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Purification of antibody Fab fragments by cation-exchange chromatography and pH gradient elution

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Abstract

The use of a pH gradient as opposed to conventional salt gradient for elution in cation-exchange chromatography was explored. pH gradients were found to be very effective in separating Fab fragments and other proteins with differences in isoelectric point as low as 0.1. To determine the efficiency of purification, the separated peaks were collected and further analyzed by capillary electrophoresis.

1. Introduction

With the increasing use of immunoaffinity chromatography, the separation and purification of antibodies has been an ongoing challenge. In addition, the recent work on capillary electrophoresis (CE)-based immunoassays have stretched the need for highly purified antibodies or antibody fragments [1–4]. To improve sensitivity and specificity in CE, either the antibody or the antigen is labeled with a dye. Prior to labeling, it is desirable that the antibody be purified since the various isoforms that recognize the antigen will generally all be labeled. The micro heterogeneity of an antibody complicates the purification and in most cases, the various isoforms of the antibody are only partially resolved. Purification of an antibody therefore requires extremely powerful separation techniques. Preparative isoelectric focusing (IEF) has been shown to resolve the isoforms of monoclonal antibodies [5,6]. However, the low product yield and the laborious extraction of sample from the gel pose a major limitation.

Chromatofocusing is an alternative to IEF [7–9]. In chromatofocusing a weak ion-exchanger is titrated with an amphoteric buffer (mobile phase). Upon titration, a pH gradient that is established in the column focuses the proteins into sharp bands. Proteins with minor differences in isoelectric point (p*I*) are easily resolved and the separations are comparable to preparative IEF. Chromatofocusing is ideal for preparative-scale purification of components with closely related p*I* values. However, in cases where chromatofocusing is used in a semi-preparative or analytical format [10,11], pH gradient separation may be more appropriate since the separation time is significantly reduced.

Ion-exchange chromatography has been proven to be a more practical approach for purification of biomolecules [12,13]. Though the

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product throughput/yield is high, the resolution in ion-exchange is generally lower than with IEF, especially when salts gradients are used. More recently, ion-exchange chromatography using pH gradients has been demonstrated to effectively resolve isoforms of an antibody [6]. Though the isoforms were only partially resolved by the pH gradient, sufficient purified material could be obtained by collection of the fractions.

This report explores the use of pH gradients for resolution of Fab fragments and other proteins having closely related p*I* values. The advantage of using a pH gradient vs. a conventional salt gradient is that the collected fractions contain very low levels of salt (10–20 m*M*) thereby eliminating the necessity of desalting. Also, the resolution can be far superior to salt gradient elution because the separation is based on relative differences in p*I* of the protein (see Results and discussion).

2. Experimental

2.1. Apparatus

A gradient HPLC system (BioCAD, PerSeptive Biosystems, Framingham, MA, USA) equipped with a UV detector operating at 280 nm was used. The CE system was assembled in the laboratory and consisted of a Spellman 1000 R power supply (Plainview, NY, USA) and a PerSeptive Biosystems UV/VIS-250 detector operating at 214 nm.

2.2. Chemicals and reagents

All chemicals used for preparation of HPLC buffers were of analytical grade. 2-(N-Morpholino)ethanesulfonic acid (MES), 2-amino-2-methyl-1,3-propanediol (AMPD) and N-tris (hydroxymethyl)-3-aminopropanesulfonic acid (TAPS) and the β-lactoglobulin were from Sigma (St. Louis, MO, USA). Reagents used for digestion of the antibody were from Pierce (Rockford, IL) and the Fab fragments were produced as described in the Imunopure kit from Pierce. The mouse monoclonal anticortisol antibody (subclass IgG_2b) was purchased from Fritzgerald (Concord, MA, USA).

2.3. Cation-exchange HPLC

A strong cation-exchange (100 × 4.6 mm, 20 μm particle size, sulfonic acid, catalog No. P021M526), POROS S/M column was used for the separation (PerSeptive Biosystems). The column was equilibrated with 10 m*M*, MES, pH 4.5. After injecting the sample, a linear gradient of increasing pH (10 m*M*, MES, pH 7.5) was used for elution of the proteins. The eluted peaks were collected and further analyzed by CE to determine their purity.

2.4. CE of the collected fractions

The collected fractions were analyzed by CE using a siloxanediol/polyacrylamide (27 cm × 50 μm) capillary coated in the laboratory. The running buffer was 20 m*M* TAPS/AMPD, pH 8.8. The applied field strength was 1100 V/cm. Samples were loaded hydrodynamically for 10 s using a 10 cm differential height between the ends of the capillary.

3. Results and discussion

The principle for separation of proteins using pH gradients is quite straightforward. At the start of a cation-exchange run (loading step), pH of the mobile phase must be below the p*I* of the protein so that the protein has a net positive charge. The protein is therefore adsorbed on the cation-exchange surface. To elute a protein, the pH is gradually increased above the p*I* of the protein. At this point the protein acquires a negative charge and elutes from the column. Since elution is dependent on the p*I* of the protein, proteins with minor differences in p*I* can be very efficiently separated. A similar procedure could be used for elution from an anion-exchange surface except that the pH would have to be above the p*I* during loading and should be

gradually decreased below the p*I* of the protein for elution.

A strong cation-exchange column was used for separation to maintain a constant charge on the column surface throughout the pH gradient while changing the surface charge on only the protein. If the surface charge density on the column decreases during elution, it is possible that the resolution would be compromised. It is desirable that column charge density be high to maintain maximum resolution.

The Fab fragments were generated by digestion of an anti-cortisol IgG_1b with papain. Papain cleaves one or more peptide bonds in the hinge region of the antibody producing two identical Fab and one Fc fragment. After removal of the Fc portion, three Fab isoforms were observed by gel IEF (not shown). The origin of heterogeneity in the Fab fragments is unclear. However, others have also reported on the heterogeneity obtained after papain digestion of IgG [14,15]. The three isoforms had p*I* values of 5.4, 5.5 and 5.8.

3.1. HPLC purification of Fab isoforms

During optimization of the pH gradient, it was found that slight variations in the gradient resulted in a significant change in resolution. Fig. 1 shows the separation of the various isoforms of the Fab using a pH gradient from 4.5 to 6.4 in 20 column volumes at a flow-rate of 1 ml/min. Approximately 100 μg of sample were injected and the various isoforms were easily separated within 20 mins. The fractions were collected and later analyzed by CE to determine the purity. Despite incomplete resolution of the isoforms, fractions could be collected from each peak devoid of impurities from other partially resolved peaks. The electropherograms for the individual fractions (Fig. 2) show that a single peak was obtained for each fraction. Recovery of the material was about 90%. The recovery was determined by collecting fractions off the HPLC column and measuring the UV absorbance at 280 nm vs. the absorbance of the original Fab mixture. An electropherogram of the Fab mixture is also shown at the bottom of Fig. 2. It is seen that the resolution of CE is superior to that of HPLC in this case.

In most purifications, one of the goals is to maintain the biological activity of the protein. Harsh elution conditions such as salt, organic or extremes in pH can affect the biological activity of a protein. The mild loading and elution conditions used during pH gradient elution are therefore ideal for purification of the Fab fragments. All three isoforms were shown to be active by their ability to recognize the specific antigen [4]. Any one of the three purified isoforms could therefore be used to perform immunoassays.

3.2. Separation of β-lactoglobulin A and B

To further evaluate the resolution obtained with pH gradients, a mixture of β-lactoglobulins A and B was injected. The two forms differ in p*I* by approximately 0.1 unit (p*I* of β-lactoglobulin A is 5.13 and that of B is 5.23) [16]. The difference in p*I* stems from the differences in the amino acid composition. Aspargine (64) and valine (118) in A are replaced by glycine and alanine respectively in B [17]. A gradient from pH 5 to 6.6 was run over 15 min at a flow-rate of 0.5 ml/min. The separation of β-lactoglobulin A and B by pH gradients is shown in Fig. 3. In spite of a difference in p*I* of approximately 0.1 between the two species, a reasonably good separation was obtained. Separation of β-lactoglobulin A and B using a salt gradient (0–0.5 *M* NaCl) was unsuccessful. Resolution of the peaks could not be obtained using any of the salt gradients attempted.

4. Conclusions

It may be concluded that pH gradient elution cation-exchange chromatography can be a valuable tool for preparation of a single Fab isoform of a monoclonal antibody. Although CE is of superior resolution, it has inadequate loading capacity for the preparation of sufficient antibody to be used for immunological assays.

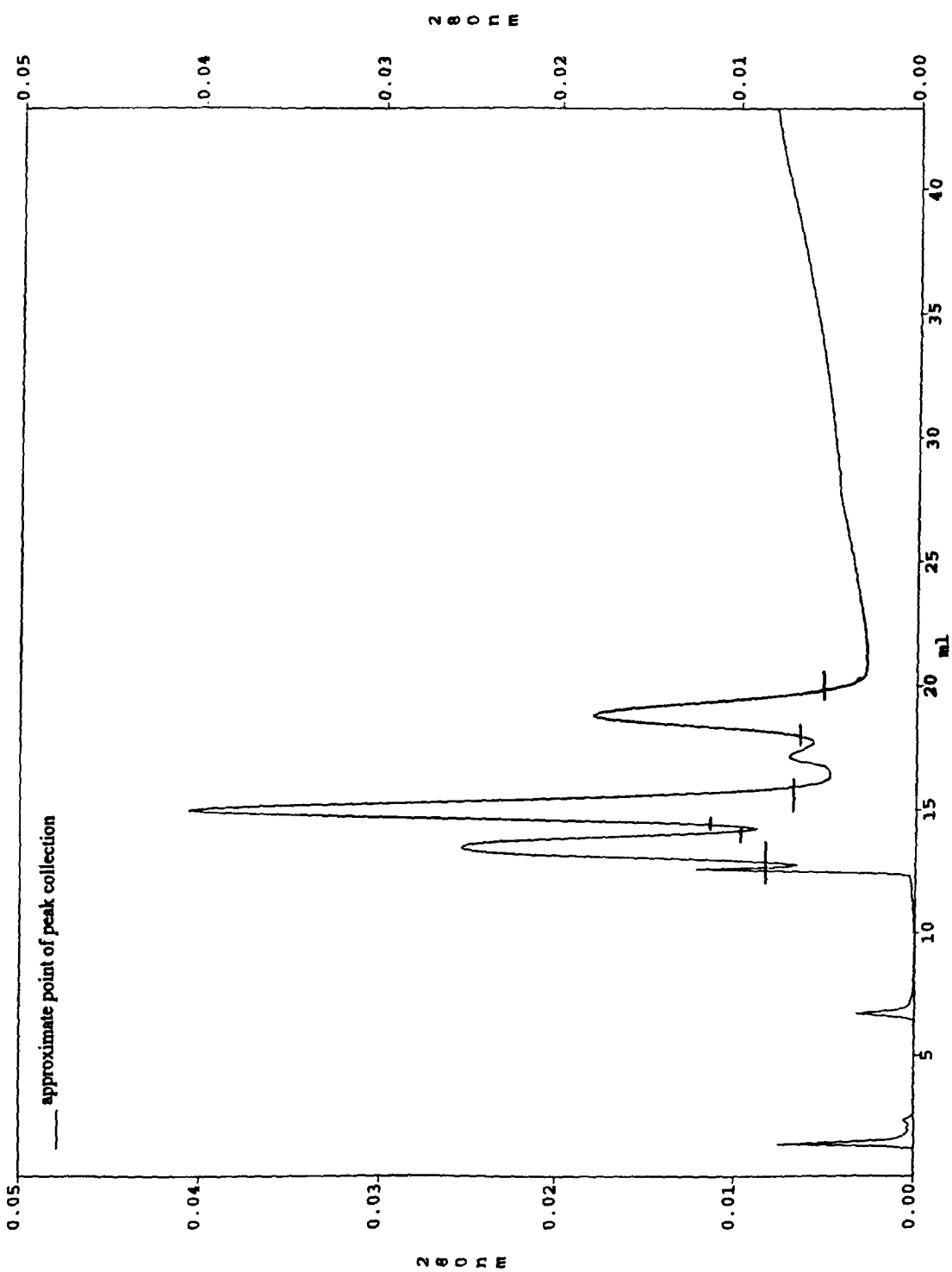

Fig. 1. Fab fragments separated by HPLC. HPLC conditions: 100 × 4.6 mm strong cation-exchange column (sulfonic acid); mobile phase A: 10 m*M* MES, pH 4.5; B: 10 m*M* MES ph 7.5; gradient 0–40% B in 20 column volumes at 1 ml/min. UV: 280 nm.

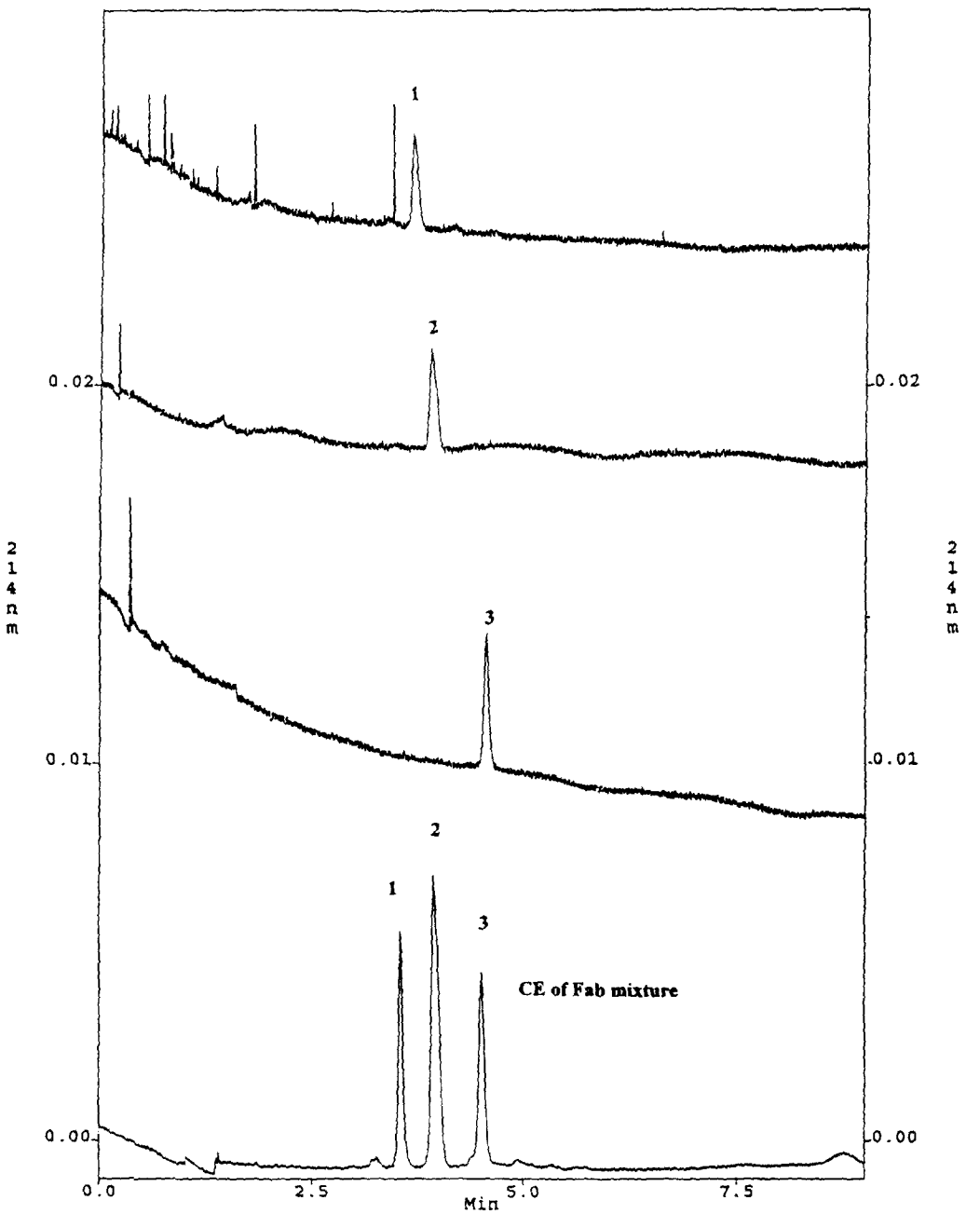

Fig. 2. CE of the collected fractions. Coated fused silica, 30 cm × 50 μm at 30 kV; buffer 20 m*M* TAPS, pH 8.8; UV detection at 214 nm.

Our initial results using pH gradients clearly demonstrate that ion-exchange chromatography with pH gradient elution is capable of separating proteins and their isoforms that differ by 0.1 p*I* unit. Separations can be achieved in less than 30 min. In spite of incomplete resolution of the Fab

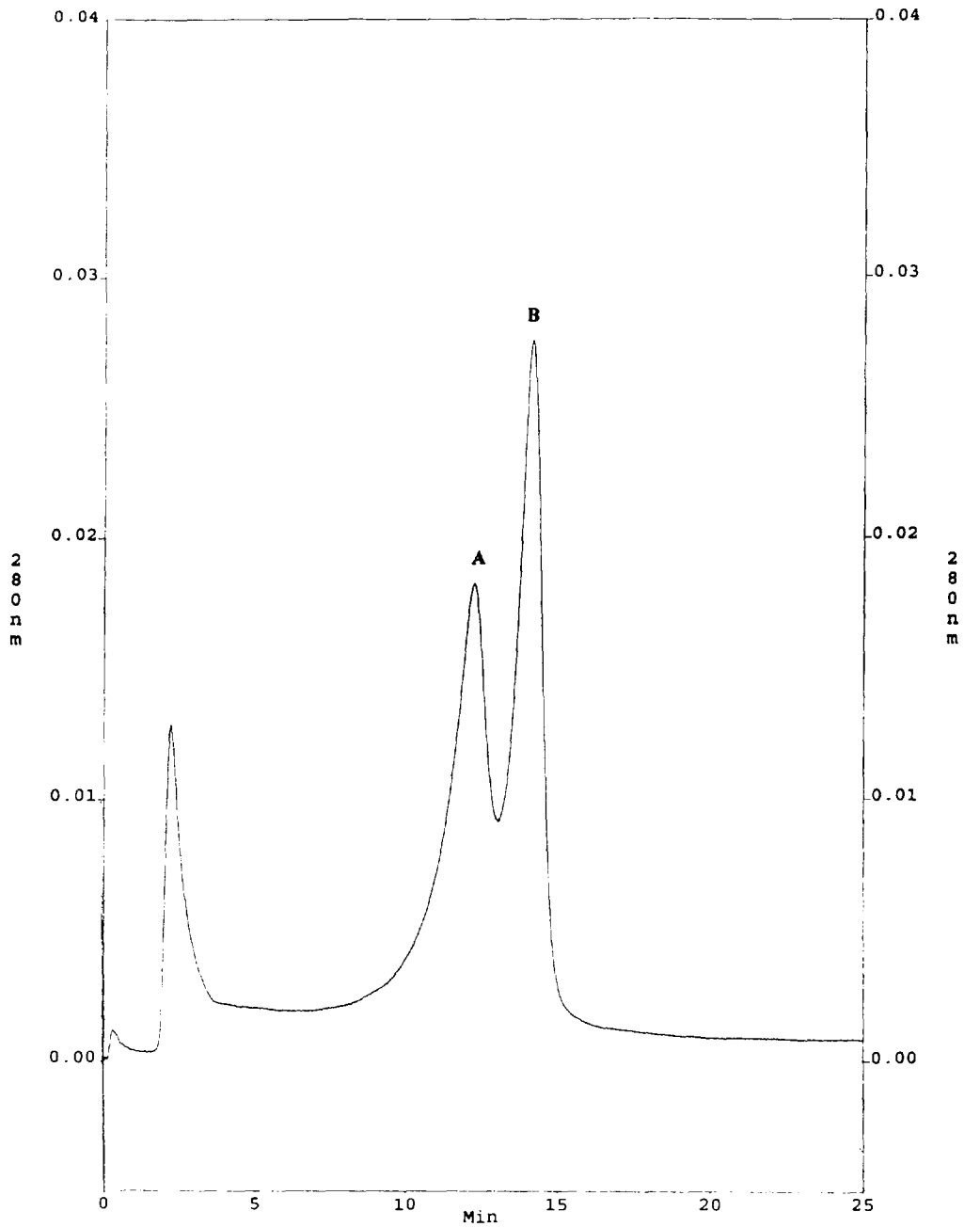

Fig. 3. Separation of β-lactoglobulins A and B using pH gradients. HPLC conditions as in Fig. 2, except a gradient of pH 5–6.6 was run over 15 min at 0.5 ml/min.

fragments, sufficient amount of purified material could be collected from the fractions.

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