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Capillary zone electrophoresis of a recombinant adenovirus

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Abstract

Adenovirus preparations are used as vectors in a number of gene therapy clinical development programs. The success of commercial production of adenovirus will strongly depend on the development of methods to define the recombinant virus product by analysis as opposed to being defined by the manufacturing process. While most analytical techniques examine portions of the virus, e.g. proteins or DNA, ion-exchange chromatography has been used to separate intact virus at low efficiency. A free zone capillary electrophoretic method was developed for high-efficiency separations of adenovirus 5. Experimental conditions such as buffer pH and concentration were explored which produced a high-efficiency separation in less than 20 min. The virus band was identified by collection of CE fractions and examination using a cell based assay. Initially, a single virus peak is found in fresh virus samples. After as little as one freeze–thaw in 1×phosphate-buffered saline with 2% sucrose, the active virus migrates as a regular series of peaks. The nature of the virus modification leading to the differing electrophoretic mobilities is presently under investigation.

Keywords: Adenovirus; Particle analysis; Buffer composition; Coated capillaries

1. Introduction

Recombinant adenovirus preparations are used for gene delivery in a growing number of clinical development programs in gene therapy. In support of the anticipated commercial scale production of recombinant adenovirus, analytical tools have to be developed to define the virus product. The goal of the analytical improvements is to allow demonstration of product equivalency (comparability) by chemical, biochemical and in vitro biological methods without (or with minimum) additional human clinical comparison studies. Such product equivalency has to be demonstrated following changes in the manufacturing process (process optimization, scale up, etc.) that potentially may affect product quality. Ultimately, the recombinant adenovirus products are expected to reach the ''well characterized–understood biologic product'' regulatory status similar to the therapeutic proteins.

The adenovirus particle consists of a double stranded 36 kilobase pair DNA molecule and at least 11 distinct structural proteins present in multiple copies. The total number of protein molecules in the virus is estimated to be 2500, and they are held together by non-covalent interactions [1–3]. A systematic analysis should characterize the intact virus as well as its individual protein and DNA constituents. The characterization of the intact virus represents a major analytical challenge due to its complexity and its fragile nature. Intact viruses (having icosahedral shape and diameter of approxi-

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mately 90 nm) have been extensively studied by electron microscopy, crystallography [4–6] and by immunological methods to detect/verify surface antigens on the virus [7,8]. There is, however, a lack of physical and physicochemical methodologies to detect changes in the surface characteristics of the virus caused by post-translational and manufacturing related modifications, including denaturation, oxidation, deamidation of proteins, proteolysis, changes in carbohydrate structures, etc.

Capillary zone electrophoresis (CZE) has become a routinely used high resolution separation tool for proteins, peptides and DNA. The potential of CZE to analyze macrobiologicals such as virus, bacteria [9] and red blood cells [10] was demonstrated a decade ago, but has not been exploited until recently [11–13]. The most significant problems to overcome in virus analysis by CZE are adsorption of the virus particles to the capillary and the positive identification of the viral peaks in the electropherogram. These problems were solved for Rhinovirus by adding detergents to the electrophoresis buffer and identifying peaks by indirect methods such as heat denaturation, enzymatic treatments and biospecific reactions performed before CZE analysis [12]. The objective of the present study was to establish CZE method for the analysis of recombinant adenovirus type 5 (Ad5). We sought to develop a separation method without the use of additives (detergents) in the separation buffers to avoid potential artifacts due to virus-detergent interactions. We also decided to approach the problem of peak identification through direct methods: collecting the peaks and subjecting them to high-sensitivity analyses, including polymerase chain reaction (PCR) and biological activity assays, to provide conclusive evidence for the presence of functional virus.

2. Experimental

2.1. Apparatus

A Beckman model P/ACE 5510 (Beckman Coulter, Fullerton, CA, USA) capillary electrophoresis system equipped with a standard UV detector was used for all CZE experiments. Polyvinyl alcohol (PVA) capillaries with a 3×bubble cell were obtained from Agilent Technologies (Palo Alto, CA, USA) and fitted into a 100×800 μm aperture Beckman cartridge. The capillary was 57 cm (effective length 50 cm)×50 μm I.D. The temperature of the capillary was controlled at 20°C. Unless otherwise noted, the electrophoresis buffer was 25 m*M* sodium phosphate, pH 7.0 and the samples were injected by applying 3.4 kPa pressure to the inlet vial for 30 s. Reverse polarity at 29.5 kV (518 V/cm) was used for the separations, the detector was at the anodic end of the capillary. Typical current during separation was approximately 30–35 μA. The detector signal at 214 nm was recorded and processed by the P/ACE Station Software (Beckman Coulter) package.

2.2. Reagents

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris), tricine, HCl, sodium phosphate tribasic decahydrate and sodium phosphate monobasic monohydrate for CZE separations were obtained from J.T. Baker (Phillipsburg, NJ, USA). Amyloglucosidase protein standard, 3-(*N*-morpholino)propanesulfonic acid (MOPS) and *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulphonic acid) (HEPES) were obtained from Sigma. Water for all buffer and wash solutions was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All electrophoresis buffers were filtered through an Anotop 25 type 0.1 μm filter (Whatman, Maidstone, UK).

Recombinant Ad5 preparations with different transgenes were prepared at Berlex Biosciences using HEK 293 or PER.C6 packaging cells adapted to serum free medium and suspension culture, purified by anion-exchange chromatography and ultrafiltration. Unless otherwise noted, we used Ad5 with a growth factor (FGF-4) transgene in the CZE separations. The concentration of purified adenovirus was typically between 10^{10} and 10^{11} particles/ml, as measured by RP-HPLC [14]. The virus samples were stored frozen in phosphate-buffered saline (PBS)+2% sucrose+2 m*M* $MgCl_2$ and thawed before the analysis.

2.3. Procedures

A new capillary was conditioned by flushing with

water for 5 min. Between runs, the capillary was flushed with 60 m*M* HCl for 4 min by applying 13.8 kPa pressure.

All samples were filtered with Nanosep MF 0.45 μm micro-concentrators (Pall Filtron, Northborough, MA, USA). Unless otherwise noted, the samples were then dialyzed into 10 m*M* phosphate, pH 7.0 before CZE analysis using a 8000 M_r cutoff membrane in a Tube-O-Dialyzer (Geno Technology, St. Louis, MO, USA) with a 10 000-fold or greater volume excess and with a minimum dialysis time of 1 h.

Electrophoresis buffer (200 μl) was placed into the exit vial for the CZE fraction collection. The timing of collection was corrected for the time needed for the sample to reach the vial from the detector (approximately 1.14 times the migration time to the detector). In some cases, the voltage was reduced to 5 kV during the elution and collection of peaks. After collection, 20 μl 10×PBS and 20% sucrose was added to the vial. If the samples were not immediately used they were stored at −70°C.

Viral infectivity was determined in an endpoint dilution (EPD) format [15,16]. In short, HEK 293 cells were plated on a 96-well microtiter plate (Costar, Corning, NY, USA) at 5000 cells/well in 25 μl of 10% fetal bovine serum (FBS) (Hyclone Labs., Logan, UT, USA) in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY, USA). Typically, an initial dilution of virus was made based on expected titer followed by 10 twofold dilutions. The virus dilutions (replicates of 8) were introduced to the cell plate at 25 μl/well. The plates were placed in a 5% CO_2 incubator at 37°C. Additional 60 μl 10% FBS (Life Technologies) in D-MEM was added to the wells at 24 h and an additional 100 uL at 4–6 days. The wells were visually scored for cytopathic effect (CPE, cells rounded in appearance, some detached from surface) on day 10–12. The pattern of positive and negative wells was converted to apparent infectivity in infectious units per ml (IFU/ml) by statistical analysis [15,16]. The assay has been validated for precision, the standard deviation is ± 0.2 $\log_{10}$, corresponding to +58% to −37%.

For virus expansion, CZE peak fractions were collected and diluted in D-MEM supplemented with 10% FBS, then passed through 0.22 μm syringe filters (Millipore), and the filtrates were used to infect HEK 293 cells cultured in DMEM with 10% FBS in 12-well (22 mm diameter) tissue culture plates (Corning, New York, USA). When significant CPE was observed the remaining attached cells were dislodged by repeated pipetting. The resulting cell suspensions were exposed to two freeze–thaw cycles (−70°C, 37°C), and used to infect HEK 293 cells cultured in 225 cm^2 cell culture flasks (Costar). When CPE was significant, flasks were struck to dislodge remaining attached cells. Cell suspensions were centrifuged at 1000 rpm for 5 min in a microcentrifuge. Supernatants were removed by aspiration, cell pellets were washed with Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies), and cells were pelleted by centrifugation as before. Washes were removed by aspiration, cell pellets were resuspended in phosphate buffered saline with 2 m*M* $MgCl_2$, and 2% sucrose, and processed through two freeze–thaw cycles. The resulting cell lysates were then centrifuged at 1000 rpm for 5 min. The virus in the supernatant was purified using an analytical ion-exchange procedure [17]. In short, the samples were loaded onto a 1 ml Resource Q (Pharmacia Biotech, Uppsala, Sweden) column equilibrated in 50 m*M* HEPES, 300 m*M* NaCl, pH 7.5. The virus was eluted in a linear gradient to 50 m*M* HEPES, 600 m*M* NaCl, pH 7.5. The virus fractions (eluting between 400 and 500 m*M* NaCl) were collected.

Quantitative PCR was performed using the TaqMan Gold PCR kit (PE/ABI, Foster City, CA, USA), with the standard mix assembled according to manufacturer's instructions. PCR were run in duplicate on an ABI 7700 Sequence Detection System (PE/ABI) with each reaction containing 2 μl of viral stock solution. The primer and fluorogenic probe combinations were designed using Primer Express software (PE/ABI) to the FGF-4 transgene inserted into Ad5. The primers/probe sequences used for the FGF-4 transgene were: forward 5′-caagctctatggctcgccc-3′, reverse 5′-ggcgttgtagttgttgggaag-3′, and probe 5′FAM-accgatgagtgcacgttcaaggagattct-TAMRA 3′. Reactions were run in a total volume of 50 μl, with 10 pmol of each primer per reaction. Thermocycling was performed for 40 cycles at 95°C for 15 s, 63°C for 1 min, after an initial denaturation of the virus for 10 min at 95°C. Analysis was performed using SDS

software and relative concentrations determined using a standard curve constructed by sequential dilutions of FGF-4 DNA standards.

3. Results and discussion

3.1. Capillary Selection

A variety of CZE conditions were unsuccessfully tried with bare silica capillaries. No peaks were detected. The loss of virus was most likely due to adsorption to the silica surfaces. Using a PVA coated capillary, several peaks could be observed and therefore we selected this capillary type for further development. Fig. 1 shows a typical electropherogram obtained with a PVA coated capillary. The major peak at approximately 9–10 min and the minor, (''series'') peaks in the 7–9 min interval were consistently present in all batches of Ad5 tested independent of the transgenes present. The peak at approximately 3 min was determined to be a ''system'' peak. This was also present in the buffer blank runs. In some electropherograms we observed small, broad peaks at about 12 and 14 min.

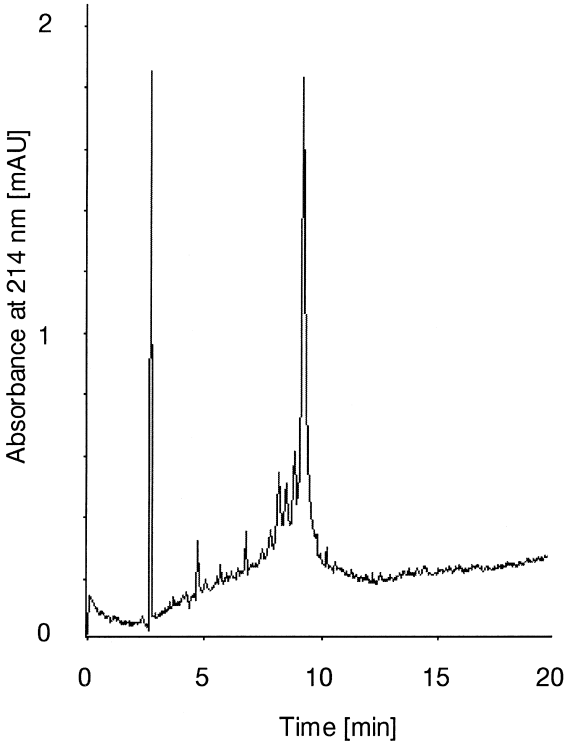

Fig. 1. Typical electropherogram of a recombinant adenovirus type 5 carrying FGF-4 transgene (Ad5FGF-4). Conditions: 57 cm (50 cm to detector)×50 μm PVA capillary with 3×bubble cell; electrophoresis in 25 m*M* sodium phosphate pH 7.0; 29.5 kV (518 V/cm) in reverse polarity; detection UV 214 nm; pressure injection for 30 s at 3.4 kPa; virus in 10 m*M* Tris pH 7, $5 \cdot 10^{10}$ particles/ml.

3.2. Recovery of adenovirus during CZE

The recovery of viral activity using the PVA coated capillary, as measured by the infectivity titer, was first determined in a separation experiment using a 27 cm capillary, applying low pressure (3.4 kPa), a separation voltage in reverse polarity (555 V/cm) and collecting the anionic and neutral fractions. The injected sample contained $3.8 \cdot 10^4$ infectious units (IU), the anionic and the neutral fractions had infectivities of $4.8 \cdot 10^4$ IU (126% recovery) and $3.9 \cdot 10^3$ IU (10% recovery), respectively. Within the error of the infectivity assay, these results suggest that adsorption of the virus to the capillary inner wall is very low (negligible) under the experimental conditions and that the anionic species are responsible for the infectious activity.

An experiment was conducted to assign viral activity to the peak(s) in the electropherogram. Eight CZE runs were performed using the same virus sample and fractions were collected for various lengths of time starting between 0 and 10 min. Fig. 2 shows the trace of the cumulative viral activity superimposed upon the electropherogram. Viral activity was found between 7 and 10 min where most of the UV peaks are located.

In an identical experiment, peaks in the 0–10 min interval were tested for their DNA content. The biologically active fractions were subjected to a quantitative PCR amplification. 84% of the injected viral DNA was recovered in the eluted fractions by this assay. Again, the fractions showing the most UV peaks contained viral DNA as determined by PCR.

An adenovirus concentration of 10^{11} particles/ml is equivalent to 12.9 μg protein II/ml (720 copies/virus [3], 108 000 M_r, [18]) and to 21.5 μg total protein/ml (protein II is approximately 60% of the protein content of the virus, [3]). We used the A_{214} corrected peak area of amyloglucosidase as a protein

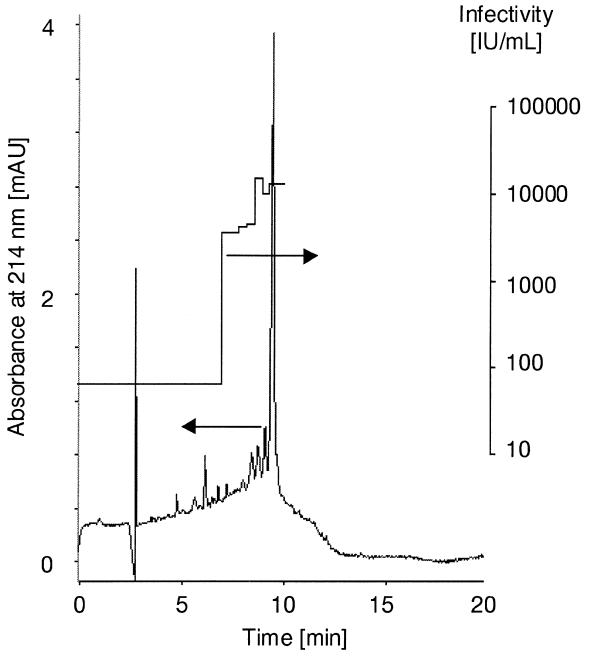

Fig. 2. Electropherogram of Ad5FGF-4 with superimposed cumulative viral activity of collected fractions. Separation conditions as in Fig. 1. Fractions collected and viral activity determined as given in section 2.3.

calibration standard to get an approximate quantitation of the virus in the main peak and the ''series'' peaks. The absorbance of a protein at 214 nm is primarily determined by the number of amide bonds [19]. In this approximation, the contribution of some amino acids and the viral DNA to the absorbance at 214 nm is omitted. The amyloglucosidase protein migrates mainly as a single band at about 10.5 min under the same experimental conditions as used for the virus. An adenovirus sample with a concentration of $5 \cdot 10^{10}$ particles/ml, determined by a RP-HPLC analysis [14], was analyzed by CZE in triplicate. Calculating the corrected peak areas (main and series peaks), the protein based estimates by the CZE for the virus concentration were $5.9 \cdot 10^{10}$, $2.4 \cdot 10^{10}$ and $6.4 \cdot 10^{10}$ particles/ml (average $4.9 \cdot 10^{10}$ particles/ml, standard deviation $\pm 2.2 \cdot 10^{10}$).

The recovery of the virus for the critical sample preparation steps: filtration and dialysis was determined independently. Virus peaks in the electropherograms of filtered and unfiltered samples were found to be indistinguishable (data not shown) indicating no detectable loss of virus. (Filtration was required for most samples to remove particles which resulted in noise spikes in the electropherograms.) We obtained a virus recovery of 87% for the dialysis step using RP-HPLC [14] to measure virus concentration.

These results indicate (a) high recovery of the intact virus during CZE with PVA coated capillary, including sample preparation and (b) separation of pure virus peaks (virus isoforms) appearing in the 7–10 min region of the electropherogram. The electropherogram did not show any major UV peak that could not be associated with viral activity.

3.3. Optimization of the CZE separation

Several experiments were performed to establish optimal pH and ionic strength of the electrophoresis buffer and optimal sample buffer composition. We evaluated peak efficiencies and corrected peak areas (relative recoveries).

Electrophoresis buffer concentrations were varied from 5 to 50 m*M* at pH 7.0. We selected electrokinetic injection of virus from 20 m*M* MOPS, pH 7.0 in order to eliminate the effect of varying sample ionic strength. Fig. 3 shows the expected linear relationship between the electrophoretic mobility of the main virus peak and buffer concentration. Increasing ionic strength is expected to mask the surface charge on the virus and to decrease mobility [20]. Since the inner wall is neutral, the endoosmotic flow is expected to be very low in this capillary.

No change was observed in the pattern of the viral peaks between buffer concentrations 5–35 m*M*, while significant peak broadening was observed at 50 m*M* (data not shown). This may be explained by the detected increase in the separation current to 65–70 μA where Joule heating could become significant. The corrected peak area of the major peak increased with increasing buffer concentration between 5 and 25 m*M* and remained stable between 25 and 50 m*M* likely indicating adsorption of the virus to the capillary wall at lower ionic strengths (data not shown). This behavior is characteristic of large proteins [21]. The recovery of the minor virus peaks as measured by the corrected peak areas showed the same behavior as the main virus peak in response to the varying ionic strength (data not shown).

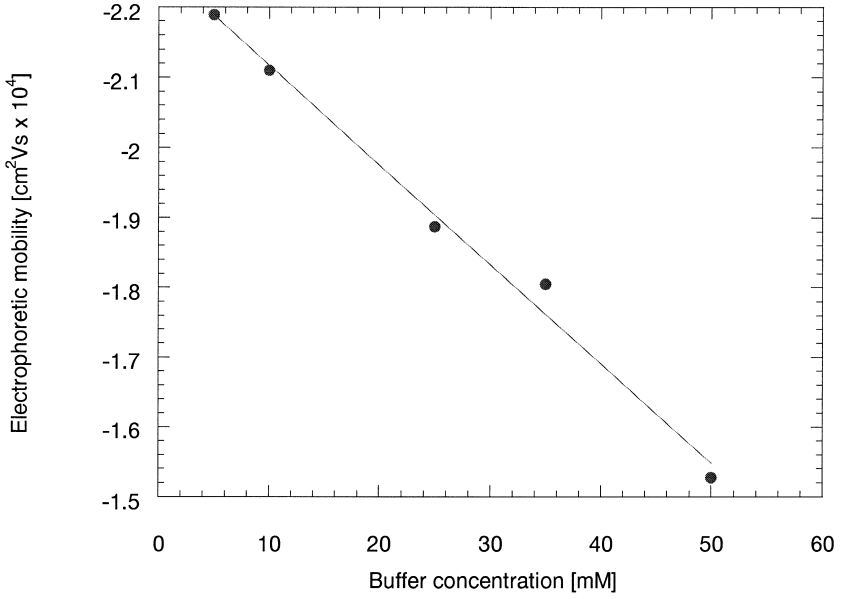

Fig. 3. The dependence of the electrophoretic mobility of the main adenovirus peak on the concentration of the electrophoresis buffer (phosphate pH 7.0). Virus was electrokinetically injected at 10 kV for 60 s from 20 m*M* MOPS. Other conditions as in Fig. 1.

The effect of pH on the electrophoretic mobility of the main virus peak is shown in Fig. 4. Below pH 5.8 no virus signal could be detected. This is consistent with the expectations based on published pH stability data of the virus: at this pH the adenovirus spontaneously dissociates [22]. There is a small increase in the electrophoretic mobility with increasing pH between 5.8 and 8.3 and the mobility

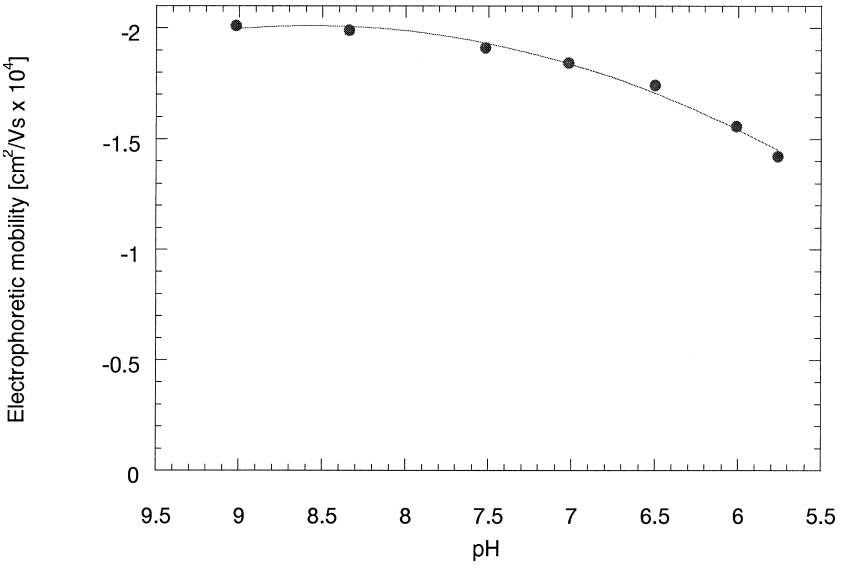

Fig. 4. The pH dependence of the electrophoretic mobility of the main adenovirus peak in 25 m*M* phosphate buffer. Virus was electrokinetically injected at 10 kV for 60 s in 20 m*M* MOPS. Other conditions as in Fig. 1.

stabilizes between 8.3 and 9.0. This behavior is consistent for a virus particle charged negatively at around pH 6.0 and above. The acid labile nature of the virus did not allow the assignment of an apparent isoelectric point (p*I*) value in this assay. The data only suggest that it is below the lowest valid experimental point of pH 5.8. If the p*I* were 5.8, the virus should be nearly completely deprotonated at pH 8.3 and therefore no further change in the electrophoretic mobility would be expected. Based on these data, we selected pH 7.0 for routine CZE separations.

Experiments were performed using different dialysis buffers for sample preparation: Tris, tricine and sodium phosphate (all 10 m*M* pH 7.0). The mobility of the major and minor virus peaks and their relative intensities were found to remain constant. Reducing the concentration of the Tris buffer to 5 m*M* gave identical results(data not shown). Increased buffer concentrations were not tried because less efficient sample stacking would occur and lead to lower resolution [23]

Based on the results of the optimization experiments, we selected 10 m*M* Tris, pH 7 for virus dialysis and 25 m*M* phosphate, pH 7 as electrophoresis buffer for routine CZE (see Experimental section). Typically, an efficiency of 20 000 plates/m was observed for the main virus peak.

3.4. Origin of the minor virus peaks (series peaks)

An important outcome of the optimization experiments was also the finding that the relative intensities of the major and minor virus peaks remained stable over a wide range of conditions during sample preparation and electrophoresis. This serves as indirect evidence that the observed putative viral isoforms were present in the original sample and were not artifacts of the analytical procedure. If one or more of the investigated parameters caused the distribution between the major and the minor virus peaks, then modification of the experimental parameters should have resulted in variable CZE profiles.

We investigated if the apparent heterogeneity of the virus samples is caused by (a) clonal or (b) by phenotypical reasons (surface modifications). CZE fractions were collected in three segments. The first two segments contained groups of minor virus (series) peaks while the last segment contained the major peak. The viral fractions were amplified to approximately 10^{12} particles, purified by ion-exchange chromatography and then immediately prepared for CZE separation. The electropherograms of all amplified virus fractions were identical showing a single peak with an electrophoretic mobility equal to the main peak in the parent sample (Fig. 5a). Only negligible traces of minor viral (series) peaks were present on the baseline of the electropherogram. These data strongly suggested that the minor virus (series) peaks were not the result of clonal differences in the parent virus samples and that the minor virus peaks must be the result of modifications caused by events after virus purification.

Since the virus preparations were always frozen between the purification and CZE analysis in our previous experiments, an aliquot of freshly purified virus in the ion-exchange eluent buffer (approximately 540 m*M* NaCl, 50 m*M* HEPES pH 7.5) was cryoprotected by the addition of sucrose to a final concentration of 2%. The sample was frozen to −70°C overnight, and then thawed at room temperature immediately prior to CZE analysis. The electropherogram of this virus preparation (shown in Fig. 5b contained the minor (series) viral peaks. This experiment was repeated several times with identical results.

The integral shift in electrophoretic mobility among the different minor (series) peaks suggests that discrete charge/size modifications had to occur to the virus surface. One would have expected that arbitrary damage to the capsid surface would have resulted in multiple species producing broadening of the virus peak in the electropherogram. The nature of these specific modifications is subject to further investigation in our laboratory.

4. Conclusions

A CZE method for the analysis of intact adenovirus has been demonstrated. The use of a coated capillary eliminates the need for detergent additives in the electrophoresis buffer. Adenovirus was separated and collected without loss of infectivity. The system resolution and efficiency are high enough to allow the observation of new viral peaks appearing

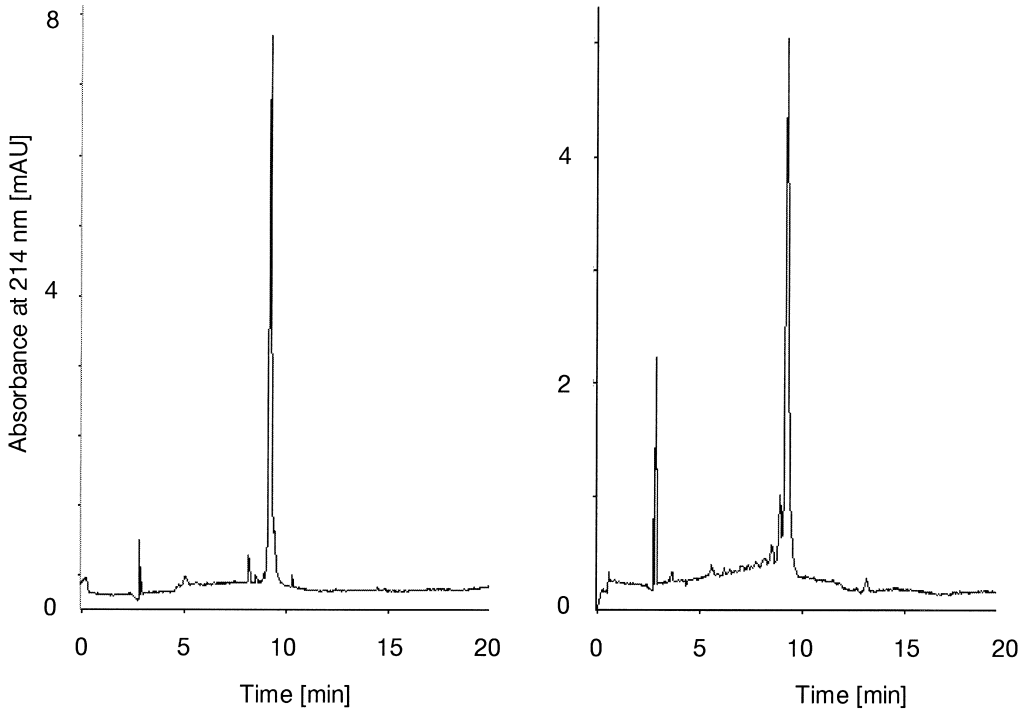

Fig. 5. Electropherograms of Ad5FGF-4 (a) freshly isolated from cell lysate and (b) freeze–thawed from −70°C. Separation conditions as in Fig. 1.

after a freeze–thaw cycle indicating discrete (not yet characterized) modifications to the viral surface. This fingerprint, although not understood, is a useful tool for the comparison of virus lots and the development of new cryoprotectant formulations.

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