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Analysis of bovine immunoglobulin G by capillary gel electrophoresis

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Abstract: A method for the analysis of bovine immunoglobulin G (IgG) using sodium dodecyl sulphate capillary gel electrophoresis (SDS–CGE) has been described. Under the electrophoretic conditions employed, monomeric and dimeric IgG were readily resolved, as were light chain and heavy chain subunits, and heavy chain dimers in reduced samples. Molecular weights determined by SDS–CGE compared favourably with those measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and published values. Reproducibility of protein quantitation was achieved resulting in a relative standard deviation of approximately 13% and calibration was linear in the range of 0.2–3.5 mg ml^{-1} protein under the conditions used.

Keywords: *Capillary gel electrophoresis; bovine IgG; molecular weight analysis.*

Introduction

Immunoglobulins (Ig) are glycoproteins which occur in the serum and secretions of vertebrates and represent one of the major protein classes present in blood. The IgG class is the most abundant of the immunoglobulin classes found in serum, milk and colostrum. An immunoglobulin molecule comprises two heavy (H) and two light (L) polypeptide chains connected by disulphide linkages to produce a characteristic Y-shaped structure. Functionally, these proteins contain variable amino acid sequence regions which configure to generate specific binding sites in response to antigenic stresses. Recombinant DNA technology applied to the preparation of monoclonal antibodies and general strategies of 'harvesting' therapeutic immunoglobulins are of increasing scientific and medical interest. As such, concomitant analytical technologies of improved convenience and automation are required to characterize and quantitate these molecules.

Capillary gel electrophoresis (CGE) represents a recent advance in capillary electrophoresis (CE) which has allowed successful analysis of oligonucleotides [1], DNA fragments [2] and proteins [3]. The use of gel-filled capillaries addresses several practical problems encountered in conventional CE analysis as gels are anti-convective, minimize solute diffusion, prevent solute adsorption to the capillary wall and suppress electroosmosis [4].

The interdependence of gel properties on practical performance and separation characteristics is a result of the crucial role of the sieving polymer concentration and chain length in determining both viscosity and mobility while simultaneously dictating pore size and molecular resolution [5–8]. Gel mobility is dependent on the polymer concentration and/or the presence of chemical cross-links. The resulting viscosity characteristics finally dictate whether the sieving matrix may be removed and replaced under pressure and so improve analytical reproducibility [3]. The molecular sieving effect has been demonstrated using both cross-linked polyacrylamide, or 'chemical gels', and a range of linear polymers, or 'physical gels', [3, 5] indicating that both types of pore structure are effective in differentiating molecular weight.

The technology developed for separation of oligonucleotides and proteins has diverged recently in response to the intrinsically differ-

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ing analytical requirements of each species with respect to gel pore size. Oligonucleotides are typically smaller than proteins and high resolution is desirable for separation of fragments potentially differing in mass by a single base. For oligonucleotides, polyacrylamide gel-filled capillaries, either cross-linked [1, 9] or non-cross-linked [2], are prepared using additives to manipulate pore size, or improve separation and detection [10]. Higher polyacrylamide concentrations are employed to achieve a smaller pore size which renders such gels physically, if not chemically, immobile to displacement. While resolution is excellent [1, 11], limited column lifetime [6, 9] inhibits full automation of this technology. The use of larger mesh, lower concentration, molecular sieving polymer matrices in a 'replacement' mode have also been reported for separation of higher molecular weight (M_r), double-stranded DNA fragments [10].

CGE analysis of typically larger protein molecules necessitates the use of larger pore, less viscous polymeric sieving agents which are conducive to replacement between analytical runs [5]. Coated capillaries are generally utilized to lower or reverse the endoosmotic flow [3] so that the gel is not displaced during electrophoresis and to reduce protein interactions with the capillary wall [12]. In a recent study describing protein analysis using non-cross-linked polyacrylamide, the dependence of the M_r resolution range on acrylamide concentration and viscosity was shown [3]. Also, the tendency for electrophoretic gel displacement by electro-osmosis could be offset by increasing the gel viscosity which obviated the need for use of a coated capillary [3]. Other molecular sieving polymers used in the 'replaceable' gel mode have been recently reported [5]. For example, Hjerten *et al.* demonstrated a combined effect of hydrophobic interaction using stearyl dextran in conjunction with the molecular sieving agent, ethylene glycol, to achieve electrophoretic resolution of proteins based on size [13].

This paper presents an application of SDS–CGE for the qualitative and quantitative analysis of bovine IgG together with details of method validation. Electropherograms illustrate the characteristic molecular size distribution profiles of bovine IgG in both non-reduced and reduced forms revealing monomeric and H and L chain subunits, respectively.

Experimental

Materials

HEPES (BDH Chemicals, Poole, England), MES (The Sigma Chemical Co., St Louis, MO), other buffer salts and chemicals were of analytical reagent quality. Sodium azide (general purpose reagent) was obtained from BDH Chemicals, (Poole, UK), polyethylene glycol 4000 (PEG, laboratory reagent) from BDH Chemicals Australia (Kilsyth, Victoria) and purified agar (code L28) from Oxoid Ltd, (Hampshire, UK). Dithiothreitol (DTT) was obtained from Pierce (Rockford, IL), SDS (electrophoresis grade) from Eastman Fine Chemicals (Rochester, NY) and Orange G and molecular weight protein standards were from Beckman Instruments (Palo Alto, CA). Water was obtained from a Milli-Q water purification system (Millipore, Milford, MA).

Bovine colostrum was collected and processed as described by Davidson *et al.* [14] and was supplied in powder form by Northfield Laboratories (Hillcrest, South Australia). IgG was isolated from the colostrum (cIgG) by first removing casein proteins by isoelectric precipitation at pH 4.6 [15], readjusting the pH to 7.0 and then extracting IgG from the remaining whey protein fraction by Protein G Sepharose immunoaffinity chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). IgG was bound to the Protein G matrix using pH 7.0 buffer (20 mM sodium phosphate) and eluted (after washing of unbound material) with pH 2.7 buffer (100 mM glycine). Elution buffer salts were removed using a PD10 Sephadex G-25M gel filtration column (Pharmacia). The aqueous, salt-free IgG solution was then freeze-dried and stored under dessication at 4°C.

The purity and biological activity of cIgG was verified by several techniques comparing results, where appropriate, with standard bovine IgG extracted from serum (sIgG, The Sigma Chemical Co.). The biological activity of cIgG was verified by immuno-double-diffusion assay with precipitation observed on reaction of cIgG with anti-bovine IgG serum (raised in sheep, Silenus Laboratories, Melbourne, Australia) [16]. The diffusion matrix comprised 1.5% (w/v) agarose, 3.0% (w/v) PEG 4000 and 0.1% (w/v) sodium azide preservative in phosphate-buffered saline, pH 7.0.

Instrumentation

Capillary electrophoresis was performed on a Beckman P/ACE system 2100 (Beckman Instruments, Palo Alto, CA) using the proprietary 'eCAP' SDS 200 coated capillary (47 cm × 100 μm i.d.). The polarity of the instrument was reversed with the capillary inlet and outlet connected to the negative and positive terminals, respectively. UV detection at 214 nm was employed, monitoring at 40 cm from the capillary inlet, capillary temperature was maintained at 20°C and separations were performed using an applied voltage of 14.1 kV. Samples were injected by 50-s pressure injection which corresponded to approximately 600 nl. Data acquisition and analysis was controlled by Beckman System Gold software (version 6.01).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted using a Phast-System automated separation and development unit (Pharmacia). Gradient 4–15 gels and SDS buffer strips (both Pharmacia) were used in conjunction with recommended separation protocols. Gels were stained using Coomassie Brilliant Blue R350 (Pharmacia) according to the recommended development procedures.

Sample preparation and analysis

SDS–CGE. The determination of molecular weights of cIgG and sIgG, quantitation of protein concentration and method validation studies were performed by SDS–CGE as follows. Proteins stock solutions were prepared in either 10 mM HEPES-MES, pH 7.0 buffer or water and filtered (0.2 μm) before dilution in CE sample buffer (240 mM Tris–HCl, pH 6.6, 2% SDS, ±2% DTT) and addition of an aliquot (10% v/v) of Orange G internal standard. Samples were heated under non-reducing conditions (−DTT) at 60°C for 15 min or under reducing conditions (+DTT) at 100°C for 10 min. The latter treatment was used to reduce monomeric IgG molecules into their component H and L chains. The SDS–CGE analytical method for IgG and standard proteins routinely included loading 60 μl of sample into 'micro' sample vials and sealing the vial with a rubber septum. The programmed method included a wash step (0.1 M HCl, 3.0 min), replacement of gel buffer (3.0 min), pre-injection electrophoresis (14.1 kV, 15 min), sample injection (pressure, 50 s) and electrophoresis (14.1 kV, 30 min).

Pre-injection electrophoresis of fresh gel buffer was found to be necessary to ensure a flat, non-drifting baseline. Following pre-injection electrophoresis and before sample injection, a brief rinse of the inlet capillary end in water was included to avoid sample dilution by 'clinging' droplets of gel buffer remaining on the capillary. Following analysis, the capillary was rinsed with 0.1 M HCl and loaded with fresh gel buffer. Capillary 'aging' after several months of intensive use was observed as baseline noise and peak broadening, particularly if a higher concentration of HCl (e.g. 1.0 M) was used during the wash step. This resulted in a significant reduction in the qualitative and quantitative capacity of the method and the ultimate need to replace the capillary. Negligible column deterioration was observed following more than 400 analyses when 0.1 M HCl was used for washing.

The capillary was calibrated using molecular weight standard proteins ranging from 29 to 250 kDa. The standards were prepared as recommended by the manufacturer and frozen aliquots were thawed and treated as required. Measured migration times were corrected to that of the internal standard, Orange G, to account for between run variation.

A calibration curve for IgG quantitation was obtained by dilution of a filtered stock solution of cIgG (6.7 mg ml^{-1}) in 10 mM HEPES/MES buffer, pH 7.0 to generate protein concentrations over the range 0.2–3.5 mg ml^{-1}. Duplicate samples of each concentration were treated under non-reducing conditions as described above. Within assay and between assay reproducibility were studied by preparing filtered stock solutions (3.6 mg ml^{-1}) of cIgG in water. Concentrations were adjusted to either 0.5 or 1.9 mg ml^{-1} prior to dilution in CE sample buffer and treatment under non-reducing conditions. SDS–CGE analysis was performed by five injections from each sample vial. Results presented represent averaged data from two consecutive days' analysis of each of two fresh protein stocks.

SDS–PAGE. The molecular weights of cIgG and sIgG were determined by SDS–PAGE as follows. Protein solutions containing final reagent concentrations of 1.2 mg ml^{-1} protein, 15 mM Tris–HCl, pH 8.0, 1.5 mM EDTA, 1.5% SDS and ±4% mercaptoethanol, were prepared. Samples were heat-treated under reducing or non-reducing conditions as for SDS–CGE samples. Molecular weight protein

standards, as used for calibration of the SDS–CGE system, were prepared as recommended and run concurrently with cIgG and sIgG.

Results and Discussion

Molecular weight determination by SDS–CGE

The replaceable gel employed for the assay of bovine IgG contained a proprietary molecular sieving agent and SDS. The reversed polarity mode of CE operation ensured that, in the presence of SDS, negatively charged protein species migrated from the negative inlet to the positive outlet of the capillary. Separation relied on SDS-denatured proteins being subject to electrophoresis whereby species of similar charge-to-mass ratios migrated at a size-only dependent rate. Thus, the generally observed linear relationship between electrophoretic mobility, E_m, and logarithm of molecular weight, M_r, enabled calibration for the determination of unknowns [8].

The response of the gel-filled capillary with respect to migration times of analyte proteins was calibrated using molecular weight standards. Reciprocal migration time, which is proportional to E_m was plotted as a function of log M_r to obtain a calibration plot over the range 30–205 kDa (Fig. 1). The plotting of calibration data as shown illustrates the linear correlation between log M_r and $1/T_m$ (correlation coefficient of 0.994). This finding contrasts with the correlation of E_m (or $1/T_m$) with $q/M_r^{2/3}$ as observed in conventional CE, where q represents the protein charge [17]. Thus, the solvation of proteins by SDS eliminates the dependence of mobility on protein charge and invokes the empirical correlation of E_m with log M_r.

Corrected migration times of standard and analyte proteins were found to be highly reproducible using SDS–CGE. Relative standard deviation (RSD) values for standard proteins was found to average 0.5% (n = 10) in comparison with an average value of 2.7% for non-reduced and reduced IgG. Thus, given reproducible migration behaviour, accurate M_r determination depends crucially on ideal, charge-independent, migration behaviour of standard proteins.

Typical electropherograms of non-reduced and reduced IgG are shown in Fig. 2(a) and (b), respectively. The profiles were analogous

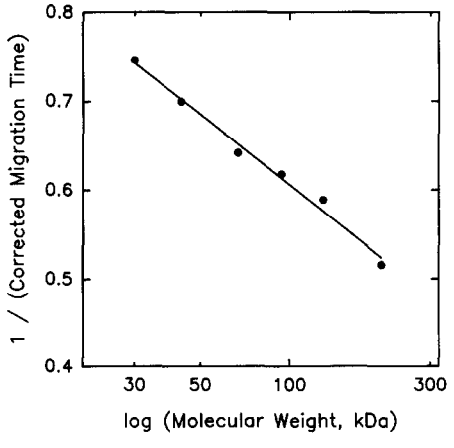

Figure 1
Calibration plot of reciprocal (migration time) vs log (molecular weight) of protein standards. See text for details of sample preparation and analysis. Molecular weight standard proteins were: carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; phosphorylase b, 94 kDa; beta-galactosidase, 130 kDa; and myosin, 205 kDa. Correlation coefficient was 0.994.

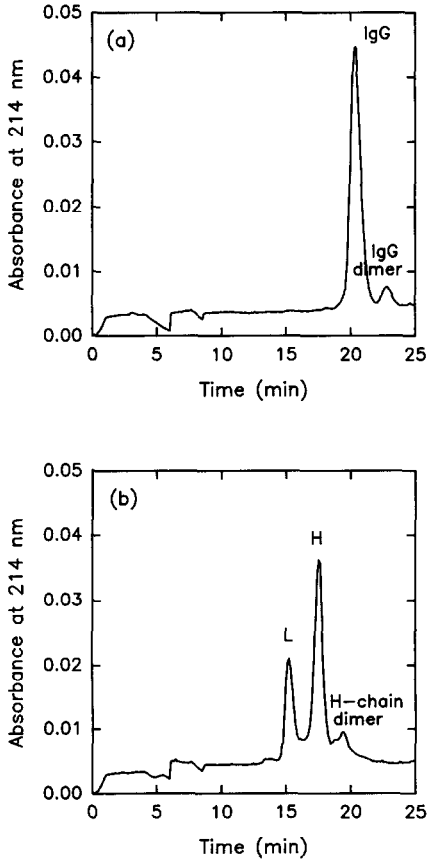

Figure 2
(a) Electropherograms of 2 mg ml^{-1} non-reduced bovine colostral IgG also showing dimeric IgG and (b) reduced bovine colostral IgG showing heavy chain (H), light chain (L) and H-chain dimer. See text for details of sample preparation and analysis.

to results obtained with SDS–PAGE gels (not shown) where larger protein species had lower electrophoretic mobilities. The smaller peaks which eluted at approximately 23 and 19 min in Fig. 2(a) and (b) corresponded to dimeric IgG ($c(IgG)_2$) and heavy chain dimers ($c(H)_2$), respectively. The presence of these species is consistent with other reports for bovine IgG where dimeric species were not disassociated by treatment with 1% SDS, indicating covalent linkages [18]. The sustained appearance of heavy chain dimers in the electropherogram of reduced IgG strongly suggested that the covalent link present in dimeric non-reduced IgG was non-disulphide in nature and was localized between the heavy chains.

SDS–CGE-determined molecular weights of cIgG, sIgG and their respective H and L chains are presented in Table 1 together with values determined by SDS–PAGE. For comparison, M_r values of cIgG, sIgG and their subunits are also presented. The nominal molecular weights of cIgG and sIgG were calculated as the mass-weighted average of the IgG_1 (162 kDa) and IgG_2 (152 kDa) subclasses where the IgG_1:IgG_2 ratio in colostrum and serum is 16:1 and 1.2:1, respectively [15]. The three sets of data are in reasonable agreement, to within experimental error, and exemplify the utility of SDS–CGE for M_r determination of bovine IgG and derived species.

SDS–CGE results for heavy and light chains of cIgG and sIgG were slightly higher than expected. These results may reflect the non-constant ratio of SDS to protein mass in standard proteins which introduces dependence of mobility upon net charge and non-ideal migration times. Molecular weights of $c(IgG)_2$ and $c(H)_2$ were determined to be 260 and 124 kDa, respectively. While the result for $c(H)_2$ is close to the expected value (114 kDa), the low value obtained for $c(IgG)_2$ (expected result, 322 kDa) exemplifies that this molecular weight region is beyond the calibration range capability of this capillary where an increasingly non-linear relationship prevails.

Quantitation of protein concentration by SDS–CGE

The suitability of SDS–CGE for quantitative analysis of bovine IgG was investigated by measuring the calibration of total peak area vs total protein concentration (monomer + dimer). A typical calibration curve obtained under standardized analytical conditions is shown in Fig. 3. The calibration was found to be linear ($r^2 = 0.9995$) over the protein concentration range studied (0.2–3.5 mg ml^{-1}).

Reproducibility of peak areas for five replicate analyses of a low (0.5 mg ml^{-1}) and high

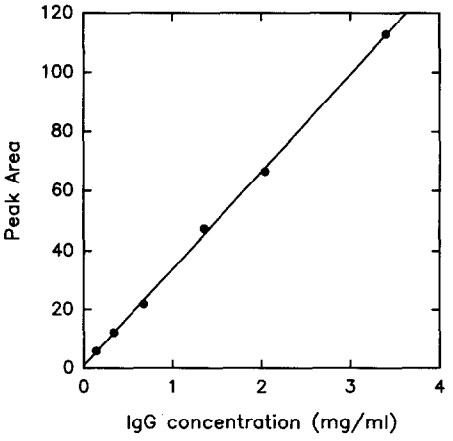

Figure 3
Calibration curve of peak area vs total concentration of non-reduced bovine colostral IgG (monomer + dimer). See text for details of sample preparation and analysis.

Table 1
Summary of molecular weights (M_r) of bovine colostral IgG (cIgG) and serum IgG (sIgG) determined by SDS–CGE and SDS–PAGE compared with published values

Protein	M_r-SDS–CGE (kDa)	M_r-SDS–PAGE (kDa)	M_r-published* (kDa)
cIgG	155	151	161
cIgG-H†	73	61	57
cIgG-L‡	32	28	25
sIgG	149	150	158
sIgG-H†	63	56	56
sIgG-L‡	32	27	24

*Calculated as the mass weighted average of IgG_1 and IgG_2 subclasses and component heavy and light chains, from the proportions of each subclass present in serum and colostrum, respectively [14].
†Reduced IgG-heavy chain.
‡Reduced IgG-light chain.

(1.9 mg ml^{-1}) IgG concentration stock solution were as follows. Within-assay and between-assay CV values were 14.1 and 15.1%, respectively, for the low and 11.0 and 10.3%, respectively, for the high IgG concentration. RSD values for peak areas of 10 replicate analyses of the standard proteins was found to average 10.0% which is comparable to that found for bovine cIgG.

The similarity of RSD values obtained for cIgG and standard proteins suggests that analytical reproducibility was not sample-related but most likely reflected variability in the sample introduction process. The use of a coated capillary and the presence of SDS in the gel and sample matrices would be expected to eliminate sample loss due to capillary wall adsorption. Discrepancy between the viscosity of the sample and gel matrices may have contributed to inconsistency of injection volumes and the correction of analyte peak area to that of the internal standard may be advisable.

SDS–CGE analysis of proteins offers advantages of speed and automation for both M_r determination and quantitation over other conventional methods such as SDS–PAGE and size exclusion chromatography (SEC). Data obtained using SDS–CGE were in good agreement with SDS–PAGE which has historically been the most frequently adopted method of M_r determination for proteins. While the data obtained with SDS–CGE were not directly compared to those obtained with SEC, interactions between protein molecules and several of the common SEC matrices can lead to non-ideal elution characteristics precluding the utility of SEC for accurate M_r determination [20]. Both SDS–CGE and SEC are highly quantitative and amenable to numerous on-line detection modes (e.g. UV, fluorescence) while SDS–PAGE relies upon staining and densitometric scanning for quantitation. As both SDS–CGE and SDS–PAGE are carried out under denaturing conditions (i.e. in the presence of SDS), neither technique is suitable for assessment of non-covalent protein association or aggregation processes. In contrast, SEC analysis in a non-denaturing mode can be used to study these phenomena.

Conclusions

Protein analysis by SDS–CGE involving a replaceable sieving gel matrix was successfully applied to analysis of bovine IgG. Structural subunits of IgG including monomer, dimer, heavy chains, light chains and heavy chain dimers were readily resolved. Molecular weights of these species compared favourably with results using SDS–PAGE and with published values. The quantitation of IgG was possible with the within assay variability of peak areas being approximately 13%.

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