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Characterization of each isoform of a $F(ab')_2$ by capillary electrophoresis

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ABSTRACT

Free solution capillary electrophoresis was investigated for the characterization of an M_r 100 000 purified $F(ab')_2$. Optimization of the experimental conditions allowed the identification of five separated peaks, suggesting the presence of isoforms which differed by only 0.2 pH unit. This heterogeneity was still detectable with 80 amol of protein. After a preparative separation by chromatofocusing, identification of each form was performed for the first time by capillary electrophoresis. A quantitative and qualitative correlation with isoelectric focusing showed that free solution capillary electrophoresis represents a sensitive method for revealing subtle differences in charge, even for large proteins.

INTRODUCTION

Wenisch *et al.* [1] described different forms of a purified immunoglobulin using isoelectric focusing (IEF). This heterogeneity was explained by the binding to carrier ampholytes or by the presence of different immunoglobulin G (IgG) complexes [2]. In contrast, Moellering *et al.* [3] attributed this physico-chemical difference to a deamidation of the monoclonal antibody studied. Before entering a protein product in clinical trials, it is important to develop different techniques to identify such heterogeneity as far as possible.

An alternative method, rapidly becoming the separation technique of choice for the analysis of polypeptides, is capillary electrophoresis. Lauer and McManigill [4] separated two small peptides of 162 amino acids for which the p*I* values differed by 0.1 pH unit, indicating that the large apparent number of theorical plates allowed discrimination between small differences in charge densities. Nevertheless, most publications on capillary electrophoresis have been concerned with oligonucleotides, amino acids or low-molecular-mass products [5,6], and only a few data have been presented on proteins [7,8].

Among the different methods, free solution capillary electrophoresis (FSCE) is well suited for analytical studies on proteins in their native state and allows an increased separation efficiency by a limited zone broadening effect [9]. However, Grossman *et al.* [10] have shown that, as the electrophoretic mobility of a peptide is related to its charge/size ratio, serious adsorption problems can alter the mobility of large proteins and prohibit proper separations by FSCE. One option for avoiding wall adsorption of proteins in solution is to operate at a pH that is near or below the point of zero charge for the fused silica (*ca.* pH 2) [11]. Unfortunately, this is a pH at which most proteins are not in their native conformation. An alternative procedure that can be used to eliminate the effect of solute–wall interactions is to operate at a pH

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above the p*I* of the protein, resulting in a repulsion between the capillary wall and the molecule [4]. Further, as FSCE is a method strongly dependent on charge differences between proteins, one can manipulate the selectivity of a separation by modifying the pH of the running buffer [12]. Recently, FSCE has been used to characterize isoforms of recombinant erythropoietin [13], tissue plasminogen activator [14] and a monoclonal antibody [15].

This paper describes capillary electrophoresis as an alternative technique to isoelectric focusing for the identification and determination of isoforms of a purified $F(ab')_2$. The effects of separation variables such as pH, voltage and buffer type on the resolution and sensitivity of the method were determined. Further, to ascertain that this $F(ab')_2$ heterogeneity was not due to the presence of residual unproteolysed IgG, a kinetic analysis of pepsination of the antibody was also followed by FSCE. As the different forms have the same molecular mass of 100 000, they should differ essentially in their charge. Thus, a comparative analysis of the product was performed using classical isoelectric focusing. Another limitation of this technique is the identification of the different peaks observed on the electropherograms. Separation of the different forms of the $F(ab')_2$ by preparative chromatofocusing [16] led to the first identification by capillary electrophoresis of each $F(ab')_2$ isoform.

EXPERIMENTAL

Kinetics of pepsination

The IgG1 anti FcγRI [17] was obtained and purified as already described [18], then buffer-exchanged into phosphate-buffered saline (PBS) (pH 7.2). The antibody was incubated at 37°C in 1.5 *M* sodium citrate (pH 3.5) with pepsin (specific activity 4770 U/mg) (Sigma) in a ratio 20:1 (w/w). Hydrolysis was stopped increasing the pH to 7.2 (with 2 *M* K_2HPO_4). The samples were then dialysed against 20 m*M* phosphate buffer (pH 6.8).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli [19]. Aliquots were diluted in sample buffer [3% (v/v) glycerol–0.125 *M* Tris–HCl–5% (w/v) SDS–0.1% (w/v) bromophenol blue] and heated for 5 min at 95°C under non-reducing conditions. Samples were placed on a 3% (w/v) stacking gel and 7.5% (w/v) separating gel using a Phast-System (Method 110.1; Pharmacia, Uppsala, Sweden), then silver stained (Method 210.2). A mixture of high-molecular-mass proteins (Bio-Rad Labs.) was used as markers. Quantification was achieved by densitometric scanning of the gels (Preference, Sebia, France).

High-performance size-exclusion chromatography (HPSEC)

The IgG and the fragments were resolved by HPSEC (Waters Model 810 liquid chromatographic system) on a 5-μm TSK G3000 SWXL column (300 × 7.5 mm I.D.) equilibrated with 20 m*M* phosphate buffer containing 0.2 *M* NaCl (pH 7). The proteins were eluted at a flow-rate of 0.5 ml/min and detected at 280 nm. Calibration of the column was performed by injection of a mixture of standard proteins (ferritin, M_r 450 000; catalase, 240 000; aldolase, 158 000; bovin serum albumin, 68 000; chymotrypsin, 25 000). The peaks were integrated with Maxima 810 software.

Capillary electrophoresis

Protein separation was performed with an automated capillary electrophoresis system (Model 270A; Applied Biosystems, San Jose, CA, USA) in a fused-silica capillary (72 cm × 50 μm I.D.) thermostated at 30°C. Before each injection, 0.1 *M* sodium hydroxide and the running buffer were passed through the capillary for 2 and 4 min, respectively. Samples were diluted to 100 μg/ml in water and injected at the anodic side for 1 s under vacuum (5 in.Hg). The absorbance at 200 nm was detected on-line at the cathodic side and recorded on a Spectra-Physics SP 4400 integrator (chart speed 1 cm/min) with a signal attenuation of 8.

The mobility (μ) was calculated according to the following equation:

$$\mu = (L_d L_t / V)[(1/t_m) - (1/t_{eo})]$$

where L_d is the length of the capillary from the anode to the detector, L_t is the total length of

the capillary, V is the applied voltage, t_m and t_{eo} are the migration times of the sample and the electroosmotic flow, respectively.

Isoelectric focusing (IEF)

The gels (PAGplates 3.5–9.5; Pharmacia) were run at 50 mA for 1.5 h and stained with Coomassie Brilliant Blue. p*I* markers (p*I* 3–10) were used for calibration (Pharmacia).

Preparative chromatofocusing

The column (mono P HR 5/20; Pharmacia) was equilibrated with two column volumes using 10 m*M* 2-(N-cyclohexylamino)ethanesulphonic acid (CHES) buffer (pH 9) and the proteins (0.5 mg) were eluted with a pH gradient using Polybuffer 96 diluted eightfold in 10 m*M* CHES buffer (pH 6) (flow-rate 0.1 ml/min). The absorbance at 280 nm and the pH were monitored on-line. Each fraction collected was dialysed against 20 m*M* phosphate buffer (pH 6.8).

RESULTS AND DISCUSSION

IgG and F(ab')$_2$ characterization

The products obtained after pepsin proteolysis of the purified antibody were characterized by SDS-PAGE (Fig. 1). A major band of M_r 150 000 (lane A) corresponding to the M_r of the IgG1 was seen before pepsination, whereas after 20 min of incubation with the enzyme, only one band of M_r 100 000 (lane B), corresponding to the F(ab')$_2$ fragment, was observed. The homogeneity of both preparations was determined by gel filtration. The two peaks (peaks 1 and 2) corresponded to polypeptides of M_r 150 000 and 100 000, respectively. The purity of each product, measured by peak integration, was 98%.

Discrimination of F(ab')$_2$ isoforms by FSCE

Effect of pH. At pH 2.5 the F(ab')$_2$ were positively charged and migrated toward the cathode, with a negligible electroosmotic flow, in a unique peak detected 78.47 min after injection (Fig. 2A). At pH 8.3, when the electrophoretic mobility of the protein and the electroosmotic flow were opposite, three peaks were detected 28.36 min after injection (Fig. 2B). The negative peak observed was due to the electroosmotic flow. At pH 9.5 five peaks characterized the F(ab')$_2$ (18 min, Fig. 2C), and a preceding peak corresponded to the sample buffer (16.8 min). Compared with the previous electropherograms,

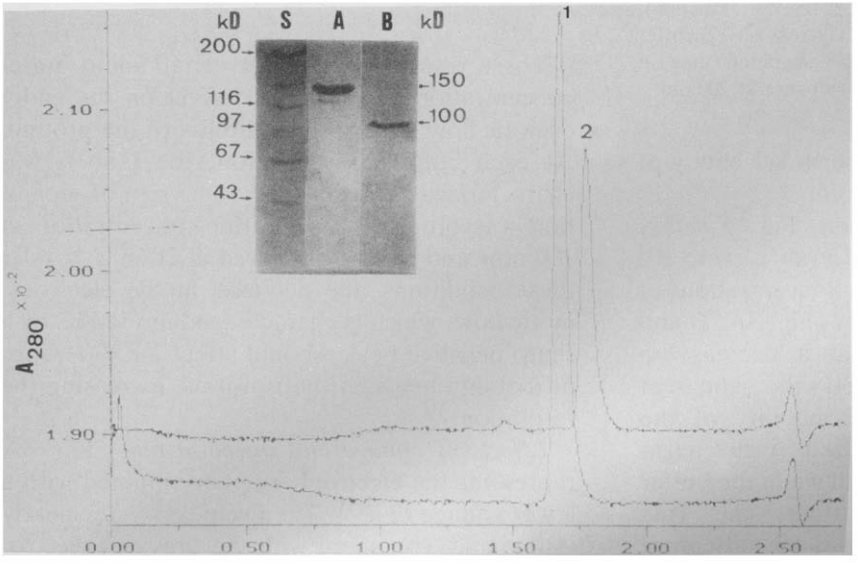

Fig. 1. SDS-PAGE and HPSEC characterization of purified IgG and F(ab')$_2$. SDS-PAGE: lane S = molecular mass standards; A = purified IgG diluted fourfold; B = pepsinated product. HPSEC: Peak 1 = purified IgG diluted fourfold; 2 = pepsinated product. kD = kilo dalton.

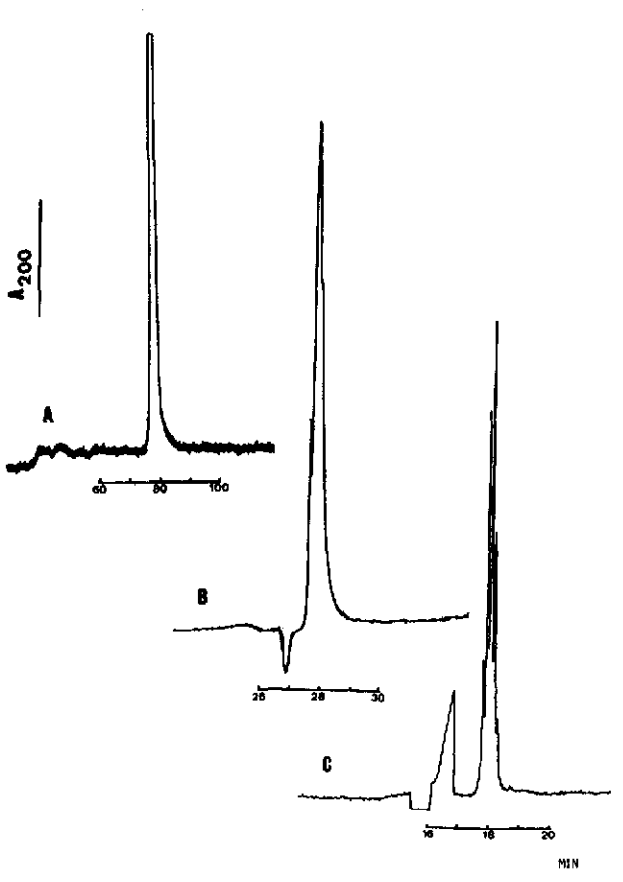

Fig. 2. Electropherograms of the purified $F(ab')_2$ at different buffer pH values. The applied voltage was 5 kV (70 V/cm). (A) 20 m*M* citrate buffer (pH 2.5) (chart speed 0.1 cm/min); (B) 20 m*M* borate buffer (pH 8.3); (C) 20 m*M* CHES buffer (pH 9.5). The horizontal axis is the migration time in minutes and the vertical axis is the absorbance at 200 nm.

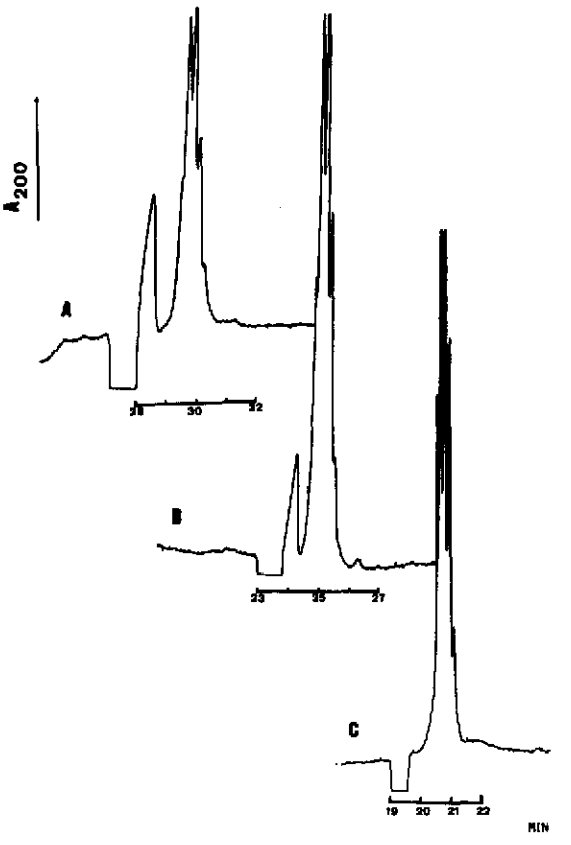

Fig. 3. Electropherograms of the purified $F(ab')_2$ at different buffer concentrations. Migration buffers: tricine of (A) 100, (B) 50 and (C) 20 m*M* (pH 8). The applied voltage was 5 kV (70 V/cm).

this result indicated an increase in selectivity at pH 9.5.

Effect of buffer concentration. Fig. 3 shows that the migration times of the $F(ab')_2$ were 30, 25 and 20 min for tricine buffer concentrations of 100, 50 and 20 m*M*, respectively (Fig. 3A, B and C). These results suggest that a decrease in buffer concentration increased the apparent mobility of the protein. The mobility of the electroosmotic flow, characterized by the negative peak (Fig. 3), also increased when the buffer concentration decreased. Further, the true mobility of the protein, which is the difference between its apparent mobility and the mobility of the electroosmotic flow, decreased.

These results suggest that variations in buffer concentration had a greater effect on the endoosmotic flow than on the mobility of the protein. At each buffer concentration, the $F(ab')_2$ was characterized by five peaks. Some sign of separation was observed at a buffer concentration of 100 m*M* and clearly improved at 20 m*M*. Under these conditions, the decrease in the electroosmotic flow, which is characterized in Fig. 3C by a sharp negative peak, should affect the difference of mobility between the isoforms, increasing the resolution.

Effects of voltage and injection time. Fig. 4A represents the electropherogram obtained with a lower voltage (3 kV). The five peaks were clearly visible and, compared with the previous electropherograms, the resolution was improved. Nevertheless, the product was eluted 34 min

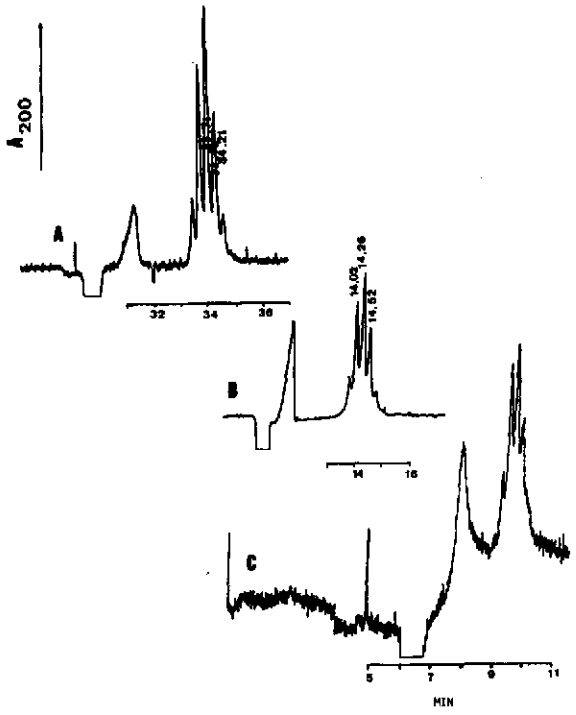

Fig. 4. Optimization of voltage and injection time in 20 m*M* CHES buffer (pH 9.5). (A) Applied voltage, 3 kV (42 V/cm); injection time, 1 s; capillary length, 72 cm; (B) electrophoresis at 208 V/cm for the first 5 min and 42 V/cm for the remainder; (C) protein diluted to 20 μg/ml and injection time 0.1 s, which corresponded to 8 pg of protein (80 amol). Capillary length, 69 cm.

after injection. In order to decrease this migration time, the electrophoresis was carried out at 15 kV for the first 5 min and at 3 kV for the remainder. Under these conditions, the $F(ab')_2$ was observed after 14 min without a significant modification of the resolution (Fig. 4B). The electropherogram obtained after injection of only 80 amol of $F(ab')_2$ (Fig. 4C) indicated that, despite the very small amount of protein injected, the different isoforms were still clearly detectable.

Kinetics of pepsination of IgG: comparison between IEF and FSCE

The kinetics of pepsination of the IgG were followed by IEF and FSCE analysis. In IEF, purified IgG was characterized by different bands corresponding to p*I* values ranging from 6.3 to 6.5 (Fig. 5, T_0). After 10 min of pepsina-

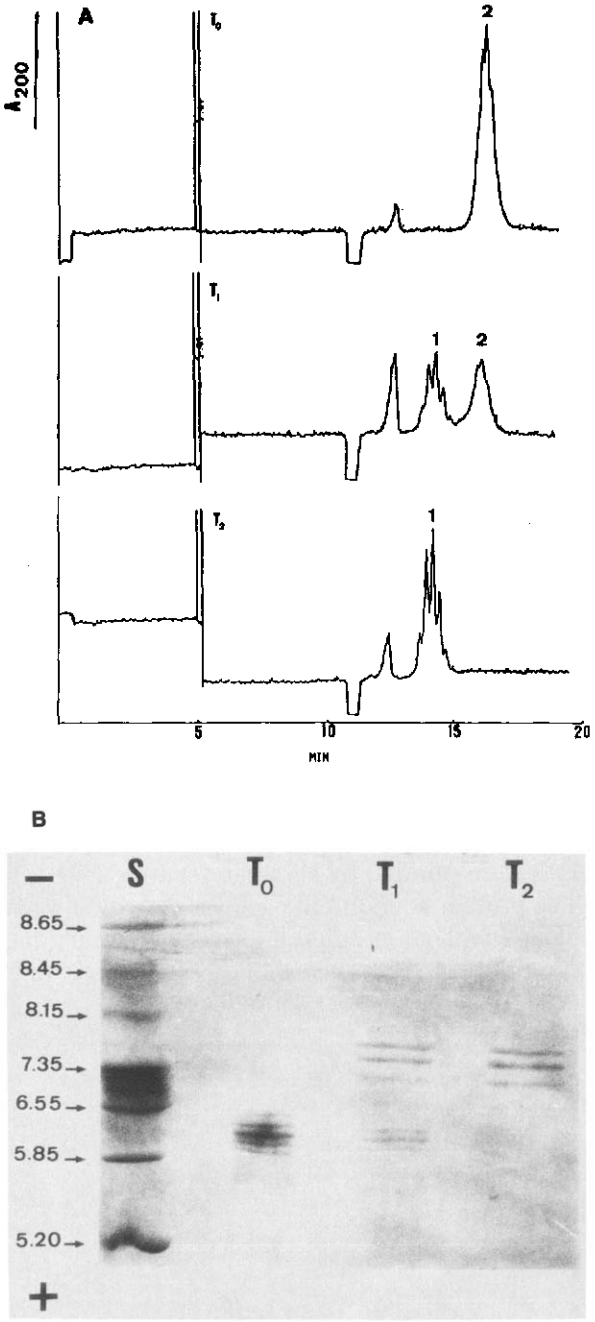

Fig. 5. (A) FSCE analysis of the kinetics of pepsination of the IgG. 20 m*M* CHES buffer (pH 9.5); applied voltage, 15 kV (208 V/cm) for 5 min and then 3 kV (42 V/cm). T_0 = starting material diluted fourfold before injection; T_1 = after 10 min of pepsination; T_2 = after 20 min of pepsination. (B) Isoelectric focusing at different times of the kinetics. Lane S = p*I* markers 3–10; T_0 = starting material; T_1 and T_2 = protein after 10 and 20 min of pepsination, respectively.

tion (Fig. 5, T_1), both IgG and the $F(ab')_2$ were visible. The $F(ab')_2$ was characterized by different bands ranging from p*I* 7.1 to 7.55. After 20 min (Fig. 5, T_2), the intensity of $F(ab')_2$ had increased whereas no residual IgG was detectable, suggesting total digestion of the antibody. In FSCE, purified IgG was characterized by different peaks eluted 16 min after injection (Fig. 5, T_0, peak 2). After 10 min (Fig. 5, T_1), the previous peaks decreased while new peaks corresponding to the $F(ab')_2$ appeared 14 min after injection (Fig. 5, T_1, peak 1). After 20 min of pepsination, no IgG was visible while the concentration of the $F(ab')_2$ increased. No contaminating peaks were visible.

These techniques allowed a clear discrimination of the IgG from the $F(ab')_2$ and confirmation that the heterogeneity of the $F(ab')_2$ was not due to unproteolysed IgG. The presence of these isoforms should be attributed to post-translational modifications of the IgG [20] and the microheterogeneity observed with the $F(ab')_2$ indicates that these modifications were not due only to the Fc fragment.

Preparative separation of the isoforms

The $F(ab')_2$ obtained after pepsination of the IgG were purified by chromatofocusing (Fig. 6). The protein bound to the gel was eluted between pH 7.5 and 6.5 in different peaks, confirming the presence of isoforms. Some eluted fractions corresponding to the different peaks observed in Fig. 6 were characterized by IEF and FSCE.

Characterization of the separated isoforms by FSCE: comparison with IEF

Fig. 7 shows the overlayed electropherograms obtained after FSCE analysis of the $F(ab')_2$ (Fig. 7F) and of fractions 18, 20, 22 and 24 (Fig. 7A, B, C and D, respectively) eluted from the chromatofocusing column. Fraction 18 was characterized by one major peak (Fig. 7A, peak 1) and a second protein more retained in the capillary (peak 2). These products corresponded to the first and second isoforms identified in the $F(ab')_2$ (peaks 1 and 2, Fig. 7F). In Fig. 7B the same isoforms are seen in inverted ratio and a

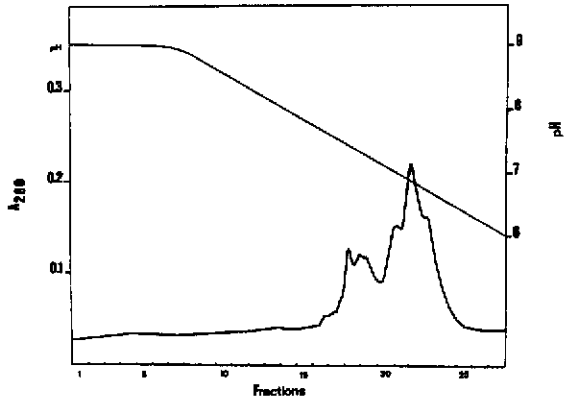

Fig. 6. Chromatofocusing separation of the $F(ab')_2$ isoforms. The chromatofocusing was performed using a Mono P column (HR 5/20). Elution was effected with a linear pH gradient from 9 to 6. The flow-rate was 0.1 ml/min.

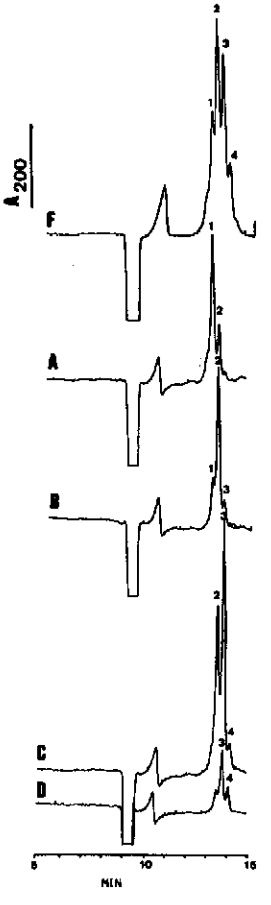

Fig. 7. FSCE characterization of each isoform. The analysis was performed with the Mono P fractions 18, 20, 22, 24 (A, B, C and D, respectively). Experimental conditions as in Fig. 4B. The $F(ab')_2$ was diluted fourfold before injection.

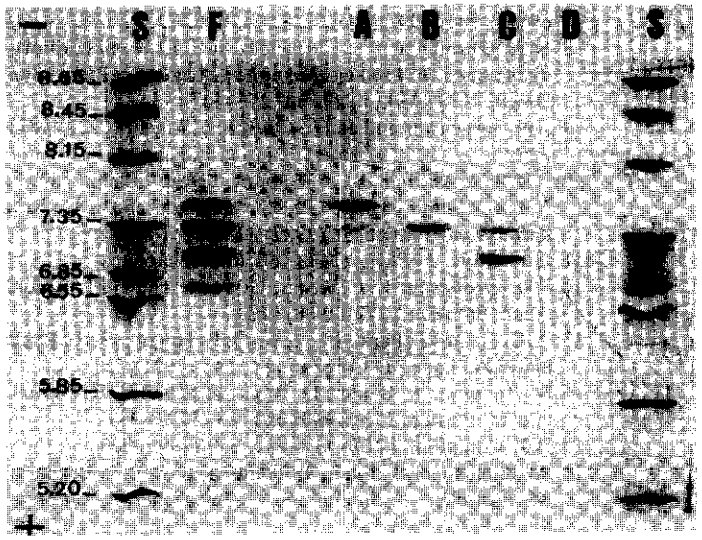

Fig. 8. Isoelectric focusing of the Mono P fractions (Fig. 6). Lane F = $F(ab')_2$; lane A–D = Mono P fractions 18–24; S = p*I* standards.

third peak is slightly detectable (peak 3). FSCE analysis of fraction 22 shows two significant peaks (Fig. 7C, peaks 2 and 3) indicating that the two isoforms were present at similar concentrations. These peaks corresponded to the second and third forms of the purified $F(ab')_2$ (Fig. 7F, peaks 2 and 3). In contrast to the previous fractions, the most electropositive form (peak 1) was absent whereas a fourth peak was visible (peak 4). In fraction 24 (Fig. 7D), the major isoform was the third one and the second and fourth isoforms were still detectable. These results show that the different $F(ab')_2$ isoforms could be separated by chromatofocusing and that each peak characterized by FSCE corresponded to one of those present in the starting material.

Compared with the FSCE pattern (Fig. 7F), the IEF characterization of the $F(ab')_2$ (Fig. 8F) shows similar repartitioning of the different isoforms. After chromatofocusing, fraction 18 (lane A) contained two forms characterized by one major band (p*I* = 7.56) and a more acidic band (p*I* = 7.35). In lane B, the concentration of the second form increased whereas the previous one decreased. Fraction 22 (lane C) contained a mixture of two isoforms with p*I* = 7.35 and 7.1. In contrast, in lane D, only the third form (p*I* = 7.1) was slightly visible and the most acidic form, identified in the starting material (lane F), was not detectable. The isoforms whose ΔpI differed by 0.23 pH unit were identified by FSCE with a Δt between each isoform of 0.24 min (Figs. 7 and 8).

These results show a qualitative and quantitative correlation between IEF and FSCE techniques which can be explained by the fact that the isoforms have the same molecular mass (Fig. 1) and were thus separated in FSCE only by their differences in charge.

CONCLUSIONS

The identification of different forms of a monoclonal $F(ab')_2$ by FSCE has been achieved. Optimization of the experimental conditions (20 m*M* CHES buffer at pH 9.5 and lowering the voltage to 3 kV) led to the identification of five isoforms. The presence of these isoforms was first confirmed by the different bands observed after isoelectric focusing analysis. Further, the kinetics of pepsination of the purified IgG, also characterized by FSCE, indicated that the heterogeneity described above was not due to the presence, with the $F(ab')_2$, of residual anti-

bodies. In order to characterize each isoform, chromatofocusing was successfully used for a preparative separation of the $F(ab')_2$ isoforms. The reproducibility of the capillary electrophoresis analysis allowed each isoform to be identified and to be correlated with the $F(ab')_2$ polypeptides previously defined. Capillary electrophoresis represents an attractive complement to conventional analytical techniques for the characterization of subtle differences in charge of proteins, with the advantage that it is quicker, more sensitive and requires very small sample amounts.

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