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Capillary zone electrophoresis for monitoring r-DNA protein purification in multi-compartment electrolysers with immobiline membranes

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ABSTRACT

Isoforms of human monoclonal antibodies against the gp-41 of AIDS virus and of human recombinant superoxide dismutase have been purified to homogeneity by isoelectric focusing (IEF) in a multi-compartment electrolyser with isoelectric, immobiline membranes. This system allows the processing of large sample volumes and gram-scale protein loads and can resolve isoforms as close as 0.001 in p*I* difference. The purification progress was usually monitored by analytical IEF in immobilized pH gradients (IPG). Capillary zone electrophoresis (CZE) was applied to the monitoring of the content of each chamber of the electrolyser. CZE was found to be superior in terms of speed of analysis and quantification (but only by UV reading at 200–210 nm, *i.e.*, in the region of the peptide bond) but, notwithstanding the millions of theoretical plates reported, was no match for the resolving power of IPGs, at least for protein analysis. When compared also with chromatofocusing, the resolving power decreases in the order IPG > CZE ≫ chromatofocusing.

INTRODUCTION

Capillary zone electrophoresis (CZE) is rapidly becoming a method with great potential for high-speed and high-sensitivity analysis of biological substances, including proteins produced by recombinant-DNA (r-DNA) techniques and targeted for human consumption as pharmaceuticals. Review papers on CZE have already appeared[1–3], and an international meeting has recently been held[4].

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In reality, the CZE technique has been extensively used for producing peptide maps and in general for peptide analysis, as the conditions adopted (typically high-molarity buffers at low pH) greatly help in quenching electrosmosis and reducing potential adsorption to the capillary wall. For proteins, CZE separations are more difficult, because in untreated capillaries a substantial fraction of the protein macroion can be adsorbed in the Debye–Hückel layer of the silica wall[5]. Even though some proteins have been analysed in the same buffer used for peptide fractionations[6] (100 m*M* phosphate, pH 2.5), it should be borne in mind that this pH is denaturing for most proteins. As an alternative method one could use alkaline buffers, at pH higher that the isoelectric point (p*I*) of the sample proteins: as the capillary wall and the protein will bear a net negative charge, adsorption will be minimized[7,8]. This problem is particularly severe with basic proteins, as the buffer pH would have to be so high as to destroy the silica capillary. For this particular problem Green and Jorgenson[9] advocated the use of moderately alkaline buffers (pH 9.0) supplemented by high concentrations (0.25 *M*) of potassium sulphate, which minimized most efficiently protein adsorption, and least interfered with UV readings. More recently, Bushey and Jorgenson[10] suggested the use of buffers containing high concentrations of zwitterions, as these compounds should not contribute to the conductivity of the operating buffer, but should be able to associate with the negatively charged capillary surface on the one side, and with the charged protein sites on the other, thus minimizing protein adsorption in the Debye-Hückel layer. The other alternative, as proposed by Hjertén's group[11], is to coat the capillary wall with a viscous agent (generally methylcellulose or bonded linear polyacrylamide) able to minimize the zeta potential at the wall. In coated capillaries, Kilar and Hjertén[12] were able to separate by zone electrophoresis and isoelectric focusing (IEF) transferrin isoforms and many other proteins.

Based on an original idea of Faupel *et al.*[13] and Righetti *et al.*[14], we have recently developed a multi-compartment electrolyser, able to purify to homogeneity large amounts of proteins (on the gram scale), particularly useful for removing the most tenacious contaminants from r-DNA proteins[15,16]. In this apparatus, a single protein is kept isoelectric in each chamber of the electrolyser by two flanking membranes, able to titrate continuously the macroion to its p*I* value. By carefully engineering the different membranes, ideally in each compartment one can isolate a pure protein species. The membranes are made with the Immobiline technology, *i.e.*, they consist of neutral monomers [acrylamide and N,N′-methylenebisacrylamide (Bis)] and charged acrylamido derivatives (known as Immobilines) mixed in such ratios as to determine unequivocally and very precisely a given p*I* value. If the p*I* values of the two flanking membranes encompass the p*I* value of a given protein component, this species will be kept isoelectric in such a chamber (the membranes, by virtue of their high buffering capacity, act as pH-stat units) and will not be able to leave it even in the presence of a strong electrosmotic flow. We have purified by this technique large amounts of r-DNA proteins, including eglin C, monoclonal antibodies (Mab) against the gp-41 of AIDS virus, human growth hormone and superoxide dismutase (SOD). The rate-limiting step in monitoring the purification progress (which could be rapid if the contaminants have very different p*I* values, but slow with very small Δp*I*) is the analytical method itself, which is IEF in immobilized pH gradients (IPG). Because in general the contaminants have small Δp*I*, and thus focus over a narrow pH range, the analytical IPG step is generally performed over 1 pH unit (and sometimes even less).

In such ultra-narrow ranges, long focusing times are required[17], so that it might well take 10–12 h before the results of the purification progress are available. Meanwhile, the purification will have further progressed in the electrolyser, so that the "picture" obtained has already aged. With CZE, owing to the short analysis time (usually 15–30 min even with protein mixtures), one could hope to monitor the progress of purification in real time. We have applied CZE to such analytical problems and compared its resolving power with that of IPGs and of chromatofocusing in resolving the same protein mixtures. In addition, we had an interesting problem to solve, as one of our proteins (the anti-gp-41 monoclonal antibody) was resolved into a series of bands with very high p*I* values (in the pH range 9.0–9.6).

EXPERIMENTAL

Instrumentation

CZE analysis of the basic monoclonal antibodies (Mab) anti-gp-41 was performed in a Bio-Rad Labs. (Richmond, CA, U.S.A.) HPE 100 unit. Capillaries were supplied mounted in cartriges with an integral flow cell for on-column optical detection. All capillaries were covalently coated at the internal wall with a hydrophilic polymer. Detection was by UV absorbance at 200 nm and the capillary was not thermostated. For human superoxide dismutase (hSOD), CZE analysis was performed in an Applied Biosystems (Foster City, CA, U.S.A.) Model 270A unit, equipped with an uncoated, air-cooled capillary. Chromatofocusing was carried out on a Mono P column (Pharmacia–LKB, Uppsala, Sweden) connected to a fast protein liquid chromatographic (FPLC) system and to an on-line pH monitor. Analytical IEF in IPGs was operated in a Multiphor II chamber, connected to a Macrodrive power supply and to a Multitemp Thermostat (Pharmacia–LKB). Preparative protein purification in a multi-compartment electrolyser equipped with isoelectric immobiline membranes was executed in the appparatus designed by Faupel[13] and Righetti[14].

Materials

Anti gp-41 Mabs were prepared according to Jungbauer *et al.*[18]. Recombinant human (r-h) SOD was produced as described by Weselake *et al.*[19]. All samples were lyophilized from volatile buffers (10 m*M* ammonium formate). Immobilines and carrier ampholytes were purchased from Pharmacia–LKB. The membranes for the multi-compartment electrolysers were made and used according to Righetti *et al.*[15].

Capillary zone electrophoresis

Analysis of Mabs in the Bio-Rad HPE 100 unit was carried out in 100 m*M* phosphate buffer (pH 5.8), generally at 10 kV, 20 μA, 20 mAU full-scale (200 nm). For sample loading, a 10-μl volume of the sample (with an ionic strength about one tenth that of the electrophoresis buffer) was injected into the anodic reservoir just ahead of the capillary inlet. The power supply was then turned on and the sample electrophoresed into the capillary for 6–8 s at 8 kV. The anodic reservoir was then flushed with the electrophoresis buffer, and the CZE run made at 10 kV. For analysis of r-hSOD, CZE was performed in the Applied Biosystems apparatus (Model 270CE, standard 72-cm capillary) in 20 m*M* citrate buffer (pH 4.0). The conditions for sample loading were a 2-s vacuum injection, run at 21 kV, 21 μA.

Analytical IPGs

Mab analysis was done in IPG gels in the pH range 8.5–10. The samples were loaded in slots preformed at the anodic side, in a pH 8.0 plateau gel segment (2-cm long). About 20 μl, containing up to 50 μg of protein, were loaded and focusing was continued for up to 30 000 V h; r-hSOD analysis was made in IPG pH 4.5–5.5 gels, using the above conditions (except for the cathodic sample application). All gels were stained with Coomassie Brilliant Blue R-250 in Cu^{2+}, according to Righetti and Drysdale[20].

Chromatofocusing

Mono P chromatofocusing columns (Mono HR5/20 and HR10/30) from Pharmacia–LKB were connected to an FPLC system equipped with an on-line pH flow electrode. The columns were operated at a flow-rate of 0.1 ml/min. About 1 mg of lyophilized monoclonal antibodies was dissolved in 500 μl of equilibration buffer and loaded onto a 3.4-ml column. Elution was performed at a flow-rate of 0.1 ml/min in a linear pH gradient from 9.5 to 7.0.

Preparative IPGs

Preparative IPGs in multi-compartment electrolysers were run according to Righetti and co-workers[15,16]. For Mab purification, the electrolyser was assembled with six flow chambers (two electrodic and four sample chambers). The compartments were separated by five Immobiline membranes (supported by Whatman GF/D microfibre filters) having p*I* values of 9.11, 9.25, 9.36, 9.49 and 9.64. The membranes facing the cathodic and anodic reservoirs were made more robust (10% T, 4% C)[a], whereas the membranes in between the sample flow chambers were more porous (5% T, 8% C), so as to allow passage of the large Mab molecules (molecular mass 150 000). For r-hSOD purification, the electrolyser was assembled with eight chambers (two electrodic and six sample chambers), delimited by seven isoelectric membranes having p*I* values of 4.60, 4.82, 4.90, 4.94, 5.05, 5.09 and 5.30. The anodic and cathodic membranes were made 12% T, 5% C, whereas the sample membranes contained 5% T, 5% C gel matrix. The content of each chamber was analysed by CZE and by analytical IPGs.

RESULTS

Fig. 1A and B compares the pattern of isoforms of purified human Mab against the gp-41 of AIDS virus. It is seen that, notwithstanding the fact that the antibodies produced are monoclonal, and purified to homogeneity, they are resolved in a narrow IPG interval (pH 8.5–10) into a fine spectrum of as many as 21 bands (B). The IPG profile agrees fairly well with the CZE peak spectrum, except that the resolution is much lower (at best fifteen peaks can be counted) and that in the central portion, where three major components are present, no baseline resolution can be obtained in CZE (whereas ample, empty gel space can be seen among the same zones in the IPG profile). As a comparison, Fig. 1C shows the chromatofocusing chromatogram of the same preparation; even under the best conditions and by applying a shallow pH

[a] C = g Bis/% T; T = (g acrylamide + g Bis)/100 ml solution.

gradient, not more than ten peaks are resolved and again no baseline resolution is achieved in the region of the most abundant Mab isoforms.

This Mab preparation has been subjected to purification in a multi-compartment electrolyser[15,16], equipped with isoelectric membranes able to define isoelectric conditions for a single species in each chamber. The purification progress has been monitored in parallel with CZE and IPGs. Fig. 2A–C compares the results of the two techniques in analysing the content of chambers 2, 3 and 4, respectively, of the electrolyser. Here too the two techniques compare fairly well except that, in cases in which a few large peaks are present, small zones in between tend to disappear in the CZE profile. This is clearly illustrated in Fig. 2B; as there is no baseline resolution

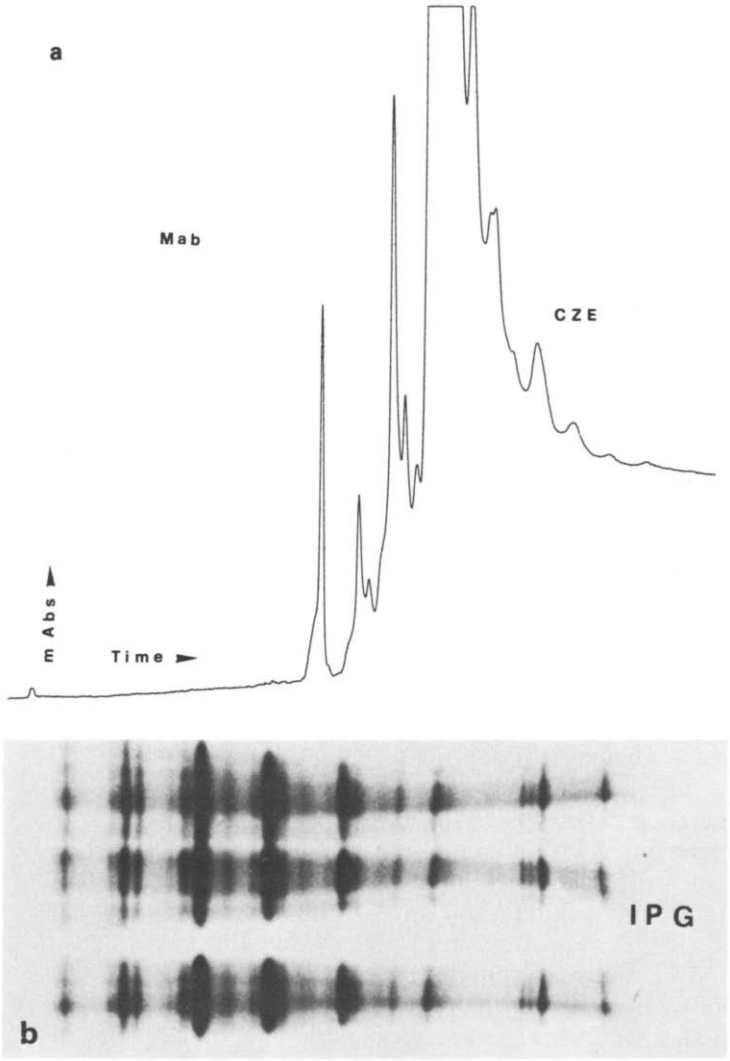

Fig. 1.

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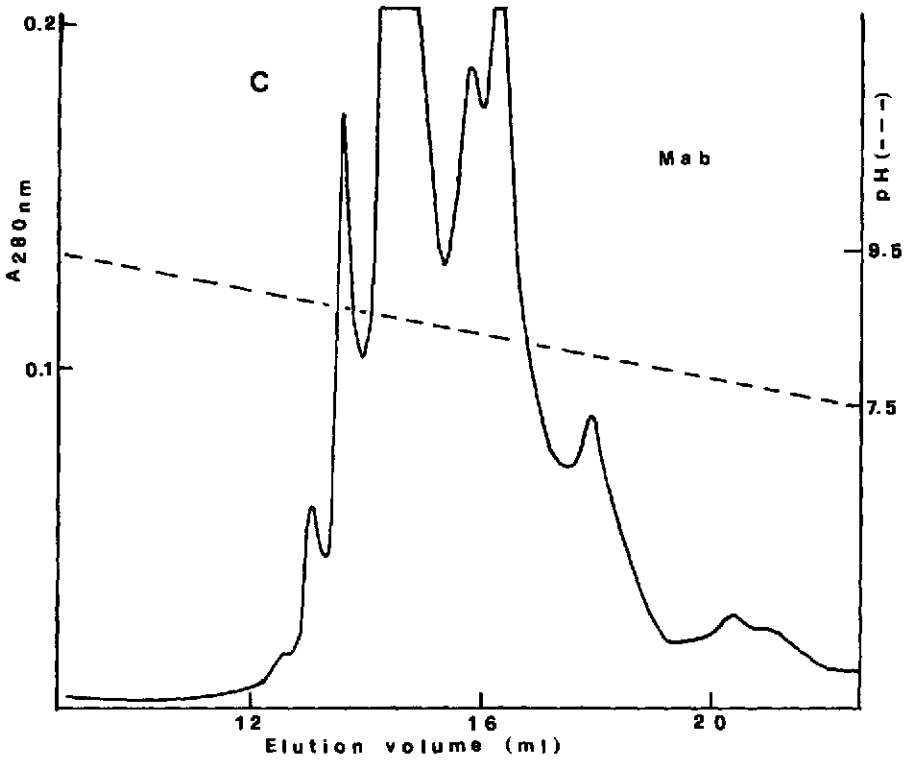

Fig. 1. Comparison among CZE, IPG and chromatofocusing. A pure preparation of human monoclonal antibody isoforms against the gp-41 of AIDS virus was analysed by (a) CZE, (b) IPG and (c) chromatofocusing. (A) CZE with the Bio-Rad HPE 100 unit in 100 m*M* phosphate buffer (pH 5.8). Sample loading: 8 s at 8 kV. Run: 20 kV, 20 μA, 20 mAU full-scale. (B) IPG in the pH range 8.5–10 . The sample was loaded in a pH 8.0 plateau in slots precast in the gel. Run: 30 000 V h at 10°C. Staining with Coomassie Brilliant Blue R-250 in Cu^{2+}. Three sample tracks are shown, for a visual assessment of pattern reproducibility in IPGs. (C) Chromatofocusing in a Mono P column; elution with a linear pH 9.5–7.0 gradient. Sample flow-rate: 0.1 ml/min.

between the two major peaks, the intermediate proteins bands are lost in the CZE profile but are clearly seen in the IPG pattern. This is possibly due to the fact that, in all focusing techniques, even under overloading conditions (often necessary to detect minor contaminants), there is a built-in force counteracting diffusion, whereas this mechanism is not operating in CZE.

We next investigated the purification progress of r-hSOD, produced in *Escherichia coli*. The protein was purified in a multi-chamber apparatus assembled with eight chambers (six for sample collection and two electrolyte reservoirs). Fig. 3 shows the isoform profile of r-hSOD, as analysed in a 1 pH unit wide IPG gel, of the starting material (control) and of the zones collected in each chamber. Fig. 4A–C shows representative runs in CZE *vs.* IPG of the contents of chambers 2, 3 and 4, respectively. Here too it is seen that, whereas the overall pattern is similar, some protein zones tend to disappear from the CZE profile. For example in Fig. 4C the spectrum of fine bands (as many as four) between the two major SOD zones, clearly visible in the

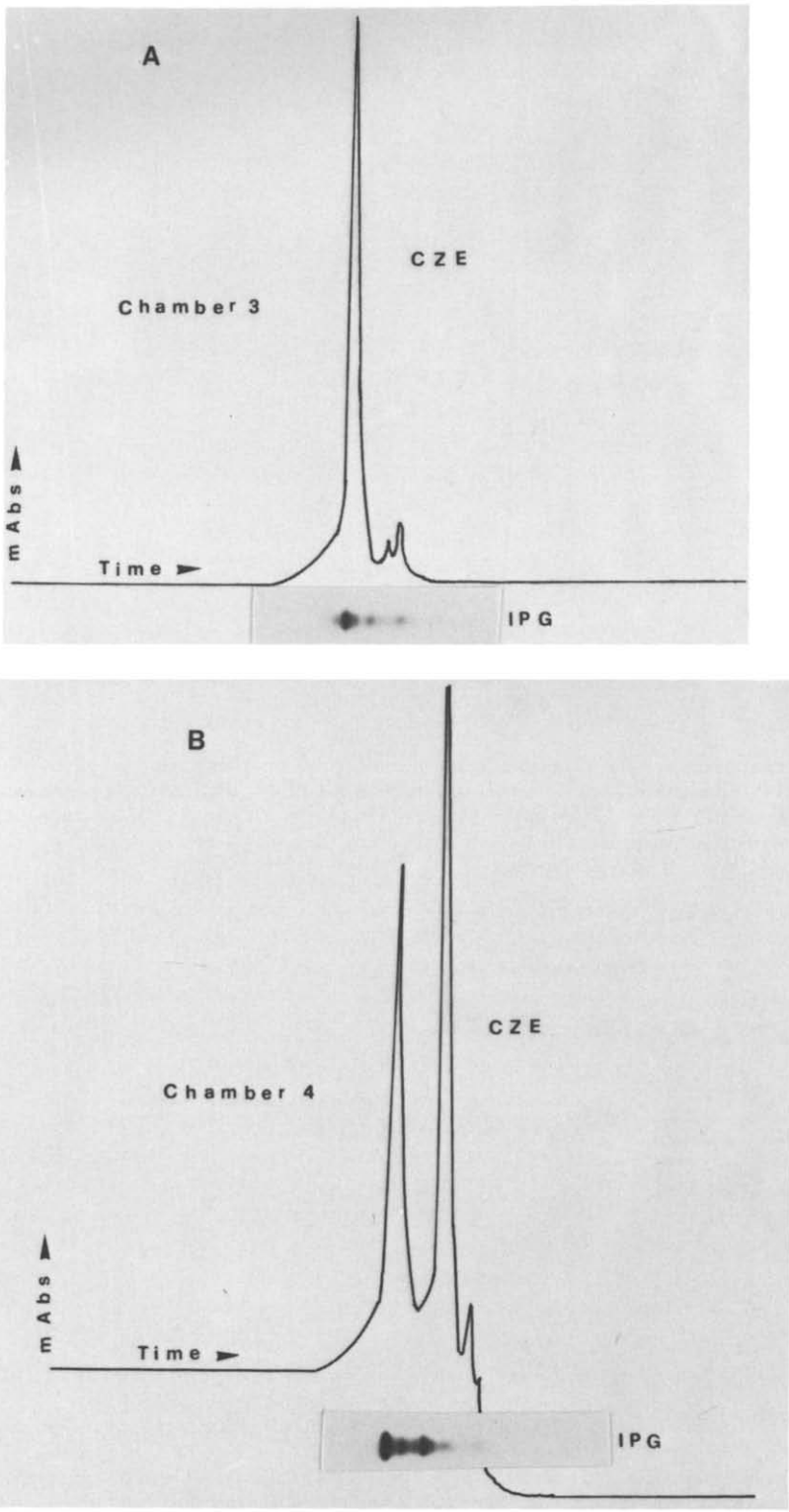

Fig. 2.

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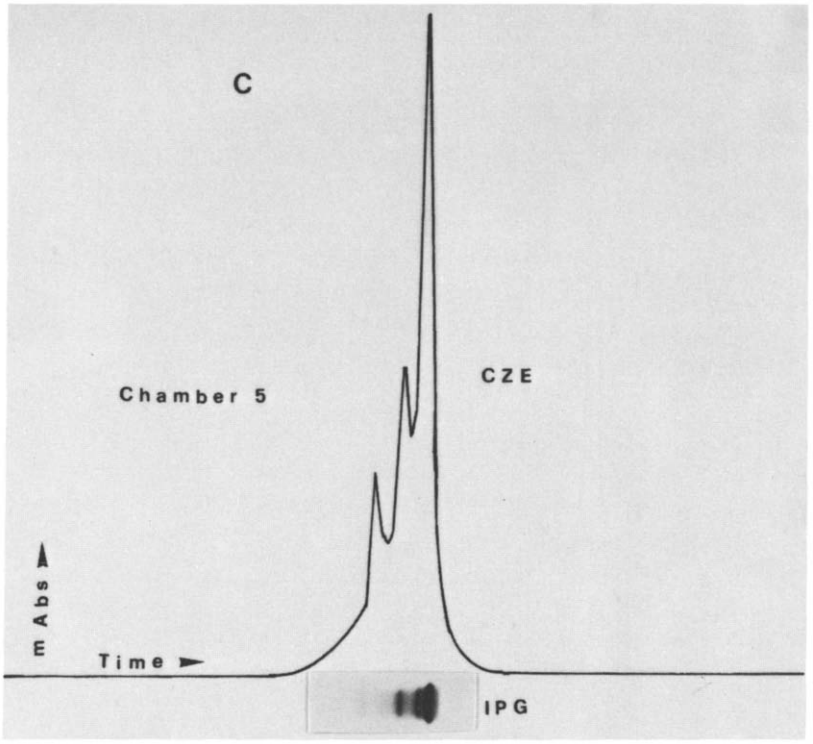


Fig. 2. Progress of purification of Mabs in a multi-compartment electrolyser. After running the electrolyser for 1 day at 1000 V/cm, the content of each chamber was analysed by CZE and IPG. (A) Comparative data for the content of chamber 3; (B) analysis of the content of chamber 4; (C) screening of the content of chamber 5. The purification progress can easily be compared with the same patterns shown in Fig. 1 (21 bands by analytical IPGs). All other conditions as in Fig. 1.

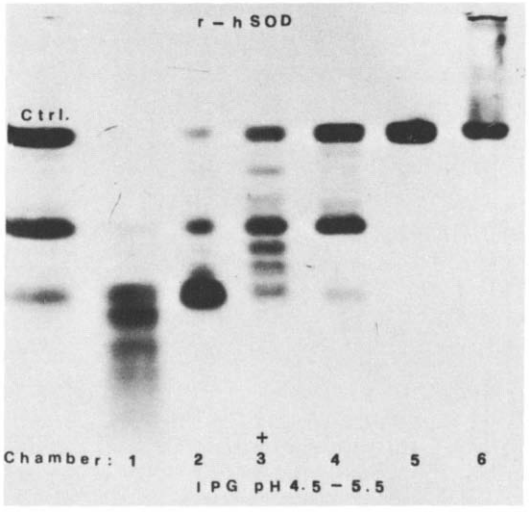
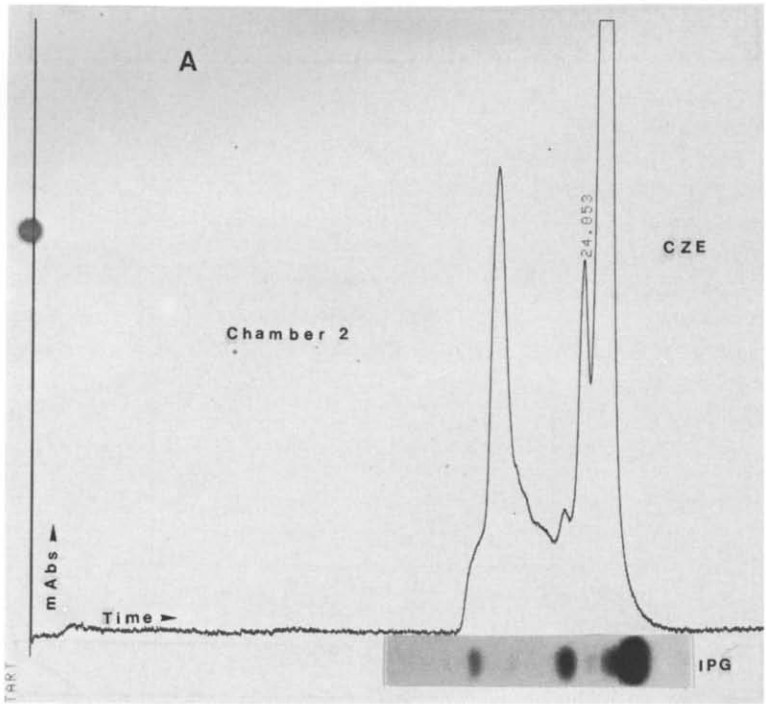


Fig. 3. Analytical IPG gel of a preparative r-hSOD run in the multi-compartment electrolyser. IPG gel: 5% T, 3% C, pH range 4.5–5.5, run for 20 000 V h at 10°C. Staining with Coomassie Brilliant Blue R-250 in Cu^{2+}. Ctrl. = Control, unfractionated r-hSOD. Tracks 1–6: content of chambers 1–6 in the electrolyser. The cathode is uppermost. Note that chamber 5 contains a single homogeneous SOD band.



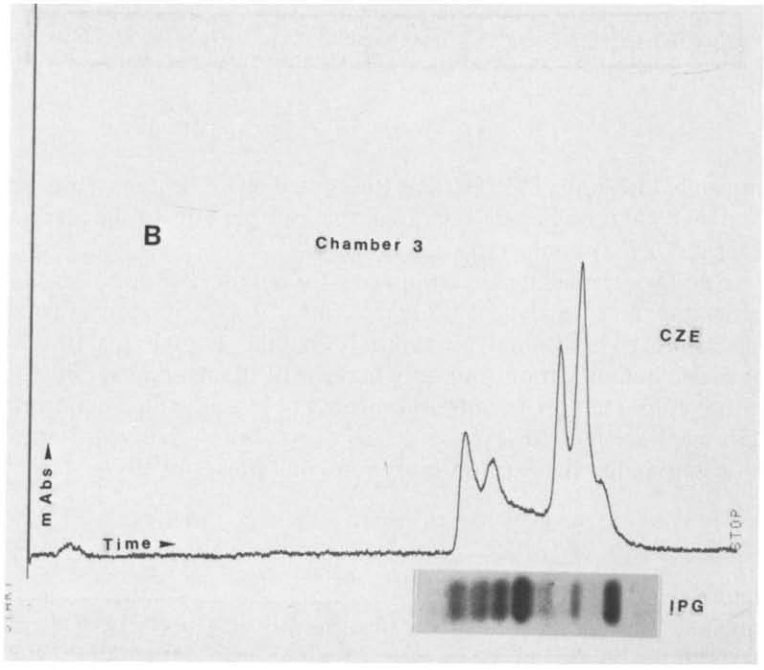


Fig. 4.

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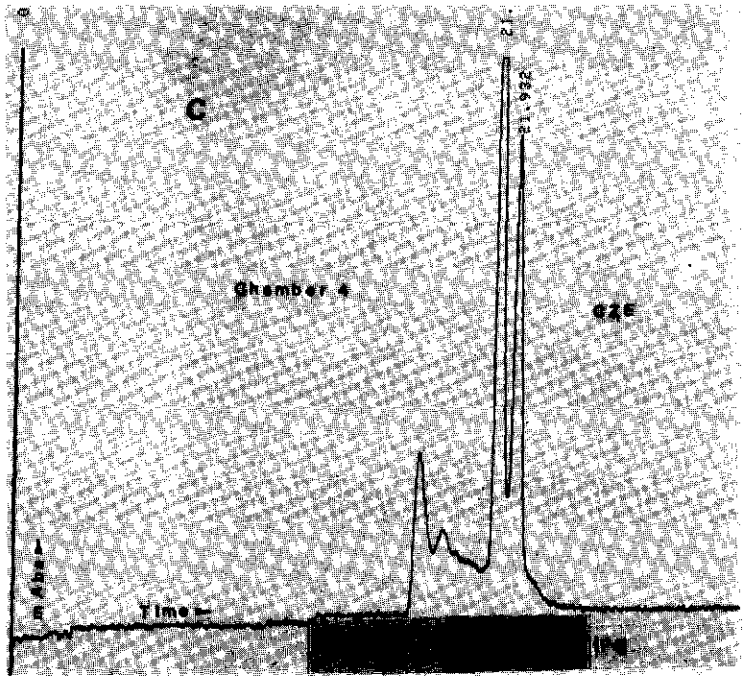

Fig. 4. Progress of purification of r-hSOD in a multi-compartment electrolyser. After running the electrolyser for 1 day at 200 V/cm, the contents of each chamber were analysed by CZE and IPG. (A) Comparative data for the content of chamber 2; (B) analysis of the content of chamber 3; (C) screening of the content of chamber 4. The purification progress can easily be compared with the same patterns shown in Fig. 3. Conditions for CZE: Applied Biosystems Model 270CE unit (standard 72-cm capillary) in 20 m*M* sodium citrate buffer (pH 4.0); sample loading: 2-s vacuum injection, run at 20 kV, 21 μA, 25°C. All other conditions as in Fig. 1.

IPG profile, is completely lost in the CZE pattern (this could again be due to the fact that there is no baseline resolution between the two zones, or perhaps to the reversal of the mobility order in CZE as compared with IPG).

Our experience so far accumulated in comparing CZE with IPG indicates that CZE has a clear advantage over analytical IPGs in terms of speed of analysis (each CZE run takes *ca.* 20 min, whereas narrow-range IPGs take as many as 10 h to process) and also in zone quantification (but only because of the reading at 200–210 nm; with fluorescent or coloured tags, quantitation would be just as problematic as in conventional gel electrophoresis). However, in terms of resolution, it seems that at present no technique can match the extraordinary resolving power of IPGs.

DISCUSSION

Advantages and limitations of CZE

"Are the Russians Ten Feet Tall?" predicates the title of a book by Keller[21] meant to destroy everything created in the modern Russian society. We do not want to be so vicious and unscrupulously hard as Mr. Keller, but we feel that a proper

assessment of the performance of CZE, especially with regard to protein analysis, is now due and we listing here a series of points worth examining.

Speed

There is no doubt that speed of separation is by far the greatest advantage of CZE, when compared to all other electrophoretic techniques. For example, both sodium dodecyl sulphate (SDS) electrophoresis and conventional isoelectric focusing[22] take at least 2–3 for the migration step alone (to this must be added the time for preparing the gel, staining, destaining and densitometry). In the case of IPGs, the situation is the same when running wide pH gradients, but in narrow and ultra-narrow pH ranges (*e.g.*, 1 pH unit or less) it might take up to 10 h to reach steady-state conditions[17]. The only system which can substantially reduce these times (and which also represents an instrumental approach to electrophoresis) is the Fast System of Pharmacia, where both SDS and IEF can be run in *ca.* 45 min and where gel staining and destaining have been completely automated. This has been made possible by an idea proposed long ago by Kinzkofer and Radola[23], *viz.*, running IEF in postage stamp-size gels. For example, in gels barely 1–3 cm long, equilibrium IEF is obtained in only 2 min in 1-cm long gels and in only 10 min in 3-cm long gels. In addition, in such miniature, ultra-thin (50–100 μm) gels, staining is accomplished in 5 min and destaining in 2–3 min. This compares favourably even with CZE, where protein run times as long as 20 min are required (in addition, in such miniature gels, at least ten samples can be run simultaneously, which further reduces the analysis time to *ca.* 1 min per sample).

Sensitivity

Here too CZE scores fairly high, as extremely high sensitivities have been reported. For example, Chung and Dovichi[24] reported the detection of 2–7 amol of eighteen fluorescein isothiocyanate derivatives of amino acids. In the best cases, they could detect as little as 0.05 amol of arginine, which corresponds to *ca.* 5700 molecules. This is almost frightening and one wonders if one day we could scrape the bottom of Avogadro's barrel and dig out the last remaining molecule. This compares favourably with the best detection conditions obtained in gel electrophoresis: by Coomassie Brilliant Blue staining the lowest detection limit for proteins is of the order of 10–15 ng per band[23] and by silver staining it is *ca.* 1 ng per spot[25].

Nevertheless, even this should be taken with caution, as in real-world detection at such minute levels may not be feasible as extreme care would be required with respect to sample manipulation to minimize losses due to adsorption and the effects of contaminants on the signal. In addition, such sensitivity levels have been obtained with the use of a highly sophisticated detection system, *e.g.*, an ultratrace laser detector, not available in any commercially available equiment. With the UV detection system currently available, such sensitivity in protein analysis is drastically reduced. With a universal conductivity detector (which does not require any sample derivatization) the limit of detection is *ca.* 10^{-6} *M* (ref. 26). As a last comment, it should be noted that, whereas the mass sensitivity is good, relatively high sample concentrations are needed because, in most electrophoretic loading systems, only a fraction of the sample injected into the electrolyte reservoir enters the capillary[27].

Quantification

One of the major drawbacks in the quantification of protein zones in conventional gel electrophoresis is that the colour yield varies greatly with different proteins, so that the slope of the colour intensity curve can differ by as much as one order of magnitude. This applies to both Coomassie Brilliant Blue[28] and silver-stained patterns[25]. It has been claimed that only CZE offers correct quantification of the different protein zones, and this is very important when assessing the level of impurities in, *e.g.*, r-DNA proteins. Thus, Frenz *et al.*[6] demonstrated that, when determining the extent of deamidation of recombinant human growth hormone (r-hGH), they could determine 2.6% deamidated r-hGH by anion-exchange high-performance liquid chromatography and 3.1% by CZE, which is fairly good, considering also that part of the difference could result from the electrophoretic sample loading procedure in CZE, which could allow relatively more loading in the capillary of the faster migrating deamidated r-hGH. We fully agree with these data, but only with the proviso that they apply to UV sample detection at 200–210 nm, where protein adsorption is largely due to the peptide bond. However, when this statement is extended to proteins labelled with fluorescent dyes or with any other coloured tag, it is hard to understand why the signal should be quantitative in CZE and not in, *e.g.*, SDS electrophoresis or IEF or IPG.

Reproducibility

There are three problems to be addressed in this respect: reproducibility in sample loading, in sample migration time and in total sample eluted from the capillary. Our main problem in protein analysis has been reproducibility in sample migration (or transit) time, as also reported by Frenz *et al.*[6], as well as in sample elution. CZE equipment is generally "user-friendly", *i.e.*, very easy to operate even by inexperienced technicians. In addition, in some systems, such as the Bio-Rad unit, the coating in the inner capillary wall greatly helps in reducing protein adsorption, and this has been found by us to be excellent for the analysis of alkaline monoclonal antibodies (p*I* values in the pH range 9.1–9.6). Our major problem, however, has been the lack of reproducibility in transit times: often, after a few runs, no further protein was eluted and the capillary became clogged. Perhaps one problem, as pointed out by Nelson *et al.*[29] and Terabe *et al.*[30], could be the lack of adequate cooling of the capillary. In contrast, IPGs afford unique reproducibility; *e.g.*, in two-dimensional maps, the uncertainty in spot position is reduced to a minute ellipse, with a variation in absolute spot location never greater than 1–3 mm, over a gel area of 14 × 16 cm (IPG × SDS dimensions)[31].

Resolving power

This is perhaps the most ambiguous aspect of CZE. According to Bushey and Jorgenson[10], in protein separations over 100 000 theoretical plates can be obtained, which seems reasonable. However, theoretically, Jorgenson and Lukacs[2] have predicted, in diffusion-limited cases, several million theoretical plates. In the case of DNA restriction fragments, Cohen *et al.*[32] have obtained an effective plate count of 600 000 (for a capillary length of 13 cm). More recently, in separations of oligodeoxynucleotides, Karger *et al.*[1] have claimed plate numbers, in gel-filled capillaries, as high as $15 \cdot 10^6$, for lengths of 50 cm. We do not understand the significance of all these

numbers, but a close inspection at Fig. 1 could be instructive. Our anti-gp-41 monoclonal antibodies (a pure family of isoforms) are separated in an IPG pH 8.5–10 gradient into 21 fully resolved and clearly visible zones. In the best run obtained by CZE, only fifteen peaks can be counted. In chromatofocusing, only ten peaks are visible. There is more to it: in order to detect minor components, the run has to be overloaded with respect to the major fractions; this results in CZE in spread-out peaks (Fig. 1A) with no baseline resolution in the central portion of the electropherogram. In the IPG gel profile (Fig. 1B), because there is a built-in force which counteracts diffusion in focusing techniques, there is ample resolution even among the major peaks, with clear gel zones in between.

However, there is even more to it: had we wanted, we could have further improved the resolution in IPGs by narrowing the pH gradient to about 1/2 (in fact, most of the zones are isoelectric between p.H 8.9 and 9.6, so a pH gradient between pH 8.5 and 10 is indeed much too wide). Thus, if we have to speak in terms of resolution, it is clear that the order should be IPG > CZE ≫ chromatofocusing. This could give a clue as to why the spreading of IPGs has been relatively slow: too much resolution can be just as hard to deal with as too little resolution. There is no problem in handling chromatofocusing (moderate resolution); there are already some problems in adapting to CZE (high resolution), but there are definitely major difficulties in accepting the extremely high resolution of IPGs.

CONCLUSIONS

CZE, when used for monitoring the progress of protein purification in multicompartment electrolysers, has the definite advantage of very short analysis times and good quantification of proteins bands via UV readings at 200–210 nm. However, the technique still lacks reproducibility and is definitely not a match for the very high resolving power of immobilized pH gradients.

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