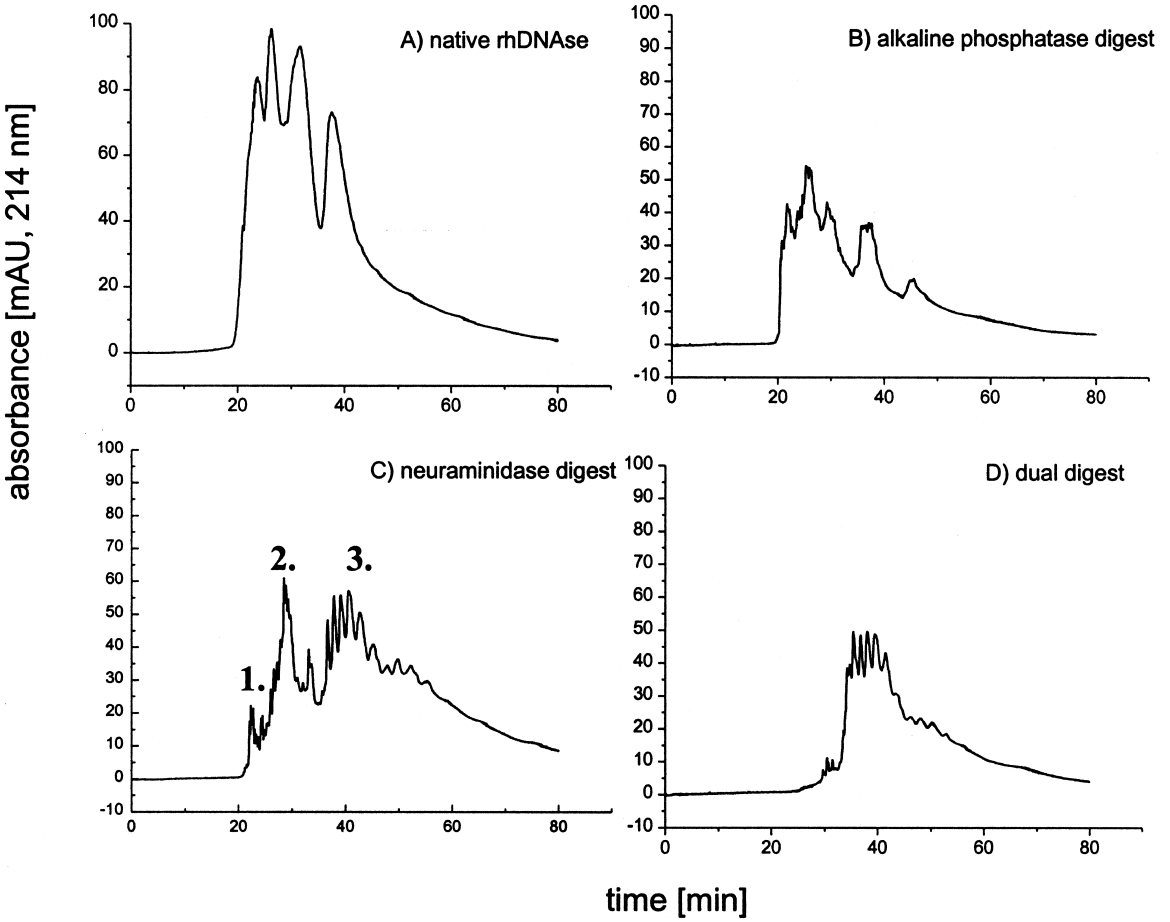

Fig. 9. Electropherogram of enzyme treatments at pH 8.0: (A) rhDNAse, undigested; (B) alkaline phosphatase digest; (C) neuraminidase digest; (D) dual digest=neuraminidase+alkaline phosphatase. For separation conditions see Fig. 6 (3 m*M* Ca^{2+}). (1) diphosphorylated, (2) monophosphorylated, (3) no phosphorylation.

geneity remained the primary source of the observed multipeak charge and mobility differences (Fig. 1A), consistent with the multiple acidic charged bands visible on the IEF gel (Fig. 1). In contrast, at pH 8.0 (Fig. 10B), the same level of calcium ions had less influence on the mobility of the protein than at acidic pH, since the protein remained negatively charged despite multiple calcium ion interactions at both high and low affinity binding sites. Glycosylation-based charge contributions were also less significant to the overall protein charge at basic pH, producing a more compact CE profile that distinguished broad zones of different charged states.

For the profiles at the two pHs, the neutral and negatively charged glycoforms of native rhDNAse were separated (see Fig. 10). Evidence from the removal of glycosylation based charges in the doubly digested sample profiles at acidic and basic pH (Figs. 8D and 9D, respectively) indicated that the neutral glycoforms (no post-translational charge heterogeneity) of the protein had the lowest mobility. In the comparison of profiles of the intact molecule (Fig. 10), the neutral species migrated from approximately 36 to 45 min at acidic pH (Fig. 10A) and from approximately 40 to 48 min at basic pH (Fig. 10B). The more mobile charged species preceded the neutral glycoforms and, based on the evidence from the removal of phosphate only charges (Figs. 8B and

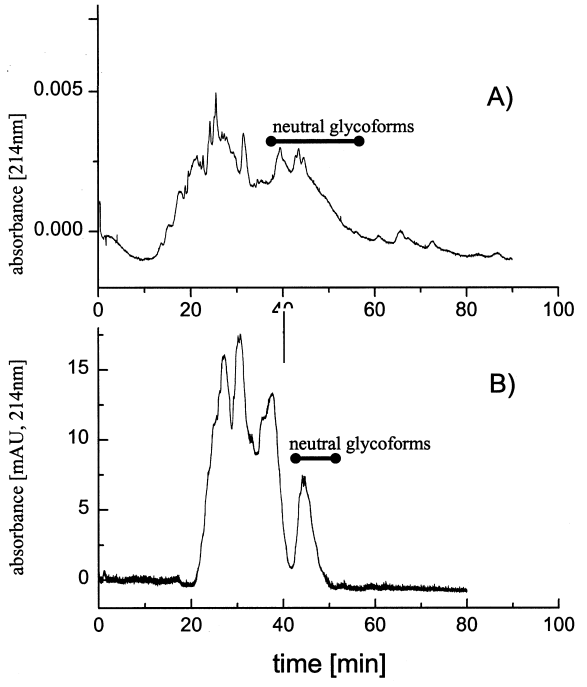

Fig. 10. Comparison of CZE separations at pH 4.8 and pH 8.0: (A) pH 4.8, 3 m*M* Ca^{2+}, for separation conditions see Fig. 4; (B) pH 8, 3 m*M* Ca^{2+}, for separation conditions see Fig. 6.

9B), mimicked the charge state of sialic acid heterogeneity. The four broad zones resolved at basic pH (Fig. 10B) were most likely the −3, −2, −1 and 0 charge states, in order of increasing migration time, similar to the IEF gel pattern of increasing acidic-charged bands (Fig. 1). The corresponding proposed pattern was not readily apparent at acidic pH (Fig. 10A), due to the increased presence of glycoform fine structures.

4. Conclusions

This work describes the analysis of rhDNAse by capillary zone electrophoresis, demonstrating the enhancement of separation of a complex phosphoglycoprotein by the selection of a protein specific buffer additive, in this case calcium. Divalent metal ion interactions with the large, polyelectrolytic protein surface increased protein conformational stability. The acidic protein became less negatively charged and therefore decreased electrophoretic mobility, resulting in an increase in the degree of resolution of glycosylation based microheterogeneity. Selectivity and separation enhancement as a function of pH also proved to be essential to the analytical characterization of rhDNAse; two different pH profiles yielded resolution of both neutral and negatively charged glycosylation based microheterogeneity. Decreased protein mobility at acidic pH allowed superior resolution of the more acidic glycan heterogeneity (overall glycosylation that had two or more sialic acid and/or phosphate residues), while increased protein mobility at basic pH permitted complex resolution of the more neutral glycan heterogeneity (overall glycosylation that had zero or one sialic acid and/or phosphate residue). High recovery values and good migration time reproducibility were achieved at both acidic and basic pH in the presence of 3 m*M* Ca^{2+}, assuring a quality analysis of protein heterogeneity.

The charged, glycosylation based microheterogeneity was further analyzed by selectively reducing the degree of heterogeneity through enzymatic digestions. Low pH analysis of an alkaline phosphatase digest of rhDNAse showed clearly the high degree of microheterogeneity resulting from sialic acid residue content, indicating that the overall separation profile was predominantly due to the sialic acid residues. The high pH separation of neuraminidase digested rhDNase indicated that only a minor part of the heterogeneity present in undigested protein originated from phosphorylation. The removal of sialic acid residues resulted in a profile that was shifted toward the more basic region containing uncharged glycans, and that was quantitatively consistent with the results from HPLC analysis for the distribution of phosphorylated glycans [4]. The double pH analysis of the charge removal digests clarified the intact protein profile, outlining the neutral glycan containing region, indicating the charged basis of separation.

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