Journal of Chromatography, 539 (1991) 177–185
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 953

Separation of antibody–antigen complexes by capillary zone electrophoresis, isoelectric focusing and high-performance size-exclusion chromatography

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(First received June 6th, 1990; revised manuscript received October 2nd, 1990)

ABSTRACT

The separation of antibody–antigen complexes by free-solution capillary zone electrophoresis (CZE) has been demonstrated. The antigen, a monoclonal antibody specific for the antigen, and the complex were well resolved. The entire separation was achieved in less than 10 min using on-column UV detection. The p*I* values for the three species were estimated separately by isoelectric focusing (IEF) experiments on polyacrylamide gels. Reasonably good agreement was found between the relative migration times measured by CZE and the p*I* values. Both IEF and high-performance size-exclusion chromatography of the antibody–antigen mixtures confirmed the formation of the complexes observed by CZE. This study demonstrates the utility of CZE as a new and complementary technique for the characterization of antibody–antigen complexes.

INTRODUCTION

Capillary zone electrophoresis (CZE) in free solution has gained widespread recognition as a powerful separation tool that is complementary to gel-electrophoretic and chromatographic techniques. Recent literature describes several applications, along with information on basic principles, instrumentation and methodology [1–11].

This paper describes the separation of antibody–antigen complexes formed by an immunochemical reaction by free-solution CZE; the antigen is a recombinant human growth hormone (hGH) that reacts with a monoclonal antibody [MoAb, immunoglobulin G (IgG) class] specific to hGH. The complexes were well resolved from the unreacted (or free) antibody and antigen in solution. Recently, Chen *et al.* [12] also reported a successful separation of antibody–antigen complexes by CZE. However, they employed an isoelectric focusing experiment that requires a stabilizing medium and ampholytes in capillary tubes. In our work, the separation was achieved in a free-flowing buffer solution without ampholytes or stabilizing media. Preliminary results of this work were reported elsewhere [6]. We also present data comparing CZE to conventional isoelectric focusing (IEF) and high-performance size-exclusion chromatography (HPSEC).

The monoclonal preparation employed in this study, at least in theory, contains

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a single, homogeneous population of antibody molecules specific to a well-defined epitope on the antigen. Thus, only two types of complexes [IgG–hGH and IgG–$(hGH)_2$] will be formed, that correspond to reaction at the two antigen binding sites of each antibody molecule:

$$IgG + hGH \rightleftarrows IgG\text{–}hGH \quad (1)$$

$$IgG\text{–}hGH + hGH \rightleftarrows IgG\text{–}(hGH)_2 \quad (2)$$

We will refer to these complexes in general as IgG–$(hGH)_n$, where n is the valency of the antibody. These complexes are non-covalent in nature, tightly held together by various combinations of electrostatic, hydrophobic, dipole–dipole, hydrogen bonding and Van der Waals interactions [13].

EXPERIMENTAL

Chemicals and reagents

Highly purified recombinant DNA-derived hGH was obtained from Lilly Research Labs. (Indianapolis, IN, U.S.A.). The anti-hGH monoclonal antibody (part No. 20065) was purchased from Hybritech (San Diego, CA, U.S.A.). This preparation was a murine IgG made by ammonium sulphate fractionation and subsequent purification by DEAE ion-exchange chromatography. Calibration standards (p*I* 3–9) for IEF and the precast polyacrylamide gels were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Tricine was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were analytical-reagent grade, and the solutions were made in distilled water, deionized using the Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus

CZE experiments were performed using an Applied Biosystems (Foster City, CA, U.S.A.) Model 270A instrument. The fused-silica capillary was 100 cm long (80 cm to the detector) with 50 μm I.D. and 360 μm O.D. (Polymicro Technologies, Phoenix, AZ, U.S.A.). The capillary was rinsed with the 0.10 *M* tricine buffer (pH 8) between injections. New capillaries were rinsed with 0.10 *M* NaOH, water and 0.10 *M* HCl prior to use. In all experiments, the detector end of the capillary was negatively polarized relative to the injector end. The IEF experiments were carried out on the Pharmacia PhastSystem (Pharmacia LKB Biotechnology). HPSEC was performed using a high-performance liquid chromatographic system consisting of a Hewlett-Packard Model 1050 series gradient pump (Waldbron Analytical Division, F.R.G.), ISS-100 sampling system (Perkin-Elmer Norwalk, CT, U.S.A.) and a Spectroflow Model 757 absorbance detector (Kratos, Ramsey, NJ, U.S.A.).

Electrophoretic conditions

CZE separations were performed in 0.1 *M* tricine buffer, pH 8, using an applied voltage of 30 kV, with a current of about 19 μA. Injection volumes were *ca.* 9 nl in all cases (3-s injection using a 127-mmHg vacuum), and the separated proteins were detected at 200 nm. Operating temperature was maintained at 30.0 ± 0.1°C.

In the IEF experiments, 1-μl volumes of solutions containing individual proteins or protein mixtures (prepared as described below) were directly applied to the precast polyacrylamide gels (5%) using special sample application combs provided for the Pharmacia PhastSystem. The gels were pre-focused for 75 V · h at 15°C, 2000 V, prior to sample application for 15 V · h (200 V, 2.5 mA, 3.5 W). Focusing was carried out at 15°C for 410 V · h during which time the voltage rose from 200 to 2000 V (2.5 mA, 3.5 W). Gels were fixed in 20% trichloroacetic acid and stained with Coomassie Blue R-350, following standard procedures described in the PhastSystem manual.

Chromatographic conditions

Separations were achieved with a GF-250 (25 cm × 9.4 mm) size-exclusion column (DuPont, Wilmington, DE, U.S.A.) at ambient temperature using a 0.20 *M* Na_2HPO_4 (pH 7.6) mobile phase flowing at 0.60 ml/min. Eluted peaks were detected at 214 nm. Sample injection was carried out using a 20-μl loop.

Sample preparation

Both hGH and the anti-hGH MoAb (IgG) were dissolved directly into or dialyzed against 0.1 *M* tricine buffer (pH 8). The final concentration of IgG was 3.03 mg/ml ($2.35 \cdot 10^{-5}$ *M*), and that of hGH was 0.508 mg/ml ($2.29 \cdot 10^{-5}$ *M*), based on molecular weights of 150 000 dalton for IgG and 22 124 dalton for hGH. These concentrations were determined by UV absorbance at 276 nm for hGH (a = 0.74 ml/mg · cm) and IgG (a = 1.40 ml/mg · cm), where a is the absorbance of a 1 mg/ml solution in a 1 cm path length cell. Appropriate volumes of hGH and IgG solutions were mixed to initiate the reaction. The approximate molar ratios of IgG:hGH in the mixtures used for CZE and HPSEC were 1:4, 1:2, 1:1, 2:1 and 3:1.

For the IEF experiments, hGH and the monoclonal IgG were dissolved in 0.05 *M* potassium phosphate buffer, pH 7.6. The final concentration of IgG was 2.2 mg/ml, and that of hGH was 1.0 mg/ml. Two solutions containing IgG and hGH were made by mixing appropriate volumes of the above protein solutions. The approximate molar ratios of IgG:hGH in the above mixtures were calculated to be 1:1.5 and 1:3.1.

RESULTS

The model antigen (hGH) and the monoclonal anti-hGH antibody (IgG) were readily available in highly purified preparations. They were well characterized in-house to establish purity, identity and binding properties. Results [14] showed that both preparations contained a single protein component of appropriate molecular mass by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (hGH *ca.* 22 124 dalton, IgG *ca.* 150 000 dalton). No significant protein impurities were detected when stained with Coomassie Blue R-350. The apparent affinity constant for the reaction (K_f) was *ca.* $1.0 \cdot 10^9$ l/mol by cold competition radioimmunoassay [14]. This strong binding affinity of the antibody for hGH results in strong, stable antibody–antigen complexes that form very rapidly in solution. Mixtures containing various molar ratios of IgG:hGH were analyzed by CZE, IEF and HPSEC to study the properties of the complexes formed. These techniques separate species based on differences in electrophoretic mobility, isoelectric point and size, respectively.

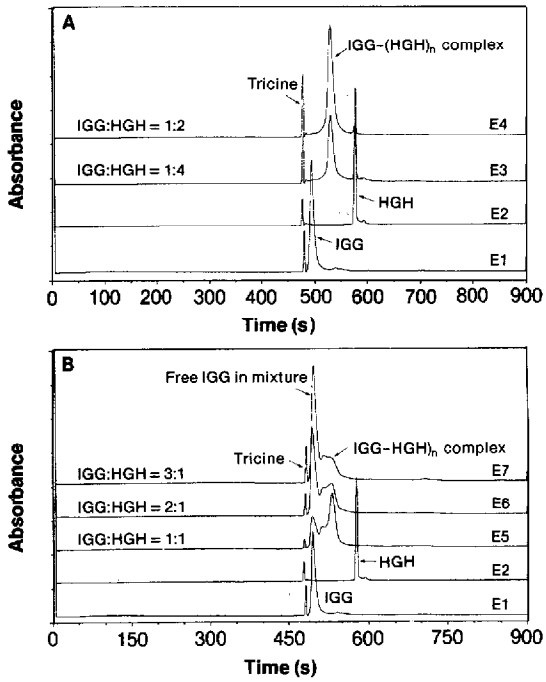

Fig. 1. Separation of IgG, hGH and IgG–$(hGH)_n$ complexes by CZE. (A) Electropherograms of IgG, hGH and mixtures containing an excess of hGH. (B) Electropherograms of IgG, hGH and mixtures containing an excess of IgG. Experimental conditions are described in the text.

CZE experiments

Fig. 1 shows seven electropherograms (E1–E7) representing separate injections. Electropherograms E1 and E2 are for individual injections of IgG and hGH, respectively. They show that these species have different migration times and are well resolved under the experimental conditions employed. Note that IgG migrates much faster than hGH, due to the fact that the p*I* for hGH is 5.2 and that for the monoclonal IgG is in the range 7.2–8.0 (multiple bands in Fig. 2). Therefore, hGH molecules have much greater negative charge than IgG molecules in the CZE separation buffer (0.1 *M* tricine, pH 8). This results in faster migration of the IgG molecules towards the negatively polarized detector end of the capillary. The first peak in all electropherograms is due to a slightly higher tricine concentration in the sample buffer compared to that of the separation buffer.

Electropherograms E3 and E4 show the results for 1:4 and 1:2 molar ratios of IgG:hGH, respectively (Fig. 1A). Based on migration-time comparison, the peak at *ca.* 531 s is a new molecular entity, most probably corresponding to one or more IgG–$(hGH)_n$ complexes. The peak at *ca.* 576 s corresponds to free hGH present in excess in both mixtures. As the IgG:hGH molar ratios decrease in the mixtures, one observes distinct changes in the peak profiles (Fig. 1B, electropherograms E5, E6 and E7). In electropherogram E5, the free IgG peak was observed at *ca.* 495 s along with two distinct peaks for IgG–$(hGH)_n$ complexes at *ca.* 513 and *ca.* 531 s, respectively.

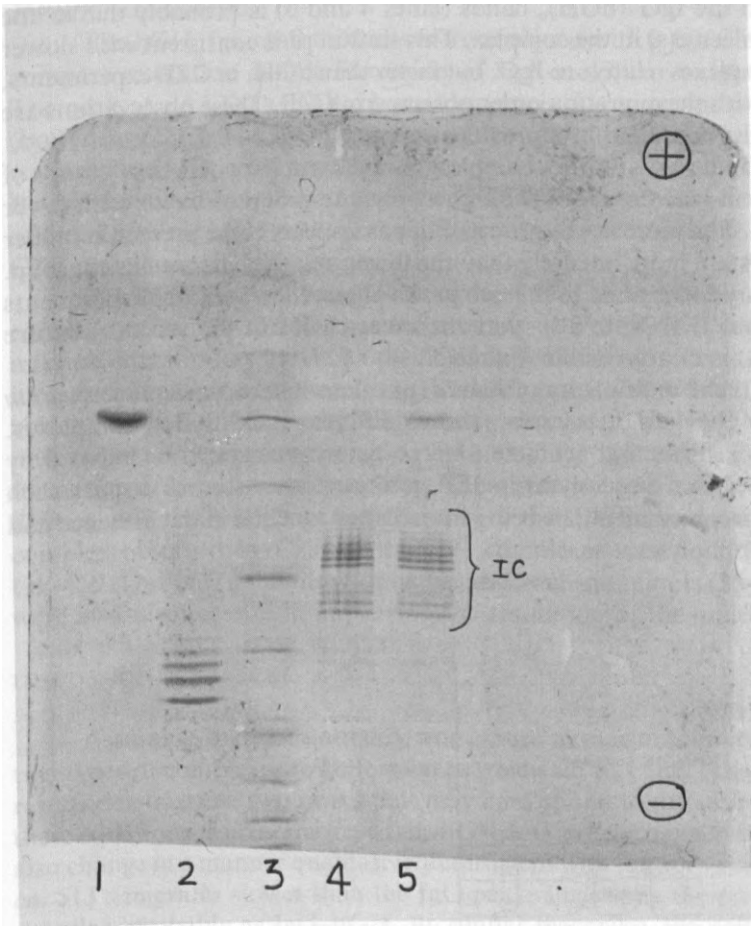

Fig. 2. Separation of IgG, hGH and IgG–$(hGH)_n$ (IC) complexes by IEF. Lanes: 1 = hGH; 2 = IgG; 3 = p*I* markers (p*I* 3–9); 4 = IgG:hGH mixture, molar ratio = 1:1.5; 5 = IgG:hGH mixture, molar ratio = 1:3.1. Experimental conditions are described in the text.

Note that the peak at *ca.* 513 s remains the same, but the late migrating peak for the second IgG–$(hGH)_n$ complex decreases rapidly as the hGH concentration is decreased (E6, E7). No excess hGH was detected in electropherograms E5, E6 and E7. IgG, hGH and various mixtures of IgG–hGH were analyzed on IEF gels (p*I* 3–9) and by HPSEC to further explore the nature of IgG–$(hGH)_n$ complexes observed in CZE experiments.

IEF experiments

IEF results in Fig. 2 show that the IgG–$(hGH)_n$ complexes were well resolved from free hGH, and appear not to dissociate significantly during the experiment. In addition, hGH shows a single band on the gel (p*I* 5.2), while IgG and the complexes show multiple bands with a p*I* range of 7.2–8.0 and 6.1–7.0, respectively. The acidic

shift associated with the IgG–$(hGH)_n$ bands (lanes 4 and 5) is probably due to the presence of hGH molecule(s) in the complex. This shift in p*I* is consistent with slower migration of the complexes relative to IgG, but faster than hGH, in CZE experiments, and correlates well with the migration order observed in CZE. These observations are consistent with results described in a previous report [4].

In lanes 4 and 5, the IgG–$(hGH)_n$ complexes are present in two distinct groups of bands, whereas IgG in lane 2 was resolved into only one group of bands with fewer components (Fig. 2). The more acidic group of bands appears to be present in higher amounts, since they stain more intensely than the lower, relatively more basic, group. These two groups may correspond to the two peaks observed in the CZE experiments in Fig. 1 (E5, E6 and E7). Note also that the excess hGH in the second mixture (IgG:hGH = 1:3.10) is clearly visible in lane 5.

It is not clear if the multiplicity of bands (or micro-heterogeneity) seen *within* these two groups on IEF gels does correspond to different IgG–$(hGH)_n$ complexes. Previous work [15,16] shows that artifactual micro-heterogeneity can be induced by proteins binding to carrier ampholytes in IEF gels, but other studies dispute such observations [17]. This phenomenon is being investigated by CZE in the presence and absence of carrier ampholytes.

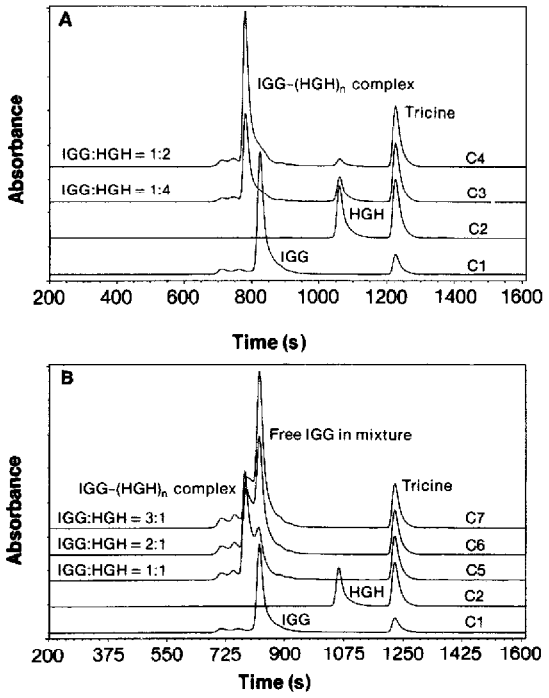

Fig. 3. Separation of IgG, hGH and IgG–$(hGH)_n$ complexes by HPSEC. (A) Chromatograms of IgG, hGH and mixtures containing an excess of hGH. (B) Chromatograms of IgG, hGH and mixtures containing an excess of IgG. Experimental conditions are described in the text.

HPSEC experiments

The hGH, IgG and mixtures were analyzed with a GF-250 HPSEC column to compare resolution and/or formation of high-molecular-weight complexes (Fig. 3). The peak at *ca.* 1220 s in all chromatograms is due to tricine present in the samples (buffer for CZE). The IgG peak elutes at *ca.* 820 s (chromatogram C1) while hGH is well resolved at *ca.* 1050 s (chromatogram C2). Chromatograms C3–C7 in Fig. 3A and B represent various molar ratios of the IgG:hGH mixtures that show varying amounts of free IgG, free hGH and the complexes. These were the same mixtures analyzd by CZE and shown in Fig. 1A and B.

Chromatograms C3 and C4 show free hGH and probably a single IgG–$(hGH)_n$ complex at *ca.* 770 s; note that the amount of free hGH decreases in C4 since the mixture contains less hGH. As the hGH:IgG molar ratio decreases, one observes an increased peak area for free IgG (chromatograms C5, C6 and C7 in Fig. 3B); the amount of IgG–$(hGH)_n$ complex decreases accordingly. Note that HPSEC resolves one major peak corresponding to IgG–$(hGH)_n$, whereas CZE clearly shows the formation of two distinct complexes. Ionic strength and buffer composition changes did not sufficiently improve the efficiency of the HPSEC separation so that the second complex observed by CZE or multiple complexes seen by IEF could be resolved. GF-450 (DuPont) columns with higher size-exclusion limits (25 000–900 000 dalton) were also unsuccessful in improving the resolution of the multiple complexes.

DISCUSSION

Assuming that the antibody was a pure monoclonal preparation, we expected two types of complexes to be formed as predicted by eqns. 1 and 2. Indeed, the CZE results demonstrate two peaks that may correspond to the stoichiometry assumed in these equations. Furthermore, as the hGH:IgG ratios change, the relative peak ratios also change in a manner qualitatively consistent with the above equations. The peak at *ca.* 513 s migrates slower than the IgG peak, suggesting the presence of hGH in the complex, probably as IgG–hGH. By similar reasoning, the peak at *ca.* 531 s should contain more hGH and, therefore, corresponds to IgG–$(hGH)_2$. The IEF results support the formation of two types of complexes, but does not reveal the stoichiometry. Analysis by HPSEC demonstrates the formation of a major complex peak, larger than the antibody itself, but does not conclusively demonstrate the formation of multiple complexes observed in CZE and IEF experiments. Attempts to estimate the molecular weight of the IgG–$(hGH)_n$ complex uisng high-molecular-weight protein calibrators were unsuccessful because the optimum separation conditions for the calibrators were not the same as that for the antibody–antigen complexes. Thus, we were unable to verify the molecular size or stoichiometry of the complexes observed in CZE, IEF and HPSEC.

In addition, we found that the total peak area by CZE for all peaks containing IgG is proportional to the amount of added IgG. Since the absorption at 200 nm is essentially independent of complex formation, this implies that (1) no material is lost by adsorption or other processes, and (2) all IgG is distributed between the three species observed. It is also apparent that the CZE peak at *ca.* 513 s is never a predominant species. These observations indicate that the complex at *ca.* 513 s may have a lower equilibrium constant (*i.e.*, lower stability) than the complex at *ca.* 531 s.

Note that the peaks for IgG and the IgG–$(hGH)_n$ were relatively broad in CZE. Multiply charged IgG species with different electrophoretic mobilities or analyte interactions with the wall may cause such broadening. It is worth noting, however, that the complexes (*ca.* 172 000–194 000 dalton) and IgG (*ca.* 150 000 dalton) were much better resolved by CZE than by HPSEC, and that the CZE separations were much faster (Figs. 1 and 3). Also, to our knowledge, these are the largest protein moieties separated by free solution CZE in less than 10 min with reasonable resolution.

CONCLUSIONS

CZE has been successfully employed to separate antibody–antigen complexes. Formation of stable IgG–$(hGH)_n$ complex(es) were confirmed by IEF and HPSEC experiments, but CZE clearly supports the formation of two types of complexes as predicted by theory. The IEF results show that the p*I* values for the complexes were more acidic than for free IgG, but more basic than hGH. The relative migration times for the three species in CZE experiments are consistent with the p*I* values observed. The CZE experiments were simple, fast, and can be readily automated with instruments that are commercially available [18].

In the long run, CZE may be a useful tool to study not only the formation of antibody–antigen complexes, but also the dynamics of other macromolecular interactions where biologically significant complexes of varying "stability" are formed. The advantage of free-solution CZE is that no supporting media such as chromatographic packings or gels are employed, which may enhance the dissociation of weak, non-covalent complexes during separation [19–21].

ACKNOWLEDGEMENTS

We thank David R. Smith for his patient and expert technical assistance during the course of this work. We also express thanks to Applied Biosystems Inc., Santa Clara, CA, U.S.A., for providing equipment used in this study.

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