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Review

On-line post-capillary affinity detection of immunoglobulin G for capillary zone electrophoresis

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Abstract

To address the quality issues of antibody manufacturing, post-capillary affinity detection of immunoglobulin G (IgG) is developed for capillary zone electrophoresis. In analogy to a two-dimensional separation system, capillary zone electrophoresis (CZE), as the first dimension, resolves IgG variants based on their differences in molecular structure. IgG variants separated by CZE are discriminated against other serum and cellular proteins by affinity complex formation with protein A binding fragment in a post-capillary reactor. The analytical power of post-capillary affinity detection is demonstrated for rapid and selective heterogeneity analysis of human IgG subclasses and monoclonal antibodies in complex sample matrices. By comparing with pre-capillary formation of affinity complexes between IgG and protein A, post-capillary affinity detection clearly exhibit greater resolving power for examining IgG microheterogeneity. Affinity complex formation prior to CZE analysis, however, has the advantage of lower detection limits. Detection limits suffer with post-capillary affinity detection because of the high fluorescence background contributed by the fluorescently labeled protein A in the post-capillary reactor, and the need to determine a small change in the background level upon complex formation.

Keywords: Reviews; Online post-capillary affinity detection; Immunoglobulin G

Contents

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1. Introduction

Quality control plays an important role in biotechnology industry because of its uniqueness in utilizing living organisms for manufacturing commercial products. Quality control is designed to evaluate a broad spectrum of known as well as theoretical product impurities and potential degradation products. The need for quality control of biologicals derived from recombinant DNA and monoclonal antibody technologies has stimulated the application and development of various analytical methods. These include DNA hybridization, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography, polyacrylamide gel electrophoresis, and viral assay [1,2]. Among these techniques, the sensitivity and specificity of immunoreaction makes ELISA an important tool in analytical biochemistry.

Despite the selectivity and sensitivity of immunoassays, they suffer from several disadvantages. One is that they have traditionally been manual methods, which involve multiple reagent additions, washing steps, or centrifugation steps. Another disadvantage is that these assays usually require prolonged incubation times [3]. Furthermore, a common drawback for all antibody-based immunoassays is that antibodies cross-react with structurally related compounds. This is because antibodies target epitopes, not the whole antigen. Therefore, the signals obtained from immunoassays may not strictly relate to only one compound. This is particularly the case in biopharmaceutical manufacturing, where the protein product can be contaminated with product variants. These structural variants of protein product can arise from many sources such as expression errors, faulty post-translational modifications, and proteolytic degradation during biosynthesis and purification [4]. These product variants may have the same or similar epitopes as that of the protein product recognized by the antibody [5].

The time required to determine whether a process is meeting specifications is an important issue in manufacturing. When analysis time is short relative to processing, the analytical data may be used in a feedback control system to adjust process parameters controlling product quality. To ensure protein quality during biosynthesis and purification, advanced bioanalytical methods are critically needed. In this paper, we describe and evaluate the approaches reported in the literature for the analysis of immunoglobulin G (IgG) using capillary zone electrophoresis (CZE). Pre- and post-capillary formation of affinity complexes between IgG and protein A combine the strengths of CZE in the ease and speed of analyses with the specificity of affinity interactions. The results obtained from pre-capillary formation of affinity complexes are compared with those of post-capillary affinity detection for the analysis of IgG. Post-capillary affinity detection for CZE allows rapid and sensitive heterogeneity analysis of IgG subclasses and monoclonal antibody variants in complex sample matrices.

2. Immunoglobulin G heterogeneity

Heterogeneity of IgG plays a critical role in antibody function and effectiveness. Variation sources include amino acid sequence variations, post-translational modifications, as well as 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 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 C_1 to the Fc region of IgG, varies considerably

among the subclasses, IgG_3 binds C_1 the strongest followed by IgG_1; IgG_2 binds to a lesser extent and binding of C_1 with IgG_4 has not been observed [6,7]. 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.

The quantitative identification of IgG subtypes could be an important diagnostic marker for recurrent pulmonary infection risk, particularly in children. The absence of specific subtypes IgG_1 and IgG_3 together with the presence of normal levels of other immunoglobulin classes such as IgM have been correlated with increased risk of bacterial and/or viral infection [8,9]. From a purified IgG sample, Mao et al. [10] reported the separation of IgG subclasses using an fluorocarbon-coated capillary with a fluorocarbon surfactant present in the buffer. Further development of this methodology may enable clinicians to readily ascertain the IgG subclassification and infection risk in individuals [11].

3. Pre-capillary formation of affinity complexes

Protein A (SpA) is an acidic protein (isoelectric point, p*I* 5.1) from *Staphylococcus aureus*, which binds selectively to the Fc region of immunoglobulins, especially IgG. The molecule has four binding sites for IgG, however, steric hindrance results in a binding stoichiometry of two molecules of IgG per molecule of SpA. The binding of IgG to SpA is a result of several different, noncovalent interactions and the dissociation constant is reported to be 10^{-7}–10^{-9} *M* which is in the same range as immunoreactions [12]. However, the interaction depends on the immunoglobulin class and subclass, and varies strongly with the pH value.

Recent research work involving the formation of immunocomplexes followed by electrophoretic separations has been used as the basis for performing immunoassays in capillary electrophoresis [13–15]. The CZE separation of the reaction mixture typically revealed two zones by fluorescence detection, one corresponding to the complex, and the other corresponding to free fluorescently labeled antibody. The amount of analyte (antigen) was quantified by measuring either the decrease or the increase in peak area of free antibody or the complex, respectively.

Lausch et al. [16] have evaluated SpA conjugated with a fluorescent dye such as fluorescein diisocyanate or dichlorotriazinyl-aminofluorescein as an affinity ligand for the analysis of IgG in cultivation media. The ligand formed a fluorescing complex with IgG in the sample and rapid separation from excess SpA was performed by CZE. However, only partial resolution of the zones was achieved when SpA as a whole molecule was utilized. In contrast, baseline resolution of the zones was obtained when recombinant fragments of SpA were used as affinity ligands (see Fig. 1). Immunoglobulin concentrations in the range of two orders of magnitude were determined. Due to the specificity of SpA for IgG, analysis was carried out even in the presence of high concentrations of other components and in cultivation media. Thus, CZE affinity assay was successfully

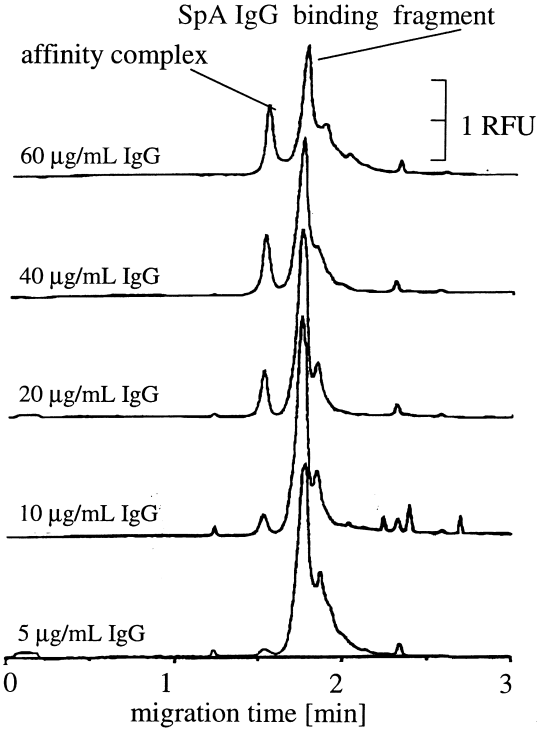

Fig. 1. Determination of increasing concentration of a monoclonal mouse antibody dissolved in cultivation medium using fluorescently labeled recombinant SpA IgG binding fragment as an affinity ligand (taken from Ref. [16]).

applied to monitor monoclonal antibody in a cultivation process.

As shown in Fig. 1, several shoulders on the peak of fluorescently labeled SpA fragment were observed during the CZE separation. This observation indicated potential microheterogeneity among SpA as the result of conjugation reaction. The electropherogram revealed two zones by fluorescence detection, one corresponding to SpA fragment–IgG complex, and the other corresponding to free SpA fragment. However, there was no clear separation resolution among various SpA fragment–IgG complexes as the requirement for the analysis of IgG microheterogeneity. The separation of SpA fragment–IgG complexes with minor variations of IgG structure is certainly more difficult than the resolution of just IgG variants. To analyze structural variants of IgG in complex sample matrices, the experience of Western Blot [17] clearly supports the separation of IgG variants prior to affinity binding with SpA, followed by the direct detection of affinity complexes in a post-capillary format.

4. Post-capillary formation of affinity complexes

We have recently developed on-line affinity detection for CZE to allow rapid identification of IgG Fc [18] variants in complex sample matrices. This technique allowed separation of analyte isoforms prior to post-capillary affinity reaction and fluorescence detection. The advantages of high efficiency and low sample consumption characteristic of CZE were combined with class selectivity of affinity reactions.

We further expanded the scope of post-capillary affinity reaction and fluorescence detection for the analysis of IgG subclasses and monoclonal antibody variants [19]. IgG variants were resolved by CZE and transferred across a 20-μm gap into a reaction capillary (see Fig. 2). A mild vacuum was applied to the outlet reservoir such that the volume-flow-rate in the reaction capillary was greater than that in the separation capillary. Fragment B of SpA conjugated with fluorescein (BF) was placed in the post-capillary reactor and was also introduced into the reaction capillary. Affinity binding between IgG and BF occurred in the reaction capillary and the complexes

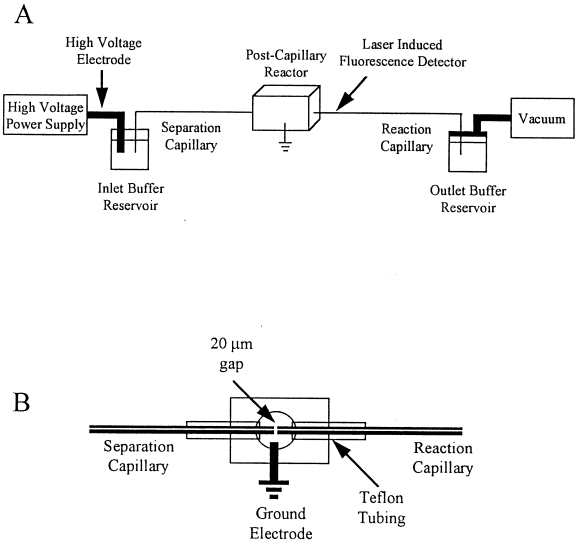

Fig. 2. Schematic diagram of post-capillary affinity detection for CZE: (A) system overview and (B) expanded view of post-capillary reactor and capillary junction (taken from Ref. [19]).

were detected by laser-induced fluorescence (LIF) due to a fluorescence enhancement upon binding [20].

4.1. Capillary zone electrophoresis of human immunoglobulin G subclasses

Due to interest in their immunological role, human IgG subclasses were studied by CZE with both UV absorbance and post-capillary affinity detection. The use of fluorocarbon-coated capillaries was essential to reduce protein–wall interactions. Minor shoulders were observed on each major peak, indicating further microheterogeneity within a given subclass (see Fig. 3A). Human IgG_4 was not employed in these studies due to difficulties encountered in the CZE separation [19]. Analysis of IgG_4 resulted in an extremely broad peak, possibly due to adsorption of IgG_4 onto the capillary.

By adding BF to the post-capillary reactor, affinity complexes between BF and IgG subclasses eluted from the CZE capillary were monitored in the reaction capillary by on-column laser-induced fluorescence (LIF) detection (see Fig. 2). The fluorescence enhancement upon binding of BF with IgG was due to a microenvironment pH increase [20]. Despite the

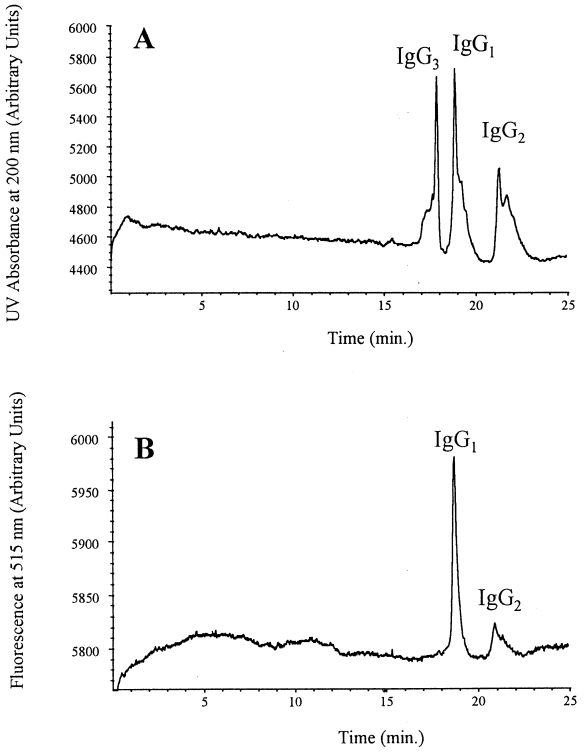

Fig. 3. Analysis of human IgG subclasses: (A) CZE–UV separation and (B) CZE–affinity detection of IgG_1, IgG_2, and IgG_3 (taken from Ref. [19]).

presence of all three subclasses, Fig. 3B revealed only two protein peaks at migration times corresponding to IgG_1 and IgG_2. Weak binding affinity between SpA and IgG_3 resulted from a single amino acid substitution at residue 435 on IgG_3 [21]. Thus, weak binding between BF and IgG_3 was expected during the post-capillary affinity reaction and could explain the lack of fluorescence detection of IgG_3. Furthermore, the peak height ratio of IgG_2 to IgG_1 was significantly decreased in the post-capillary affinity detection 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.

4.2. Capillary zone electrophoresis 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 selectively detected the same number of antibody variants in the presence of cell culture media containing 5% fetal bovine serum (see Fig. 4B). Rapid analysis of antibody heterogeneity was achieved without any interference from cell culture media containing fetal bovine serum.

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. A linear relationship was observed between total peak area and antibody concentration ranging

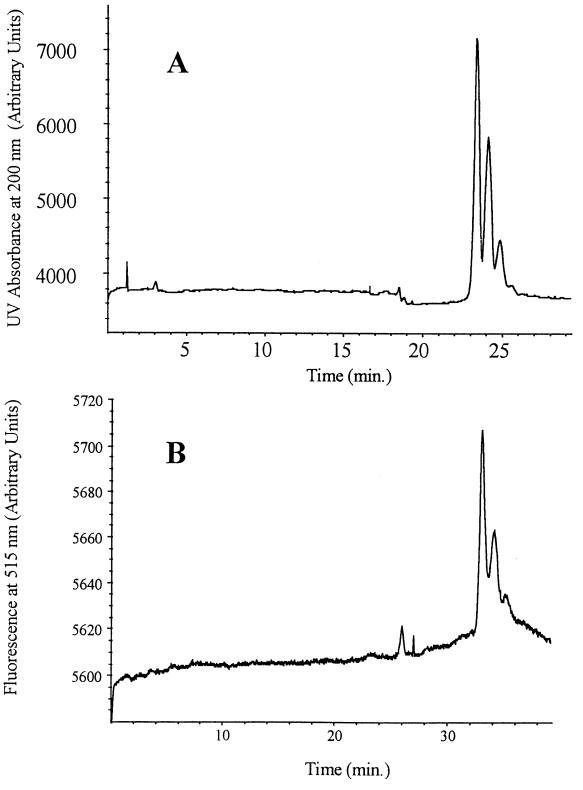

Fig. 4. Analysis of mouse anti-(human-α_1-antitrypsin): (A) CZE–UV separation and (B) CZE–affinity detection of monoclonal antibody in cell culture media containing 5% fetal bovine serum (taken from Ref. [19]).

from 20 to 100 μg/ml [19]. The concentrations analyzed were in a range commonly found in hybridoma cell culture. 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 cell culture 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. 5). Affinity complex formation with BF allowed selective detection of antibody variants despite the presence of cell culture media containing serum proteins. Both monoclonal antibodies belonged to the mouse 2a subclass and exhibited strong affinity binding with BF, demonstrated by post-capillary affinity detection.

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 ~150 000. Thus, the mobility differences among the antibody isoforms resolved by CZE are likely the result of charge heterogeneity. 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 [22–24]. Two N-linked glycosylation sites are conserved among all IgGs, one on each heavy chain in the Fc region. These oligosaccharides are known to be complex and may contain terminal sialic acid. Oligosaccharides 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 post-capillary affinity detection. However, recent studies of Mimura et al. [25] provide the evidence for suggesting the presence of IgG microheterogeneity as the result of intra- and extracellular modifications of monoclonal IgG peptide chains.

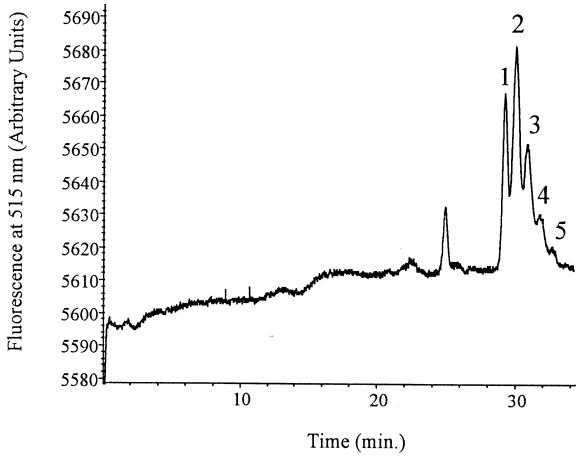

Fig. 5. CZE–affinity detection of mouse anti-human follicle stimulating hormone in cell culture media containing 3% serum (taken from Ref. [19]).

5. Conclusion

The protein quality during bioreactor operations, specifically the microheterogeneity of proteins, has arrived at center-stage with respect to manufacturing and regulatory compliance in the biotechnology industry. It is well known that the protein product in biotechnology can be contaminated with product variants in addition to other serum and cellular proteins. These structural variants of protein product result from expression errors, faulty post-translational modifications, and proteolytic degradation during biosynthesis and purification. Thus, the ability to identify protein product and product variants in complex sample matrices is extremely important for process monitoring and quality studies of protein pharmaceuticals.

The combination of CZE separation with post-capillary affinity reaction and fluorescence 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. This novel bioanalytical technique may provide in-process evaluation of antibody heterogeneity to assess purity and lot-to-lot consistency with little sample preparation.

In addition to utilize protein A as binding protein, protein G is known to bind all human IgG subclasses and also rabbit, mouse, and goat IgG [26]. The use of fluorescently-labeled protein G in post-capillary affinity detection can potentially enable specific detection and heterogeneity analysis of a wide range of IgGs of various subclasses or from different species. Perhaps the largest obstacle to more widespread use of post-capillary affinity detection technique is the lack of commercially available post-capillary detection for capillary electrophoresis. This situation can change quickly as miniaturization technologies advance.

6. Nomenclature

BF	Fragment B of protein A conjugated with fluorescein
CZE	Capillary zone electrophoresis
ELISA	Enzyme-linked immunosorbent assay
LIF	Laser-induced fluorescence
IgG	Immunoglobulin G
SpA	Protein A

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