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Journal of Chromatography B, 675 (1996) 17–25

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Antibody fragment separations by capillary zone electrophoresis☆

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First received 2 February 1995; revised manuscript received 10 July 1995; accepted 28 July 1995

Abstract

This study investigated methods of improving the separation and identification of an IgA antibody, McPC603, and its pepsin fragments. The problem presented by purification of antibody fragments (Fabs) and the antibody light chain required accurate and informative analysis of highly hydrophobic proteins, which can polymerize and fold to form secondary structures. Capillary zone electrophoresis (CZE) permits the separation of peptides and small proteins by a method which is orthogonal to the traditional method of reversed-phase HPLC. To facilitate planned studies of the antibody's biological activity, our buffer composition was kept as simple as possible. During CZE analysis, if the buffer pH is below the isoelectric point of the protein, or the protein is large (with a heterogeneous distribution of surface charges), it can irreversibly bind to the capillary wall unless the capillary is coated. We found that C_1-coatings in RP-capillaries at pH 9.5 adequately prevented the antibody fragments from binding to the wall. However, the coating did not remain stable at such high pH, so different conditions were sought. We achieved adequate separations in several buffers at nearly physiological pH, in a bare silica capillary which had been coated once with a soluble cationic polymer coating (Micro-Coat applied during column conditioning). Antibody electropherograms changed depending on the type of inorganic buffer salt used in a separation. Phosphate binds to the antigen-binding site of the IgA with low affinity, and interesting effects were observed in separations using phosphate buffer. These effects will be discussed.

Keywords: McPC603; Immunoglobulin A antibody; Antibodies; Pepsin fragments

1. Introduction

Antibodies are β-barrel type proteins with many hydrophobic residues, a tendency to aggregate, and basic N-terminal regions [1–3]. These characteristics present significant difficulties when free-solution CZE of antibodies and other serum proteins is attempted in unmodified capillaries of fused-silica. Such difficulties include lack of migration due to absorption and a lack of reproducibility. Diverse methods to overcome these difficulties in free solution have been proposed in the literature and they include: no additives, which may involve coating the capillary with the protein itself [4,5], organic solvent

☆ Presented at the 1994 Frederick Conference on Capillary Electrophoresis, Frederick, MD, October 25–26, 1994.

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0378-4347/96/$15.00
SSDI 0378-4347(95)00328-2

additives [6], and ion-pairing reagents [7]. Literature reviews of this problem emphasize that no single method is completely satisfactory in every situation [8,9].

This study examined and compared several methods which allowed the separation of an intact IgA antibody, McPC603, the antibody heavy and light chains, and its pepsin Fab fragments [10] by CZE. To facilitate planned studies of the antibody's biological activity, our buffer composition was kept as simple as possible. The pH–activity profile of McPC603 [11] provided a target range for electrophoresis buffers. Choice of a particular separation buffer and buffer concentration, antibody concentration, and the separation pH was rarely straightforward. The advantage of CZE over competing analytical methods is the small sample volume requirement (7 nl), quantitative detection (UV), and quick speed of the run (5–10 min). Another advantage to CZE separations is the large numbers of different buffer conditions which may be explored in a timely manner.

The McPC603 antibody used in our work was isolated from the ascites fluid of BALB/c mice using the method of Rudikoff et al. [10], who initially solved the crystal structure of the McPC603 Fab fragment. The availability of this, and a later high-resolution structure [3], made McPC603 an ideal antibody for synthetic and modeling studies. This antibody has an unusual structure, typical of murine IgA [1–3], where there are no covalent bonds between the heavy (H) and light (L) chains (Fig. 6). McPC603 is a phosphorylcholine (PC)-binding murine myeloma antibody which was isolated by PC-affinity chromatography. McPC603, the most abundant protein in the ascites fluid, was proteolytically cleaved into more manageable Fab fragments for our studies.

CZE permits the separation of peptides and small proteins by a method that is orthogonal to traditional methods of HPLC [12]. The results of a free-solution CZE separation of the antibody were expected to be analogous to those obtained by native gel electrophoresis. Key considerations in any CZE separation include pH, temperature and buffer effects on the stability and solubility of the protein; protein charge and the charge/volume ratio at a given pH; and the tendency of the protein to form aggregates [13]. Inorganic buffers at higher pH values and the use of coated capillaries allowed elution of the antibody, yet it was necessary to check for adsorbed protein. Post-run washing of the column with dilute alkali controlled for adsorbed protein. Several surfactant- and organic solvent-based approaches to avoid aggregation and precipitation of difficult proteins have been developed [6,7], but our concern about solvent effects on the antibody conformation led to the choice of Micro-Coat, a column-conditioning reagent. Micro-Coat was not used according to the suggested protocol, but only to coat the column initially and prevent the protein from sticking to the capillary wall (repulsion of positive charges) [14]. It was not added later, between runs.

An important issue to be considered in macromolecular CZE separations was how to identify the peaks once the separation was achieved. Choosing effective buffer conditions for a macromolecule such as McPC603 antibody, utilized a simplified model of the titration behavior of polypeptides [15]. This model was also tested as a way to predict elution order. This simple calculation of first-order theoretical titration curves, which ignored both interactions between ionized groups and solvation effects, aided in the interpretation of our electropherograms. Unexpectedly, different buffers caused different elution patterns for the same sample. The effect of buffers which bind to the antibody-binding site was explored.

2. Experimental

2.1. Chemicals

McPC603 tumor was the kind gift of M. Potter (National Institutes of Health, Bethesda, MD, USA). IgA antibody was quantitated by radial immunodiffusion performed on gels impregnated with sheep anti-murine IgA (The Binding Site, Birmingham, UK). Immobilized pepsin was 2–3 mg/ml on 6% cross-linked beaded agarose

(Pierce, Rockford, IL, USA). Micro-Coat was obtained from Applied Biosystems (Division of Perkin-Elmer, Foster City, CA, USA). Buffer salts were J.T. Baker analyzed. Electrophoresis buffers were dissolved in water that had been purified by reverse osmosis to a resistivity less than 18 MΩ/cm, and then were filtered through a membrane syringe filter (0.2 μm pore size) immediately prior to use.

2.2. Apparatus

Slab gel electrophoresis was performed on a PhastSystem (Pharmacia, Uppsala, Sweden). Capillary electrophoresis was performed on an ABI 270A instrument, (Applied Biosystems) under positive polarity at 30 kV. Capillaries were 50 μm I.D. 60–72 cm, C_1-coated fused-silica capillaries (CElect H50, Supelco, Bellefonte, PA, USA); or bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) conditioned for 30 min with 1 *M* NaOH, followed by a water wash (30 min), then conditioned again with Micro-Coat (ABD), followed by a wash with the separation buffer for 1 h. The columns were always washed with 0.1 *M* NaOH (2 min) and buffer (3 min) prior to each run. Sample detection was by UV (220 nm) through a window at 40–52 cm from the origin (anode) burned through the polyimide coating with a lighter (20 cm from the cathode, cleaned with ethanol). Dialysis of high salt samples was performed on Spectra/Por 3 membrane discs (Spectrum Medical Industries, Houston, TX, USA). Ultrafiltration required a nitrogen pressure-driven 3 or 50 ml stirred cell (Amicon, Beverly, MA, USA) and YM-1 membranes (25 and 43 mm, MWCO ≈ 1000, Amicon).

2.3. Preparation and digestion of McPC603 (IgA)

As described by Rudikoff et al. [10]; in a cold room, at 8°C; reduced and alkylated McPC603 antibody (1 mg/ml in ascites fluid from pristane-primed female BALB/c mice, which had been inoculated i.p. with M603 tumor) was purified by affinity chromatography in 0.2 *M* sodium borate with 0.16 *M* NaCl, pH 8.46; the buffer exchanged by ultrafiltration to 0.15 *M* Tris, 0.15 *M* NaCl, 2 m*M* EDTA, pH 8.0; reduced and alkylated again with iodoacetamide; concentrated by ultrafiltration to approximately 0.5–2 mg/ml; buffer exchanged into 20 m*M* acetate pH = 4.5; and then digested with immobilized pepsin at 37°C in a shaking water bath. The reaction was monitored by CZE after 0, 15, 30, 60, 90, 120, 180, 240 and 480 min by removing an aliquot, adding an equal volume of 10 m*M* Tris pH 7.5 to stop the digestion, and centrifuging the sample to remove the beads. CZE and traditional sodium dodecyl sulfate (SDS), denaturing polyacrylamide gel electrophoresis (PAGE) with β-mercaptoethanol on the Phastsystem were performed on the supernatant. The enzyme–substrate ratio was varied from 1:34 to 1:300 w/w (based on the Pierce calibration of the immobilized pepsin and assuming that 1 AU at 280 nm approximates 1 mg/ml of the IgA monomer [10]).

2.4. Separation and identification of Fab fragments

For preparative purposes, a sample prepared as described above was digested at an enzyme–substrate ratio of 1:34 (performed as described above) in 0.1 *M* acetate pH 4.5 for 2 h. The mixture was then applied to a Sephadex G-150 column (83.5 cm × 1.5 cm I.D., exclusion volume 49 ml by Blue dextran) and eluted with a buffer consisting of 20 m*M* potassium phosphate, 300 m*M* sodium chloride and 5 m*M* EDTA at pH 7.4. The three peaks which eluted (A, 48–77 ml; B, 82–100 ml; and C, 120–160 ml) were pooled separately and concentrated by ultrafiltration at 8°C to at least 0.5 mg/ml. For mass spectrometry, the buffer was exchanged by ultrafiltration into 100 m*M* ammonium carbonate pH 7.96.

2.5. Capillary electrophoresis

To allow facile monitoring of the electroosmotic flow (EOF), a neutral marker (0.1 s, mesityl oxide or benzyl alcohol) was co-injected with the sample (1 s, antibody or fragment). Vacuum injection provided acceptably reproduc-

ible peak heights, and resulted in a complete electropherogram of all the components present in a given sample. Based on the extinction coefficient of the Fab fragment ($6.7 \cdot 10^4\ M^{-1}$ [10]) and the total UV absorbance of the sample at the detector, a 1-s vacuum injection introduced approximately 7 nl of the McPC603 Fab into the capillary. This assumes no loss due to antibody absorption on the capillary walls.

To achieve the most efficient separation, we found that a low buffer concentration (usually 20 m*M* borate) allowed the protein sample to carry most of the current, and thus elute faster. A low buffer concentration also allowed the analyst to use high voltage (30 kV, 417 V/cm) with low current (8 μA), thereby increasing the resolution [12]. The presence of large amounts of salt in the sample attenuated the effective electric field strength acting on the antibody. If the salt peak was very large, the protein was desalted to obtain reproducible elution times and sharp peaks. Samples were either desalted by placing a small drop (20 μl) on a piece of dialysis membrane, and floating the membrane in water, for 1 h, or by diluting the sample with water (1:1). However, when the concentration of salt and/or McPC603 antibody in the sample became too low, the chains separated and the heavy chain precipitated. Once successfully introduced into the capillary, the sample seemed to remain in solution (no adsorbed protein, reasonably quantitative peaks were observed) even though the salt concentration in the buffer was low.

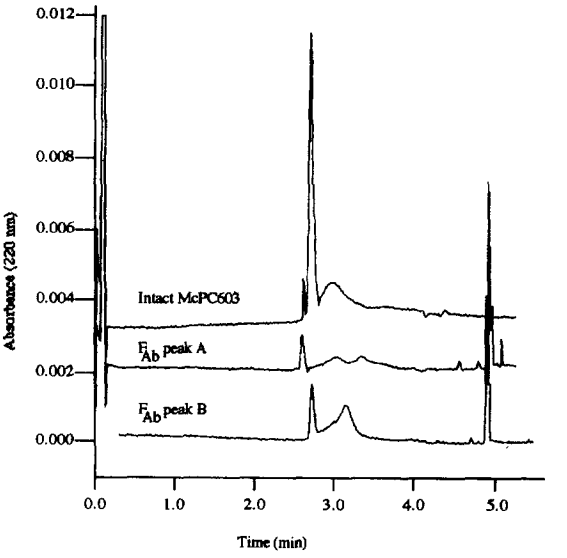

Fig. 1. Capillary electropherograms showing the observed peaks isolated after a pepsin digestion of monomeric McPC603 antibody (IgA, reduced and alkylated). Supelco CElect, C_1-coated capillary, 72 cm total length, 46.4 cm to detector, 20 m*M* sodium cacodylate buffer, pH 7.76, 30 kV (417 V/cm), 20 μA; detector wavelength 220 nm; neutral marker (mesityl oxide), eluted at 2.62 min followed by antibody peak(s).

3. Results and discussion

The techniques and conditions described successfully caused migration of an IgA antibody and its fragments (Fig. 1). The resulting electropherograms provided helpful information about the results of several pepsin digests of the antibody using immobilized pepsin at different enzyme–substrate ratios (Fig. 2). The utility and sensitivity of CZE in measuring binding phenomena was illustrated by a comparison of the results of separations performed in phosphate buffer with those in other buffers (Figs. 3 and 4).

In our preliminary experiments, basic proteins, such as the synthetic and natural N-termini of antibodies, were irreversibly retained in bare fused-silica capillaries when the buffer pH fell below their p*I* values. The surfaces of large proteins are heterogeneous in nature and at different moments a region of high hydrophobicity or high basicity might interact with the wall of the capillary. For instance, the N-terminal variable region of McPC603 (V) contains a higher percentage of basic amino acids than the constant region of the antibody (C) [1]. To complicate matters further, this Fab fragment's heavy and light chains are not covalently joined and are known to dissociate ($k_d = 1 \cdot 10^{-6}\ M$, for the variable region fragments) [16]. Also of

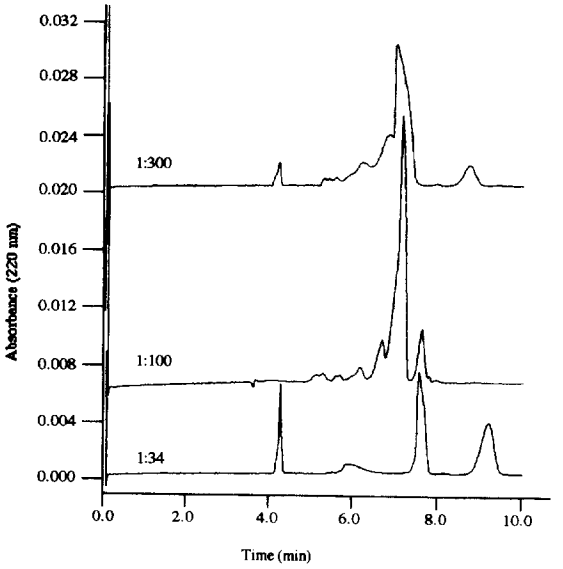

Fig. 2. Capillary electropherograms showing the observed peaks from injection of crude, quenched reaction mixtures from three different pepsin digestions of monomeric McPC603 antibody (IgA, reduced and alkylated), run at three different enzyme–substrate ratios. Supelco CElect, C_1-coated capillary, 60 cm total length, 40 cm to detector, 20 m*M* sodium borate buffer, pH 9.51, 30 kV (500 V/cm), 50 μA; detector wavelength 210 nm; neutral marker (mesityl oxide), eluted at 4 min followed by antibody peak(s).

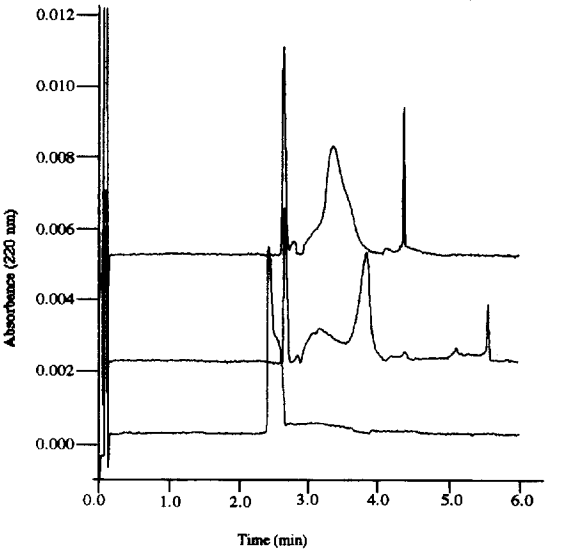

Fig. 3. Capillary electropherograms showing a purified Fab peak A sample under different buffer conditions; 30 min, Micro-Coat-coated capillary, 72 cm total length, 50 cm to detector; 30 kV (417 V/cm); detector wavelength 220 nm; 20 m*M* sodium cacodylate, pH 7.60, 15 μA; 20 m*M* sodium phosphate, pH 7.62, 33 μA; and 150 m*M* sodium borate, pH 7.67, 6 μA; neutral marker (benzyl alcohol), eluted at 2.44–2.68 min followed by the antibody peak(s).

concern was maintenance of an active, correctly folded antibody-like structure under the separation conditions so that, during the time of the separation (approx. 10 min), only one form of each species was present.

Assignment of peaks was facilitated by modeling based upon the known primary amino acid sequence of McPC603 [1]. By comparing the calculation of idealized titration curves for McPC603 and a Fab fragment containing the entire light chain and residues 1–222 (or 1–300) of the heavy chain (Fig. 5), two conclusions may be drawn: (1) that the intact chain has a lower isoelectric point (5.5) than the possible Fab fragments (≈6.1); and (2) that the difference in charge between the intact and digested antibody is greater above pH = 8, than in the physiological range (pH 6–8). Similar plots predicted that the dissociated light chain would have a p*I* of 6.26 and the potential heavy chain fragment would have a p*I* of 5.9 (not shown).

In these positive polarity CZE experiments, the positively charged species would elute first, followed by a sharp band of neutral species and the negatively charged species would elute later. Small anionic species such as chloride and acetate ions, which have a large charge/volume ratio, eluted last (>10 min). The negatively charged antibody actually electrophoretically migrated against the EOF in a positive polarity CZE experiment, but were dragged past the

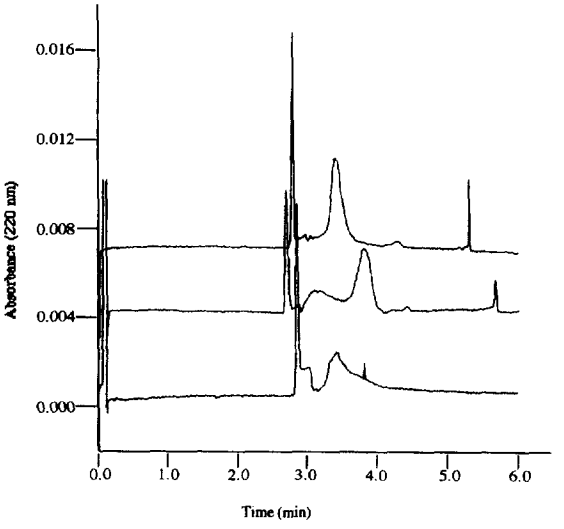

Fig. 4. Capillary electropherograms showing the same Fab peak A with salt added to raise the conductivity under several buffer conditions; 30 min, Micro-Coat-coated capillary, 72 cm total length, 50 cm to detector, 30 kV (417 V/cm); detector wavelength 220 nm; 20 m*M* sodium cacodylate, 40 m*M* NaCl, pH 7.78, 67 μA; 20 m*M* sodium phosphate, 3 m*M* NaCl, pH 7.99, 42 μA; and 200 m*M* sodium borate, 47 m*M* NaCl, pH 8.03, 107 μA; neutral marker (benzyl alcohol), eluted at 2.70–2.86 min followed by the antibody peak(s).

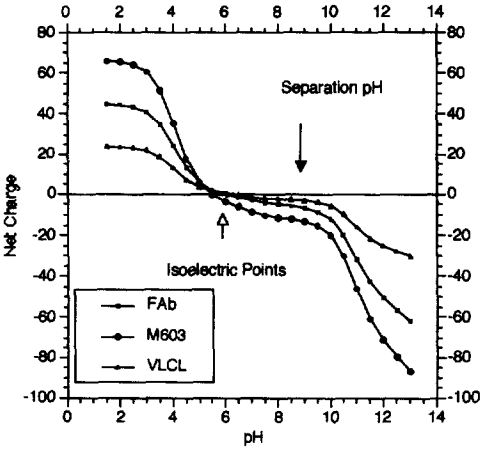

Fig. 5. Idealized calculation of titration curves for intact and pepsin cleaved McPC603 [13]

detector by the EOF. We found this orientation convenient, because it allowed all species present in the sample, positive, neutral and negative, to be viewed in one quick run. Experiments were run above the isoelectric points of all the proteins, so the first protein peak eluted after the neutral marker. An injection of mesityl oxide alone measured the EOF under the separation conditions.

The CZE methods development proceeded as follows: to interact with an electric field, the peptide must be charged at the pH of the separation. Practically, large peptides carrying a net negative charge resulted in sharper peak shapes and more predictable separations. For example, in the separation of two different peptides, it was helpful to plot the theoretical titration curves of the two peptides, and choose a pH above the isoelectric point (p*I*). In addition, by choosing a pH where the charge difference between the two species was expected to be greatest (Fig. 5), the resolution could be enhanced. Observed peaks in the electropherogram were then tentatively identified by the predicted charges on the peptides.

The antibody was initially purified in a high salt buffer (0.2 *M* borate, 0.15 *M* NaCl) to reduce adsorptive loss and aggregation. When such samples were introduced into the capillary, the excess salt caused peak distortion, and the result was irreproducibility in peak shape and elution times. In samples containing excessively high salt, small anions usually eluted as a broad band or saw-tooth near the end of the electropherogram. It proved advantageous to remove as much salt as possible before analysis. In some cases, desalting was not necessary; for instance, after digestion of the antibody with pepsin, quenched samples contained 20 m*M* acetate and 10 m*M* Tris, well within the acceptable range for

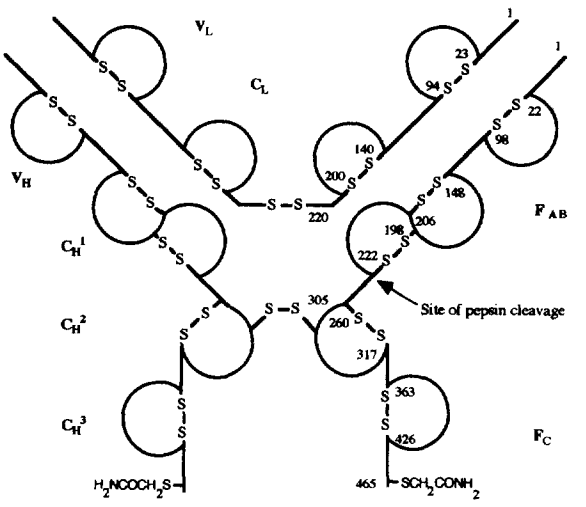

Fig. 6. Representation of McPC603, a prototype murine IgA based on sequencing information [1]; dotted lines are interstrand disulfide bonds, the arrow shows the location of pepsin cleavage. After pepsin digestion the heavy and light chains are held together by noncovalent forces only.

CZE (Fig. 2). The same fragments after Sephadex G150 purification were high in salt (20 m*M* potassium phosphate, 0.15–0.3 *M* sodium chloride) but gave acceptable results when desalted on membranes, or when diluted slightly with water (Fig. 1).

The ionic strength of the buffer determined the current in the capillary under constant voltage conditions. All runs were performed at 30 kV for maximum theoretical resolution [12]. Sodium chloride was added in one set of experiments to bring the conductivity up to a common level, to facilitate comparisons between different buffers. A comparison of buffers with and without sodium chloride is shown in Figs. 3 and 4. In the borate buffer with increased salt, the current increased, the EOF slowed down, and the electrophoresis component of the mobility (opposite to the EOF in the case of the negatively charged antibody) provided separation. As the salt concentration is increased, the separation time will also increase.

McPC603 Fab fragments did not elute from an uncoated capillary below pH 7, so the pepsin digests were initially compared in C_1-coated capillaries (Figs. 1 and 2). Post-run washing with 0.1 *M* NaOH occasionally eluted additional material. Comparison of electroendoosmotic flow (EOF) between coated and uncoated capillaries indicated that the coating was lost after a few runs. Later, the results showed that capillaries without any coating except for an initial wash with Micro-Coat may be used with antibody samples (Figs. 3 and 4). This effect was discovered inadvertently, after a capillary had been once used for Micro-Coat separations was used in the antibody work.

It appears that some residual coating is left in the capillary even after repeated washing with 0.1 *M* sodium hydroxide. The direction of the EOF appears unchanged, as the neutral marker still elutes under positive polarity. The reproducibility of this remarkable effect was confirmed by observing an equivalent sharpening of the antibody peak shape after washing a different capillary with Micro-Coat, and comparing it to previous runs (Figs. 1, 3 and 4). Results of runs with neither the C_1-coating nor the pre-coat with Micro-Coat, were not as reproducible, nor was the peak shape as good. The strength of the attractive forces between the cationic polymer and the capillary wall may have prevented all of the polymer from being removed. This effect merits further study as it may provide an inexpensive method of reducing protein-wall interaction.

To monitor the digestion of monomeric (partially reduced and alkylated) McPC603 by pepsin, which had been immobilized on agarose beads, the product Fab fragments were analyzed by SDS polyacrylamide gel electrophoresis, under reducing conditions. The product fragments were found to have different molecular masses than those reported from a non-immobilized pepsin digest [10]. Much of the heavy chain remained undigested after 8 h, and the digestion reached equilibrium by 15 min after enzyme addition. To quantitate the products of digestion,

and to study the effect of immobilized pepsin concentration on the resulting mixture, we developed a CZE method which separated the various digestion products (Fig. 2) at high pH with a coated capillary. By washing the capillary after each run (0.1 *M* sodium hydroxide), we established that no residual protein adhered to the capillary wall under these conditions (pH 9.51). Three different ranges of enzyme to substrate ratios were examined: low (1:300), medium (1:100), and high (1:34).

As demonstrated in the electropherogram in Fig. 2, increasing the ratio of immobilized pepsin to McPC603 monomer in our digestion, gave different results. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of β-mercaptoethanol showed indistinguishable bands in all three cases. This example illustrates the utility of CZE in monitoring biological processes. Our initial efforts showed that a buffer pH = 9.5 gave the best resolution of the various products of pepsin digestion. A 20 m*M* sodium borate buffer provided optimal buffering capacity in this pH range with a minimal background UV absorbance. The low range, 1:300 pepsin digests, gave incomplete digestion of the heavy chain; the leftover intact antibody caused a broadening of the product peak. In the medium range, 1:100 digest, peak sharpening indicates increasing amounts of the desired product. In the high range, 1:34 digest, intact antibody has completely disappeared, and the preparation has begun to form a new product as well as the desired one.

The successful separation of digestion products by CZE allowed monitoring of the various enzymatic digests and was also used to inform the purification process of the resulting Fab fragments. In return, a combination of modeling and analysis of the purified fragments helped to identify the CZE peaks. Electropherograms of the starting material and purified fragments from a digest in the high range (1:34) are shown in Fig. 1. Comparison of the three fractions allows a tentative assignment of the first peak eluting after the neutral marker to undigested antibody.

The "intact McPC603" is monomeric (partially reduced and alkylated, so that it only binds one mole of antigen per mole of antibody), and "Fab peak A" eluted before "Fab peak B" on a size exclusion column (Sephadex G150). Both intact McPC603 and Fab peak A exhibited binding activity by affinity chromatography; Fab peak B did not. Dissociation of the H and L chains would explain the loss of binding activity in peak B. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry MALDI of a sample of Fab peak B gave $M_r = 24{,}130.7 \pm 5$ $[M + H]^+$. This observed relative molecular mass corresponds exactly to that of the intact McPC603 light chain plus one mole of acetamide (used to alkylate Cys 220). These results showed Fab peak B to be due to dissociated light chain and thus that particular single peak in the electropherogram was positively identified (Fig. 1). In other experiments, purified, dissociated heavy and light chains were somewhat insoluble and often precipitated. One of the other, minor peaks in the peak B electropherogram may have been due to the dissociated heavy chain which had mostly precipitated. Alternatively, the heavy chain may have co-eluted from the Sephadex column with the Fab fragment in peak A. This could explain the third peak which eluted after the neutral marker and the Fab fragment in the electropherogram of peak A.

Electrophoretic mobility is a function of the charge to mass ratio of an analyte [17]. The modeling in Fig. 5 indicates that at the pH of the separation (7.8) the intact antibody should carry the greatest negative charge (p*I* = 5.5), followed by the digested heavy chain (p*I* = 5.9, not shown), the Fab fragment (p*I* = 6.1) and the light chain (p*I* = 6.3). The expected elution order based upon charge would be light chain, Fab fragment, heavy chain with intact antibody last. This is the situation observed in isoelectric focusing gel electrophoresis. In CZE, however, the mobility due to EOF is much larger than the electrophoretic mobility, and the observed elution order predominantly reflects the molecular size. Size predicts the elution of intact antibody, followed by the Fab, and then the heavy and light chains. The elution order of the similarly-sized heavy and light chains in the electropherograms of peaks A and B indeed reflects the

predicted order due to charge, with the more basic light chain eluting first.

Upon comparison of several different buffers, phosphate buffer gave a noticeably different result. In Fig. 4, a comparison of the cacodylate and phosphate runs, shows that the antibody peak shifted to a larger negative electrophoretic mobility in phosphate buffer. Antibody binding to the negatively charged phosphate anion explains this change in mobility, and demonstrates a buffer effect that is highly specific. The fraction of the sample that did not bind was adequately separated from the peak with binding activity. This preliminary result will be explored more fully.

4. Conclusions

In summary, explorations of the CZE technique with McPC603 antibody and its pepsin Fab fragments show how useful the technique can be. The separation of a large protein, containing several subunits, presents myriad difficulties, and the results of such a separation must be carefully considered. As shown by our preliminary experiments, the structure (size), charge, and association behavior of an antibody and its individual subunits (H and L) caused explicit, interpretable changes in the pattern of the CZE electropherogram. CZE was invaluable for monitoring the progress of a pepsin digestion of McPC603 antibody and the purification of the resulting Fab fragments, as very little sample was used in the analysis. CZE has proven invaluable to our research efforts towards synthetic antibodies because the UV detection provides easy quantitation of the different peaks. Correlations were observed between isolated antibody fractions which bind to affinity columns and the presence of peaks in the electropherogram which shift in phosphate buffer. This effect will allow facile determination of low-affinity binding constants and will be explored further [18].

Acknowledgments

The author thanks Professor Bruce Merrifield for inspiration, advice and helpful discussions; Dr. Klaus Schneider for the MALDI mass spectrum of McPC603 light chain; Dr. Michael Potter for kindly providing M603 tumor; Professor Milos Novotny for suggesting the dialysis membrane desalting technique; and Ken Rotondi for assisting with data collection. This work was supported by a grant from the US Public Health Service (DK01260).

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