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On-line post-capillary affinity detection of immunoglobulin G subclasses and monoclonal antibody variants for capillary electrophoresis

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Abstract

Human immunoglobulin G (IgG) subclasses each play a unique role in an immune response to foreign antigens. Three of the human IgG subclasses have distinct electrophoretic mobilities and are resolved by capillary zone electrophoresis (CZE). A post-capillary reactor is constructed to allow on-line addition of fragment B (of protein A)-fluorescein to form affinity complexes with separated IgG subclasses. Post-capillary affinity detection provides selective identification of human IgG subclasses and illustrates the effect of affinity binding constant on detection sensitivity. Additionally, post-capillary affinity detection for CZE facilitates rapid and selective heterogeneity analysis of mouse monoclonal anti-(human-α_1-antitrypsin) and anti-human follicle stimulating hormone in complex sample matrices. A constant mobility difference is observed between the antibody isoforms, likely the result of charge heterogeneity due to deamination, degradation or variation in sialic acid content. © 1997 Elsevier Science B.V.

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1. Introduction

Heterogeneity of immunoglobulin G (IgG) plays a critical role in antibody function and effectiveness. Variation sources include amino acid sequence variations, post-translational modifications, as well as chemical and proteolytic degradation. The amino acid sequence in the variable region of IgG gives rise to unique antigen binding specificity while the sequence in the constant region is characteristic of a given IgG subclass. The number of IgG isoforms is increased by post-translational modifications, notably N-linked glycosylation of the heavy chain. Deamination and incomplete C- or N-termini create additional IgG variants.

The IgG molecule is composed of two heavy and two light polypeptide chains linked together by disulfide bonds. All heavy chains are of the γ immunoglobulin class whereas the light chains are either λ or κ types ($\gamma_2\lambda_2$ and $\gamma_2\kappa_2$). In addition, four major subclasses of the γ chain have been documented in humans and termed IgG 1–4 to designate their relative abundance in serum. Over 95% of the

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amino acid sequence is homologous among the subclasses, however, major differences exist in the hinge region where the two heavy chains are joined. The numbers of both amino acid residues and interchain disulfide bonds vary in the hinge region which alters molecule flexibility and access to the Fc region. Complement activation, initiated by binding of C1 to the Fc region of IgG, varies considerably among the subclasses. IgG 3 binds C1 the strongest followed by IgG 1; IgG 2 binds to a lesser extent and binding of C1 with IgG 4 has not been observed [1,2]. Due to their unique functions in immune response, subclass levels have been characterized in an attempt to understand the significance of individual subclasses on health.

Glycosylation of proteins is known to influence protein folding, secretion and clearance of glycoproteins from the blood stream [3]. In addition, certain glycoforms have been shown to induce an immune response and concern by regulatory agencies regarding therapeutic glycoproteins is increasing [4]. Glycosylation of IgG has little to no effect on antigen or protein A binding [5]. However, IgG glycosylation is critical for effector functions including complement fixation, binding to Fc receptors on macrophages and rapid elimination of antigen bound complexes from the circulation [5].

Due to structural changes upon antigen binding, terminal galactose residues on IgG become more accessible to hepatic receptors which clear rat IgG–antigen complexes from the circulation [6]. For human IgG, a decrease in terminal galactose has been associated with rheumatoid arthritis and primary osteoarthritis [7] as well as pathological myeloma IgG [8,9]. Glycosylation of hybridoma produced IgG varies significantly with culture age and may vary to some extent with culture medium pH or proliferation state [10]. Given the importance of glycosylation, a need exists for rapid identification of IgG heterogeneity during production.

Recently, we have developed on-line affinity detection for capillary zone electrophoresis (CZE) to allow rapid identification of IgG Fc variants [11] or biotin derivatives (unpublished work) in complex sample matrices. This technique allows separation of analyte isoforms prior to on-line affinity detection. The advantages of high efficiency and low sample consumption characteristic of CZE are combined with class selectivity of affinity reactions. In this study, IgG variants are resolved by CZE and transferred across a 20 μm gap into a reaction capillary (see Fig. 1). A mild vacuum is applied to the outlet reservoir such that the volume flow-rate in the reaction capillary is greater than that in the separation capillary. Fragment B of protein A conjugated with fluorescein (BF) is placed in the post-capillary reactor and is also introduced into the reaction capillary. Affinity binding between IgG and BF occurs in the reaction capillary and the complexes are detected by laser induced fluorescence (LIF) due to a fluorescence enhancement upon binding [12].

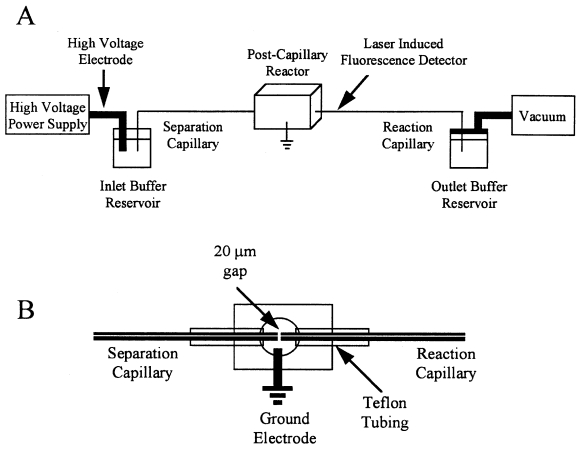

Fig. 1. Schematic diagram of post-capillary affinity detection for CZE: (A) system overview and (B) expanded view of post-capillary reactor and capillary junction.

In this paper, post-capillary affinity detection for CZE is applied to analyze human IgG subclasses and to allow rapid monitoring of monoclonal antibody heterogeneity in cell culture media. Analysis of three human subclasses reveals that assay sensitivity is highly dependent upon association constants between BF and the IgG. Selective detection of monoclonal antibody variants is achieved despite the presence of cell culture media containing serum. The lack of significant interference by complex sample matrices demonstrates the potential application of this technique for process monitoring of monoclonal antibody production.

2. Experimental

2.1. Fluorescein conjugation

Fragment B of protein A (Sigma, St. Louis, MO, USA) was reacted with 5-carboxyl fluorescein succinimidyl ester (Molecular Probes, Eugene, OR, USA). Subsequently, the mixture was separated by anion-exchange high-performance liquid chromatography (HPLC) and fractions were collected as described previously [12]. The fraction corresponding to fluorescein conjugated with lysine 154 of fragment B was diluted in run buffer for use in all studies.

2.2. CZE–UV analysis

Human IgG 1λ, 2λ and 3κ subclasses were purchased from Sigma. Mouse monoclonal IgG_{2a} antibodies against human follicle stimulating hormone and human-α_1-antitrypsin were obtained from CalBiochem (San Diego, CA, USA). All antibody samples were stored in aliquots at −10°C prior to use. For the CZE analyses, antibody aliquots were diluted with run buffer and/or de-ionized water such that the final ionic strength was equal to that of the run buffer.

Run buffer was prepared by titration of 20 m*M* monobasic phosphate with 20 m*M* dibasic phosphate (Fisher Scientific, Pittsburgh, PA, USA) to pH 7.3 and addition of neutral and weakly acidic fluorochemical surfactants at a concentration of 0.0025% each. Prior to use, fluorochemical coated capillaries (J&W Scientific, Folsom, CA, USA) were conditioned overnight in buffer containing 0.05% fluorochemical surfactants.

CZE–UV analyses were performed with a Linear UVIS 200 multi-wavelength detector (Linear Instruments, Reno, NV, USA) at 200 nm. High voltage power supplies, equipped to deliver 0–30 kV (Spellman, Plainview, NY, USA), were housed in self-contained interlock boxes and used for all CZE experiments. Data collection was performed with an HP 35900D analog-to-digital interface board with the HP G1250C General Purpose Chemstation software (Hewlett-Packard, Fullerton, CA, USA).

2.3. Post-capillary affinity detection for CZE

Antibody aliquots were diluted in DME cell culture media (Sigma) containing fetal bovine serum such that the final antibody concentration ranged from 20 μg/ml to 100 μg/ml. After preparation, these antibody samples were placed in 10 000 M_r cut-off microfiltration units (Millipore, Bedford, MA, USA) and were desalted by repeated addition of run buffer and centrifugation at 5000 *g*. During the course of dialysis, the concentrations of anti-human follicle stimulating hormone and anti-(human-α_1-antitrypsin) were increased by factors of 10 and 2.5, respectively.

A rectangular post-capillary reactor was machined out of Plexiglas with dimensions of 2.5×1.3×0.7 cm and a 50 μl reservoir (see Fig. 1). Two 1/16 in. (1 in.=2.54 cm) diameter channels accessed the reservoir from opposite sides and were fitted with PTFE tubing (0.3 mm I.D.×1/16 inch O.D.). The small I.D. of the tubing allowed secure alignment of the separation and reaction capillaries at the same height and lateral position. The end-to-end distance between the capillaries in the post-capillary reactor was adjusted manually and visualized by a custom microscope (Edmund Scientific, Barrington, NJ, USA) equipped with a 10× objective and a 10× eyepiece. The use of a micrometer disc reticle allowed measurement of the 20 μm gap between the capillaries.

For post-capillary affinity detection of IgG variants, BF was diluted in run buffer and placed in the post-capillary reactor. Vacuum was applied to the outlet reservoir via a pipet-aid (Drummond Scientific, Broomall, PA, USA) to transfer separated IgG variants and BF into the reaction capillary. The 488 nm line of an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) was used for LIF detection. A LIF detection system originally designed by Yeung et al. [13] was geometrically modified to allow the integration of a post-capillary reactor. Modifications were described in detail elsewhere [11]. Fluorescence emission at 515 nm in the reaction capillary was monitored 1 cm from the post-capillary reactor.

2.4. Homogeneous fluorescence assay

Homogeneous affinity assays for IgG subclasses

were performed using the well plate accessory for a LS50B fluorescence spectrophotometer (Perkin-Elmer, Buckinghamshire, UK). BF and IgG were diluted in 20 m*M* phosphate buffer at pH 7.3, such that the final volume was 190 μl. The BF concentration was fixed at 1.5 μg/ml while the concentration of each IgG subclass was varied from 0–10 μg/ml. Triplicate fluorescence intensity measurements at 515 nm were averaged for each IgG concentration.

3. Results and discussion

3.1. CZE of human IgG subclasses

Due to interest in their immunological role, human IgG subclasses were studied by CZE with both UV absorbance and post-capillary affinity detection. Successful baseline resolution between the subclasses (see Fig. 2A) was obtained by CZE with UV absorbance detection at 200 nm. The use of fluorocarbon coated capillaries was essential to reduce protein–wall interactions. IgG 3 eluted first followed by IgG 1 and IgG 2. Minor shoulders were observed on each major peak, indicating further heterogeneity within a given subclass. Slight differences in electrophoretic mobility allowed partial resolution of IgG isoforms within a particular subclass. Human IgG 4 was not employed in these studies due to difficulties encountered in the CZE separation. Analysis of IgG 4 by CZE resulted in an extremely broad peak, possibly due to adsorption of IgG 4 onto the capillary wall (data not shown).

Analysis of the subclasses by post-capillary affinity detection for CZE was also performed. IgG subclasses were transferred across a 20 μm gap between the separation and reaction capillaries (see Fig. 1) without significant deterioration in separation efficiency or resolution. BF was added to the post-capillary reactor and affinity complexes between BF and IgG subclasses were monitored in the reaction capillary by on-column LIF detection. The fluorescence enhancement upon binding of BF with IgG was due to a microenvironment pH increase and was discussed previously [12]. Despite the presence of all three subclasses, Fig. 2B revealed only two protein

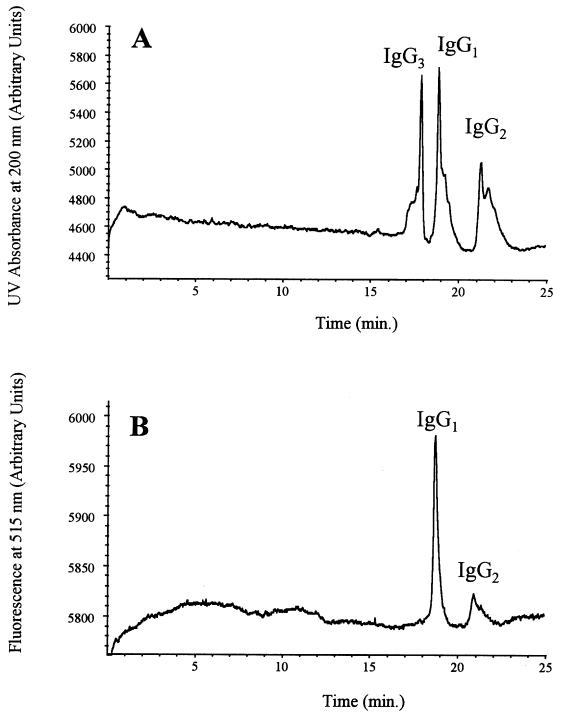

Fig. 2. Analysis of human IgG subclasses. (A) CZE–UV separation of 500 μg/ml of each IgG 1, 2 and 3. A fluorocarbon coated capillary of 58 cm (31 cm to detector)×50 μm I.D.×375 μm O.D. was filled with 20 m*M* phosphate buffer containing 0.005% fluorochemical surfactant at pH 7.3. 11.5 kV was applied across the capillary for a 5 s electrokinetic injection and maintained during the separation. (B) CZE–affinity detection of 300 μg/ml of each IgG 1, 2 and 3. Separation was performed in a fluorocarbon coated capillary of 30 cm×50 μm I.D.×375 μm O.D. filled with 20 m*M* phosphate buffer containing 0.005% fluorochemical surfactant at pH 7.3. The fluorochemical coated reaction capillary was 6 cm×20 μm I.D.×375 μm O.D.. 6 kV was applied across the separation capillary for a 5 s electrokinetic injection and maintained during the separation. BF at 2 μg/ml was placed in the post-capillary reactor and vacuum was applied to the outlet reservoir. LIF detection at 515 nm was monitored in the reaction capillary, 1 cm from the post-capillary reactor.

peaks at migration times corresponding to IgG 1 and IgG 2. As shown in Fig. 2B, the shoulders observed in the CZE–UV analysis (see Fig. 2A) were broadened during the affinity step and merged with the main IgG peaks. A reduction in separation resolution due to the affinity reaction was also observed in the analysis of IgG Fc variants [11].

Weak binding affinity between protein A and IgG 3 resulted from a single amino acid substitution at

residue 435 on IgG 3 [14]. Since fragment B contained the same IgG binding site as protein A, weak binding between BF and human IgG 3 was expected and could explain the lack of fluorescence enhancement during the analysis of IgG 3. In the post-capillary affinity detection, the peak height ratio of IgG 2 to IgG 1 was significantly decreased as compared with the ratio observed in the CZE–UV analysis. This decrease in peak ratio might again result from the difference in binding affinity between BF and IgG subclasses. Thus, fluorescence homogeneous binding assays were performed to assess the binding affinity between BF and the three IgG subclasses.

In the presence of 1.5 μg/ml BF, the increase in fluorescence intensity due to increasing amounts of IgG subclasses was measured and shown in Fig. 3. The samples were incubated for 30 min to ensure binding equilibrium was reached. In the case of IgG 1, the fluorescence intensity increased sharply and then leveled off at ca. 7.5 μg/ml IgG 1. A similar trend was observed for IgG 2, although the initial slope was somewhat lower and a larger IgG concentration was necessary to reach binding saturation. Thus, the overall assay sensitivity was lower for IgG 2 than for IgG 1. The fluorescence intensity of BF failed to change significantly as the concentration of IgG 3 was increased, however, a slight increase was observed at high IgG 3 concentrations.

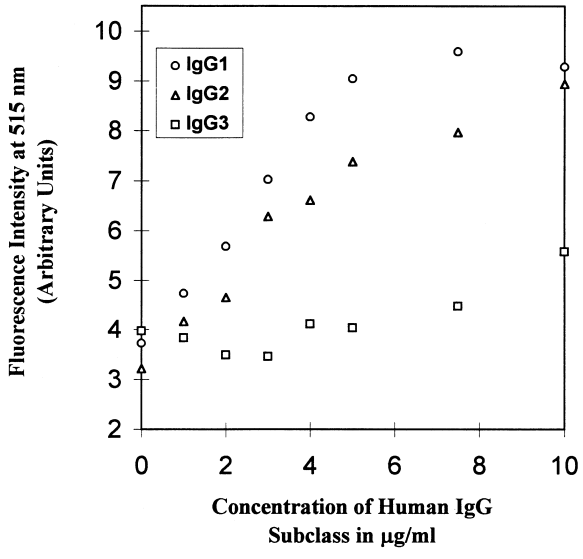

Fig. 3. Homogeneous fluorescence binding assay of human IgG subclasses.

As discussed previously [12], the magnitude of the initial slope in the calibration curve reflected the binding strength between BF and IgG. For IgG 3, the low fluorescence enhancement indicated weak affinity binding with BF. Additionally, the lower initial slope and the higher IgG concentration required to reach binding saturation suggested a slightly weaker binding constant for IgG 2 than for IgG 1. Duhamel et al. [15] observed the elution IgG 2 at pH 4.7 followed by IgG 1 at pH 4.3 from a protein A column, further supporting our observed differences in affinity binding of IgG 1 and IgG 2 for BF.

In the homogeneous fluorescence binding assays, both IgG 1 and IgG 2 reached the same fluorescence plateau at binding saturation (see Fig. 3). Thus, increases in microenvironmental pH and fluorescence intensity of BF were comparable upon binding with IgG 1 or IgG 2. Based on the results obtained from the homogeneous fluorescence binding assays, the difference in peak heights and detection sensitivities of IgG 1 and IgG 2 (see Fig. 2B) were due in part to variation in binding affinity with BF.

Similar BF concentrations were used in both the homogeneous fluorescence assay and post-capillary affinity detection. However, the concentrations of IgG differed by at least one order of magnitude due to the nature of the measurements. Fluorescence intensities were obtained after binding equilibrium was reached for the homogeneous assay. In contrast, post-capillary affinity detection was operated under kinetically limiting conditions. In post-capillary affinity detection, BF and IgG were only allowed to mix and react for about 10 s in an effort to minimize band dispersion due to the parabolic flow profile in the reaction capillary. As a result, higher IgG concentrations were employed for the post-capillary affinity reaction in order to speed up the association rate.

Among the subclasses, differences exist in the number of disulfide bonds joining the two heavy chains at the hinge region. The structure in the hinge region affects molecular flexibility and access to the Fc region of human IgG [1,2]. Given the rigid nature of IgG 2's hinge region, the association of IgG 2 with BF may be hindered. Decreased kinetics as well

as weaker binding affinity likely resulted in a decreased peak height for IgG 2 relative to IgG 1.

3.2. CZE of mouse monoclonal antibodies

In addition to IgG subclasses, CZE with UV absorbance and post-capillary affinity detection were employed to evaluate the heterogeneity of mouse anti-(human-α_1-antitrypsin) and anti-human follicle stimulating hormone. UV detection following CZE separation revealed three main variants of anti-(human-α_1-antitrypsin) (see Fig. 4A). Post-capillary BF addition was employed to selectively detect antibody variants in the presence of DME cell culture media containing 5% fetal bovine serum. As shown in Fig. 4B, the same number of antibody variants was observed in post-capillary affinity detection as in the UV measurement. Apparently, the presence of IgGs in fetal bovine serum did not adversely affect the performance of post-capillary affinity detection. Rapid analysis of antibody heterogeneity was achieved without any interference from cell culture media containing fetal bovine serum. Antibody peaks were clearly discernible with only one additional system peak at the time of the electroosmotic flow. The system peak observed in post-capillary affinity detection was due to differences in the ionic strength of the sample and run buffers, which altered the fluorescence signal. In comparison with the CZE–UV measurement (see Fig. 4A), a longer separation capillary and a lower electric field strength in CZE–affinity detection accounted for the increase in antibody migration times.

Linearity of post-capillary affinity detection for monoclonal antibody analysis was evaluated. Duplicate injections of anti-(human-α_1-antitrypsin) were analyzed and total fluorescence peak area plotted as a function of antibody concentration in cell culture media. As shown in Fig. 5, a linear relationship

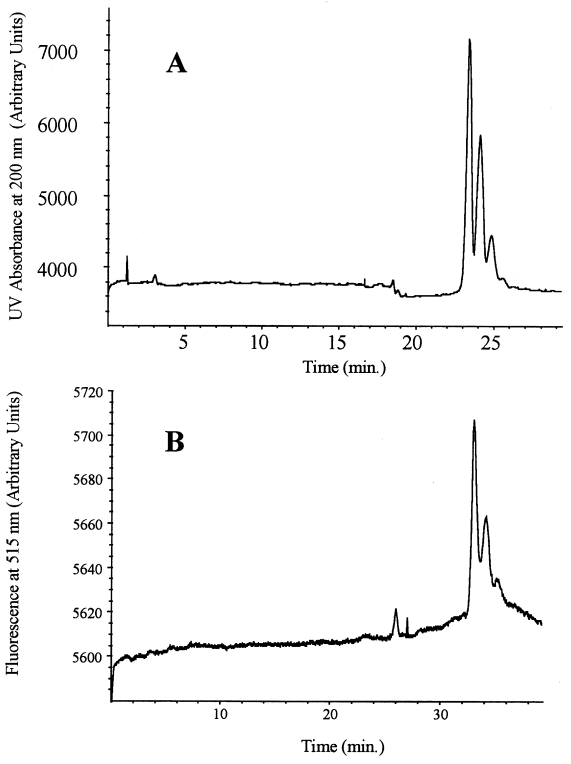

Fig. 4. Analysis of mouse anti-(human-α_1-antitrypsin). (A) CZE–UV separation of 650 μg/ml monoclonal antibody. Separation conditions were identical to those in Fig. 2A. (B) CZE–affinity detection of 75 μg/ml monoclonal antibody in DME media containing 5% fetal bovine serum. Separation was performed in a fluorocarbon coated capillary of 34 cm×50 μm I.D.×375 μm O.D.. 6 kV was applied across the separation capillary for a 5 s electrokinetic injection and maintained during the separation. BF at 0.5 μg/ml was placed in the post-capillary reactor. Other conditions were the same as those in Fig. 2B.

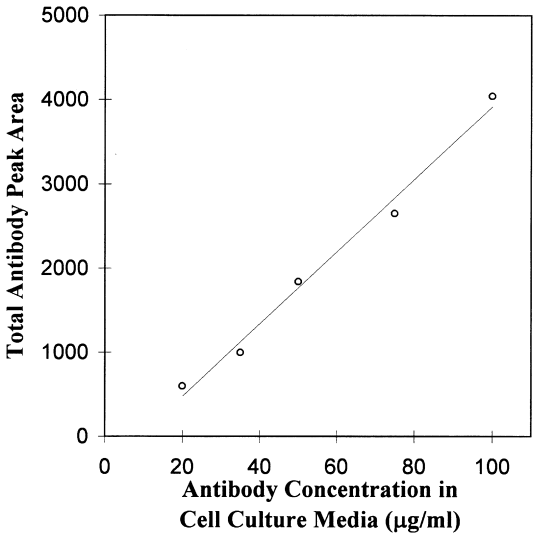

Fig. 5. Linearity of post-capillary affinity detection of mouse anti-(human-α_1-antitrypsin). Run conditions were the same as those in Fig. 4B.

between total peak area and antibody concentration was observed with a correlation coefficient of 0.989. The concentrations analyzed were in a range commonly found in hybridoma cell cultures. Therefore, CZE combined with post-capillary affinity detection has the ability to simultaneously measure antibody concentration and assess protein heterogeneity during antibody production.

Mouse monoclonal anti-human follicle stimulating hormone in DME media containing 3% serum was also analyzed by post-capillary affinity detection for CZE. Three major and two minor antibody variants were partially resolved by CZE and selectively detected in the reaction capillary (see Fig. 6). Affinity complex formation with BF allowed selective detection of antibody variants despite the presence of cell culture media containing serum proteins. Both monoclonal antibodies employed in this study belonged to the mouse 2a subclass and exhibited strong affinity binding with BF, demonstrated by post-capillary affinity detection.

Samples containing mouse monoclonal antibodies were dialyzed against run buffer prior to CZE–affinity detection (see Section 2.3). High salt concentrations in antibody samples contributed to band broadening in CZE and resulted in poor separation resolution among antibody variants. In addition, the magnitude of the system peak was proportional to the difference between sample and run buffer ionic strength. The small system peak that appeared in the electropherogram did not interfere with the affinity detection of antibody variants in this study. However, if the mobilities were such that antibody variants migrated near the system peak, further minimizing ionic strength variation would be critical.

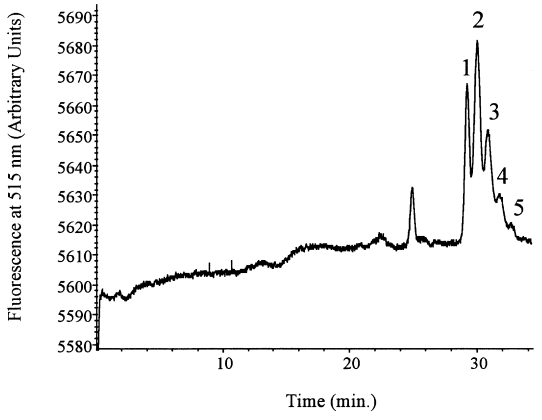

Fig. 6. CZE–affinity detection of 25 μg/ml mouse anti-human follicle stimulating hormone in DME media containing 3% serum. Other conditions were the same as those in Fig. 4B.

The peaks observed for mouse anti-human follicle stimulating hormone, were separated by similar but increasing migration times (see Fig. 6). The difference in electrophoretic mobilities between neighboring antibody peaks turned out to be a constant value of $2.92 \cdot 10^{-6}$ $cm^2/V\,s$ with a relative standard deviation (R.S.D.) of only 1.68%. The electrophoretic mobilities of peptides and proteins were successfully correlated with their effective charge (q) and molecular mass (M_r) using a function of $q/M_r^{2/3}$ by other researchers [16]. Thus, the constant mobility difference among the antibody isoforms likely resulted from charge heterogeneity.

CZE resolves protein analytes mainly on the basis of their charge and to a lesser degree on size. Electrophoretic mobility is fairly insensitive to mass changes for monoclonal antibodies with a molecular mass of around 150 000. For example, a decrease in M_r of 1000 only increases electrophoretic mobility by 0.45% and a change in M_r of 10 000 results in a mobility shift of 4.5%, assuming effective charge (q) is unchanged. For the antibody of anti-human follicle stimulating hormone, the electrophoretic mobility differed by 43% between the earliest and the latest eluting variants.

Monoclonal antibodies, by their very nature, contain a homologous amino acid sequence. On the other hand, post-translational modifications depend on cellular concentrations of modifying enzymes and substrates. A major source of antibody heterogeneity results from differences in protein glycosylation [17–19]. Two N-linked glycosylation sites are conserved among all IgGs, one on each heavy chain in the Fc region. These oligosaccarides are known to be complex and may contain terminal sialic acid. Oligosaccarides on IgG account for about 3% of total molecular mass. Based on the nature of monoclonal antibodies, variation in sialic acid content may contribute to the charge variants observed in this study. Mouse monoclonal antibody was treated with sialidase for four days in an effort to cleave all terminal sialic acid. The original antibody isoforms were still observed in CZE–UV analysis of the

digest. In addition, a small peak eluted prior to the earliest migrating variant. This decrease in negative electrophoretic mobility was consistent with a reduction in negative charge.

Additional sources of antibody heterogeneity can also contribute to the constant mobility difference observed in this study. Both deamination and ragged termini have been reported for monoclonal antibodies [19]. In conclusion, post-capillary affinity detection for CZE allows separation and selective detection of human IgG subclasses and mouse monoclonal antibody variants. Analysis of human IgG subclasses reveals the importance of affinity binding constant on detection sensitivity. By maintaining resolution of protein variants and introducing affinity selectivity, post-capillary affinity detection allows analysis of antibody variants in complex sample matrices. Mouse monoclonal anti-(human-α_1-antitrypsin) and anti-human follicle stimulating hormone are detected and quantified without any interference from DME cell culture media containing fetal bovine serum. This novel bioanalytical technique is linear with respect to antibody concentration and may provide in-process evaluation of antibody heterogeneity to assess purity and lot-to-lot consistency with little sample preparation.

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References

[1] R. Brunhouse, J.J. Cebra, Mol. Immunol. 16 (1979) 907–917.
[2] A. Feinstein, N. Richardson, M.J. Taussig, Immunol. Today 7 (1986) 169–174.
[3] N. Jenkins, R.B. Parekh, D.C. James, Nature Biotechnol. 14 (1996) 975–979.
[4] D.T. Liu, Trends. Biotechnol. 10 (1992) 114–120.
[5] M. Nose, H. Wigzell, Proc. Natl. Acad. Sci. 80 (1983) 6632–6636.
[6] R.W. Thornburg, J.F. Day, J.W. Baynes, S.R. Thorpe, J. Biol. Chem. 255 (1980) 6820–6825.
[7] R.B. Parekh, R.A. Dweek, B.J. Sutton, D.L. Fernandes, A. Leung, D. Stanworth, T.W. Rademacher, T. Mizuochi, T. Taniguchi, K. Matsuta, F. Takeuchi, Y. Nagano, T. Miyamoto, A. Kobata, Nature 316 (1985) 452–457.
[8] T.W. Rademacher, P. Williams, R.A. Dweek, Proc. Natl. Acad. Sci. 91 (1994) 6123–6127.
[9] N. Takahashi, I. Ishii, H. Ishihara, M. Mori, S. Tejima, R. Jefferis, S. Endo, Y. Arata, Biochem. 26 (1987) 1137–1144.
[10] R.J. Rothman, L. Warren, J.F.G. Vliegenthart, K.J. Hard, Biochem. 28 (1989) 1377–1384.
[11] J.K. Abler, K.R. Reddy, C.S. Lee, J. Chromatogr. A 759 (1997) 139–147.
[12] P.Y. Huang, C.S. Lee, Biotechnol. Bioeng. 42 (1993) 37–42.
[13] E.S. Yeung, P. Wang, W. Li, R.W. Geise, J. Chromatogr. 608 (1992) 73–77.
[14] B. Recht, B. Frangione, E. Franklin, E. Van Loghem, J. Immunol. 127 (1981) 917–923.
[15] R.C. Duhamel, P.H. Schur, K. Brendel, E. Meezan, J. Immunol. Methods 31 (1979) 211–217.
[16] E.C. Richard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197–207.
[17] Y. Mimura, E.A. Kabat, T. Tanaka, M. Fujimoto, K. Takeo, K. Nakamura, Electrophoresis 16 (1995) 116–123.
[18] P. Wang, K. Nakamura, Y. Mimura, K. Takeo, T. Tanaka, M. Fujimoto, Electrophoresis 17 (1996) 1273–1279.
[19] G.D. Roberts, W.P. Johnson, S. Burman, K.R. Anumula, S.A. Carr, Anal. Chem. 67 (1995) 3613–3625.