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Review

Protein purification in multicompartment electrolyzers with isoelectric membranes

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Abstract

Preparative purification of proteins under isoelectric conditions is reviewed, with particular regard to novel equipment, a multicompartment electrolyzer with isoelectric membranes, which can capture any desired protein into an isoelectric trap as the sole, ultra-pure component. This novel machine is based on the Immobiline chemistry, i.e. the novel generation of non-amphoteric buffers, based on the chemistry of acrylamides, which can be insolubilized onto polyacrylamide supports. After a description of the instrument and of its performance, a number of protein purification protocols are described, leading to truly homogeneous (by the most stringent criterion of surface charge) protein fractions. Such a high charge purity has been found to be often a fundamental prerequisite for the growth of protein crystals. Interfacing the electrolyzer with mass spectrometry has permitted the decoding of the structure of minor components generated from a parental molecule, especially ones having a higher p*I*. It was found that these species were often generated either by proteolytic cleavage or by the formation of a trisulphide bridge between two Cys residues. A unique application of the electrolyzer is finally described: its use as an immobilized enzyme reactor under an electric field. The performance of this reaction is outstanding, in that the kinetic parameters of the immobilized enzyme are identical to those of a free enzyme form. © 1997 Elsevier Science B.V.

Keywords: Reviews; Multicompartment electrolyzers; Isoelectric membranes; Proteins

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1. Introduction

Historically, chromatographic processes have always had the lion's share in the panoply of purification methods. In recent years, highly sophisticated packing materials have been developed for affinity chromatography [1], for gel permeation [2], for hydrophobic interaction chromatography [3]. Typically, the most advanced generation of chromatographic beads comprises homogeneous, spherical particles as small as 2 μm in diameter and often non-porous, so as to minimize mass transport problems. Additionally, modern perfusive supports were developed, combining throughpores and diffusive pores, allowing a combination of convection and diffusion through the packed bed [4].

Although not generally perceived by the scientific community at large, preparative electrophoresis of biological (macro)molecules has also been in vogue for long time now. Continuous zone electrophoresis by crossed velocity fields in a supporting medium goes back to early attempts in the late forties from the Institute of Biochemistry in Uppsala headed by Arne Tiselius. In 1949 Svensson and Brattsten [5] described a preparative apparatus with liquids flowing perpendicularly to the electric current, with a capillary packing consisting of glass powder. The equipment was further refined by Brattsten [6] and by Brattsten and Nilsson [7], who also proposed filter paper as support medium (although, at those times, aluminium oxide, sea sand, paper pulp, cotton, barium sulphate, barium carbonate, cellulose powder and potato starch, all in a granulated form, were tested). Large-scale fractionations of human serum and of algae proteins were carried out in a cell of 28-cm length, 30-cm height and 1-cm thickness, provided with a 56-channel collection device [8,9]. Simultaneously, and independently, Grassmann's group in Germany was developing a similar type of apparatus, with fine glass beads or quartz sand as support media [10,11]. In concomitance with this research, Porath was developing preparative zone electrophoresis in static vertical columns filled with ethanolized cellulose powder [12]. He even attempted fractionations of 'reasonable' protein quantities (10 to 100 g!) in packed, horizontal columns assembled from different sections provided with stop-cocks [13]. It must be emphasized, though, that zone stabilization can also be obtained in the absence of granulated fillings, e.g. by exploiting density gradients due to electrically neutral solutes, as first advanced by Philpot [14]. In a free liquid medium, in the absence of zone stabilization by density gradients, electrodecantation will occur: this phenomenon was exploited by Pauli [15] to build huge plants for latex creaming, thus producing the much needed rubber lattices that kept the American Army's wheels running during World War II.

As an alternative to the above methods, continuous free flow electrophoresis can be performed, while avoiding electrodecantation, if laminar flow can be maintained in thin films of liquid. This is the concept of the instruments built by Barollier et al. [16] and Hannig [17,18], still in vogue in biochemical separations.

Since this review deals, however, with preparative protein purification under isoelectric conditions, we will limit our treatise to systems exploiting this principle. It should be remembered that, when isoelectric focusing (IEF) in soluble carrier ampholyte (CA) buffers was invented, it was conceived as a preparative technique, separations occurring in a doughnut-shaped space in vertical columns supported by sucrose density gradients [19]. Soon a host of alternative apparatuses were described, incorporating even the concept of continuously flowing liquid curtains (for a review, see Chapter 2 in Ref. [20]). We will only mention here some recent developments made by Bier's group, since they have similarities with our principle of multicompartment electrolyzers. This group has pioneered the use of continuous flow, but in a recycling mode. In techniques such as IEF and isotachophoresis full separation is achieved not by a single passage, but by multiple passages through the electric field until attainment of steady-state conditions [21,22]. An apparatus based on this design, called RF3 (available from Rainin, Woburn, MA, USA), is for recycling free-flow IEF. In a related approach, the Rotofor cell (available from BioRad, Hercules, CA, USA), allows focusing in a rotating cylinder, with collection of 20-sample fractions [23]. All these approaches, however, although based on advanced instrumental engineering, rely on the old chemistry of soluble CA buffers for creating and maintaining the pH gradient. As we will show, such a preparative technique is far

from optimal: CAs are a multitude of zwitterionic compounds whose precise formulation is unknown and which will ultimately contaminate the purified protein. There is no way to ensure complete elimination of such compounds (which can reach a molecular mass as high as 1000 u and have peptide-like properties) from the protein of interest; as a consequence, the Food and Drug Administration has banned from human consumption proteins obtained by conventional preparative IEF. The striking difference between our instrument and Bier's machinery is not, of course, in the engineering of the equipment, but in the chemistry behind it: our separations are based on Immobilized pH gradients (IPG), which, albeit being a focusing technique, have completely revolutionized the field of steady-state separations [24]. This novel chemistry has been coupled to another, previously unreported, separation principle: the concept of isoelectric membranes acting as a pH-stat and able to trap a single isoelectric protein in between. This concept is shown in Fig. 1: an isoelectric trap will be operative when the condition $pI_{cm}>pI_{p}>pI_{am}$ (where the subscripts cm and am denote cathodic and anodic membranes, respectively and p is the protein having a given isoelectric point between the two membranes) is satisfied.

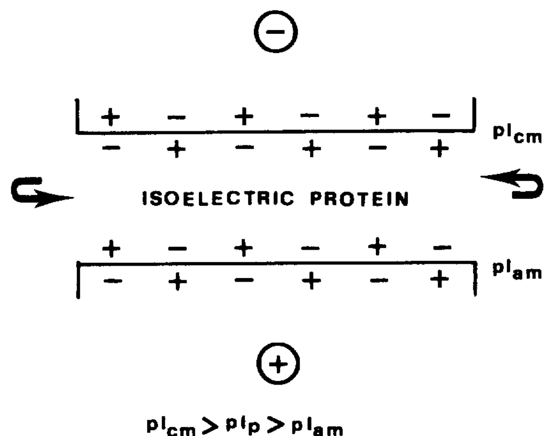

Fig. 1. Mechanism of the purification process in the multicompartment electrolyzer. The isoelectric membranes facing each recycling chamber act by titrating the protein of interest to its isoelectric point (p*I*), thus keeping its mobility constant and equal to zero throughout the purification process. For this to occur, it is necessary that $pI_{cm}>pI_{p}>pI_{am}$, where the suffixes indicate cathodic membrane, protein and anodic membrane, respectively. In addition, the two Immobiline membranes satisfy the condition of having high buffering power at their p*I* value. The curved arrows indicate protein recycling in the flow chamber (from Righetti et al., see Ref. [32]; by permission).

2. A brief excursus on the chemistry of Immobilines

The IPG technique, proposed in 1982 [25], is based on the covalent incorporation of pH-determining buffers into the polyacrylamide matrix. These buffers are acrylamido weak acids and bases (Immobiline, from Pharmacia Biotechnology), capable of being chemically bound to a polyacrylamide matrix. There are some fundamental differences between 'Ampholine' (or Pharmalyte, Biolyte etc.) and 'Immobiline' chemicals:

(a) the former are amphoteric, soluble and contain perhaps thousands of different species, of M_r values from ~300 up to about 1000 u; chemically, they are oligoamino, oligocarboxylic acids;

(b) the latter species are non-amphoteric, bifunctional acrylamido derivatives, of the general formula: CH_2=CH–CO–NH–R. Each is a single, homogeneous compound, of well-defined physicochemical properties.

For covering the pH scale useful for protein fractionation (a pH 2.5–11 interval) with high precision and high versatility only 10 compounds are needed: eight of these are weak acids and bases, with p*K* values well distributed in the pH 3.1–10.3 interval; the other two comprise a strongly acidic (p*K* 1.0) and a strongly basic (p*K*>13) titrants. With this chemistry it is possible to 'tailor' to any separation need any desired pH gradient, from extremely narrow (barely 0.1 pH unit over the entire separation distance) to wide (as much as 8.5 pH units, pH 2.5–11). Such pH gradients are characterized by a high precision of the starting and ending points of the desired interval, by well known courses along the separation column (not only linear, but also convex, concave or sigmoidal, if so desired) [26] and by highly controlled physicochemical properties (e.g., buffering power, ionic strength). Since these chemicals are acrylamido derivatives, when admixed with the two neutral monomers (acrylamide and N,N′-methylenebisacrylamide), they are grafted with a high incorporation efficiency (typically >85%),

thus producing an amphoteric matrix having monotonically increasing pH values from anode to cathode. In each infinitesimal gel layer, the correct pH value will be defined by the unique ratio of buffer and titrant ions incorporated. Clearly, the pH gradient is indefinitely stable and will not decay in the electric field, as long as the matrix is not hydrolyzed. An Immobiline matrix contains no leachables which can contaminate proteins, since all chemicals are covalently bound to form an insoluble matrix. It should be noted however that, since no chemical reaction can ever be driven to 100% conversion, the Immobiline gels (and thus the membranes in the multicompartment machine) are subjected to washing cycles so as to remove ungrafted material. In the electric field, the current will remove those traces of acidic and basic Immobilines still ionically bound to the matrix (which will thus collect in the anodic and cathodic compartments, respectively). If traces of ungrafted neutral monomers are still present (they are believed to be neurotoxins), a scavenging procedure has been described, consisting of incubating gels (or membranes) with 100 m*M* Cys (or other sulfhydryl compounds), able to add to and destroy unreacted double bonds. Matrices thus treated are inert towards proteins (unreacted monomers readily add to Cys residues in proteins at mildly alkaline pH values) [27]. It is thus clear that, if the isoelectric membranes are properly polymerized and treated, the multicompartment apparatus can be considered as one of the mildest methods for protein purification.

3. Description of the instrument

Since the inception of the IPG technique, it was apparent that the very high resolving power (proteins differing by as little as 0.001 pH unit in isoelectric points could be resolved) was coupled to an extraordinary loading capacity, estimated to be ca. one order of magnitude greater than in conventional focusing techniques in amphoteric buffers [28–30]. However, preparative IPGs were still quite cumbersome, as the original technique consisted in driving all proteins to a focusing position in a rather thick Immobiline gel, followed by: (a) protein detection; (b) gel excision; and (c) extraction of the protein zone from the gel fragment. Considering the losses in the different steps and the difficulties in washing away all the gel impurities (ungrafted monomers and oligomers, due to the rather thick gel layers used in preparative scale), the advantages of the IPG technique were largely negated by the poor preparative method devised. Additionally, the total protein load was still substantially dependent on the presence of a gel phase, limiting the protein solubility due to the large amounts of solvent coordinated around the Immobiline matrix [30].

It was thus decided to try an approach by which the protein of interest would be collected in a liquid vein, rather than in a gel phase. A series of chambers were designed to hold two segments of an Immobilized pH gradient, with a gel-free window in the middle, able to keep isoelectric in the liquid stream just a single component in a protein mixture [31]. The approach proved successful, but the chamber design was still primitive. On the basis of this initial success, we carried preparative IPGs to what could be the ultimate development: the concept of a membrane apparatus, in which the entire IPG gel is reduced to isoelectric membranes delimiting a series of flow chambers. Fig. 2 gives an exploded view of the new apparatus [32,33]: it consists of a stack of chambers sandwiched between an anodic and a cathodic reservoir. The apparatus is modular and in the present version can accommodate up to 8 flow chambers. In Fig. 2, we can see a stack of three chambers already assembled to the left, a central compartment and a thinner chamber to the right for connection to the other electrode. All flow chambers are provided with inlet and outlet for sample or electrolyte recycling, an O-ring for ensuring flow-tight connections and four holes for threading four long metal rods which can be tightened by hand-driven butterfly nuts for assembling the apparatus. The pH-controlling membranes are housed in the central depression between two 1-cm wide rings of caoutchouc (however, the commercial version, from Hoefer, San Francisco, CA, USA, is gasket-free). After assembling and tightening the apparatus, each compartment is flow-tight, so that no net liquid bulk flow ensues (except, when applicable, as generated by electrosmosis). The Pt electrodes are housed in two rectangular Perspex mountings, which also act as legs on which the electrolyzer stands. The dis-

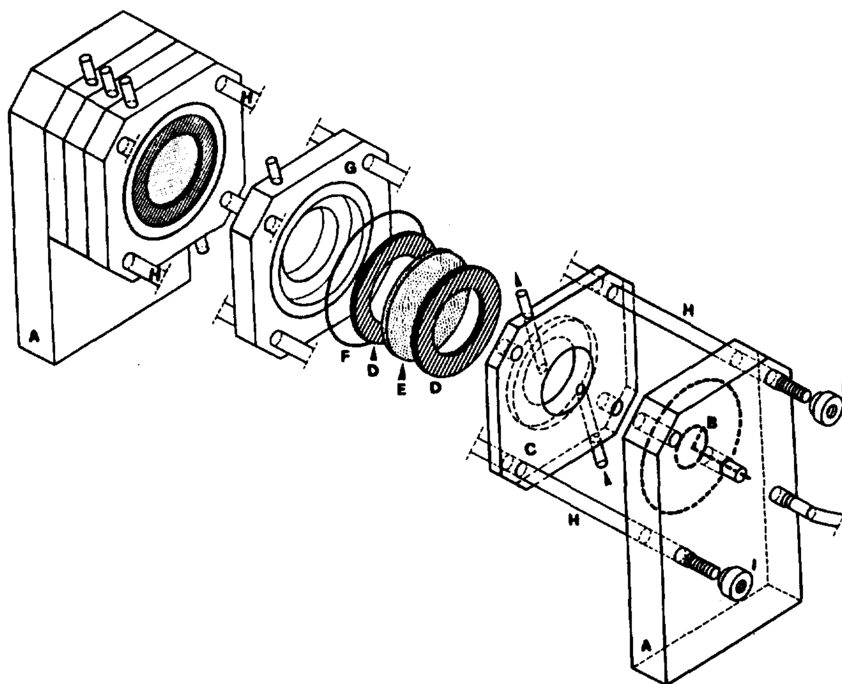

Fig. 2. Exploded view of the multicompartment electrolyzer. A; rectangular supporting legs; B: Pt electrode; C: terminal flow chamber; D: rubber rings for supporting the membrane; E: isoelectric Immobiline membrane cast onto the glass-fiber filter; F: O-ring; G: one of the sample flow chambers; H: four threaded metal rods for assembling the apparatus; I: nuts for fastening the metal bolts (from Righetti et al., see Ref. [32], by permission).

tance between adjacent cells is only 10 mm, so that each chamber holds ~5 ml of liquid. The reason why this system works is shown in Fig. 1: two isoelectric membranes facing each flow chamber act by continuously titrating the protein of interest to its isoelectric point. They can be envisaged as highly selective membranes, which retain any protein having p*I* values in between their limiting values, and which will allow transmigration of any non-amphoteric, non-isoelectric species. For this mechanism to be operative, it is necessary that the two isoelectric membranes possess good conductivity and good buffering capacity, so as to be able to effectively titrate the protein present in the flow chamber to its p*I*, while ensuring good current flow through the system.

The concept of isoelectric Immobiline membranes is quite revolutionary and deserves further comments. Such membranes act as pH-dictating assemblies in an IEF separation, much like a pH-stat unit is set up for controlling, e.g., the pH during a biochemical reaction or during in vitro tissue growth. Each species which is tangent to or crosses such isoelectric membranes is titrated to the pH of the membrane (provided it does not overcome its intrinsic buffering power, β). For amphoteric compounds, this results in a drastic change in mobility, which could reach zero if the two membranes delimiting a single flow-chamber have p*I* values just slightly lower (on the anodic side) and slightly higher (on the cathodic side) of a given macro-ion present therein (see Fig. 1). Any other amphoteric species with lower or higher p*I* value will be forced to exit from such a chamber either towards the anode or towards the cathode, respectively. Thus it is clear that, with a proper set of membranes, it is possible to define in a given chamber isoelectric conditions for just a selected component of a protein mixture, which ultimately will be arrested as the sole isoelectric species in such chamber. Moreover, if the protein concentration is high enough (e.g., 5–10 mg ml^{-1}), the macro-ion present in the liquid stream will

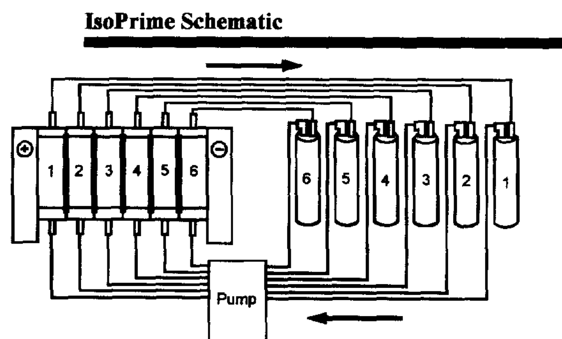

Fig. 3. Scheme of the multicompartment electrolyzer (IsoPRIME, from Hoefer) assembled with six flow chambers, six reservoirs and a multichannel peristaltic pump for recycling (the power supply is not shown). The arrows indicate liquid flow recycling (courtesy of Hoefer).

possess enough buffering power to control the pH, in the absence of exogenous ions migrating through the system. Fig. 3 shows a scheme of the assembled apparatus, with six flow chambers, six reservoirs and a multichannel peristaltic pump for recycling (the power supply is not shown).

4. Some examples of protein purification

In the production of recombinant proteins, structural variations of the final product can arise at the genetic and post-translational level or can be induced by process conditions; common changes in the covalent structure of rDNA proteins which depend on the operative conditions are for example deamidation of glutamine and asparagine side chains, scrambling of disulfide bonds, generation of ragged ends by partial proteolysis or oxidation of sensitive residues [34,35]. Other examples of in vivo post-translational modifications are phosphorylation [36], acetylation, sulfation, glycation and/or deglycation [37,38]. Microheterogeneity seems to be omnipresent in any protein preparation, no matter how extensively purified it is. When analysed by high resolution techniques, such as IPGs, all protein samples display a number of minor components both above and below the zone of the main band. Only after purification through the IsoPRIME machine could we manage to obtain single protein zones in analytical IPGs. We have applied this purification protocol to a number of proteins, including eglin C [32], monoclonal antibodies against the gp-41 of the AIDS virus [39], recombinant human growth hormone [40], the epidermal growth factor receptor [41,42], recombinant superoxide dismutase [43], interleukin 6 [44], glucoamylase [45] and hirudin [46]. For the first time, this process was applied to a rather small peptide, in fact a heptapeptide with a mass of 1800 u, a teicoplanin derivative with broad activity against highly glycopeptide-resistant enterococci [47].

We will give here an example of some unique applications of our purification protocol, which seems to be a must now in crystallography. A purified, soluble form of the epidermal growth factor receptor (sEGFR) was found, by IEF in IPGs, to consist of three major isoforms (with p*I* values of 6.45, 6.71 and 6.96, respectively) and ca. a dozen minor components. This wild type sEGFR, while producing crystals, had so far defied any attempt at decoding the structure, due to the very poor diffraction pattern. When the wild type sEGFR was purified in our multicompartment electrolyzer with isoelectric, Immobiline membranes, it yielded the three major isoforms as single-p*I* components, collected in three separate chambers of the recycling electrolyzer (see Fig. 4). The p*I* 6.71 and the p*I* 6.96 isoforms produced large crystals of apparent good quality [41]. However, while the former produced a high-quality diffraction pattern, which may lead to decoding of the three-dimensional structure, the p*I* 6.96 produced crystals which did not diffract at all. It is concluded that, in the case of 'tough' proteins (large size, heterogeneous glycosylation, high water content of crystals), purification to single-charge components might be an essential step for growing proper crystals. The unique advantage of purification via isoelectric membranes is that the protein is collected both isoelectric and isoionic, i.e. uncontaminated by soluble buffers (such as the carrier ampholytes used in conventional focusing).

5. Interfacing the electrolyzer with mass spectrometry

While we have been always able so far to obtain pure, single bands in any protein preparation after passing it through the multicompartment elec-

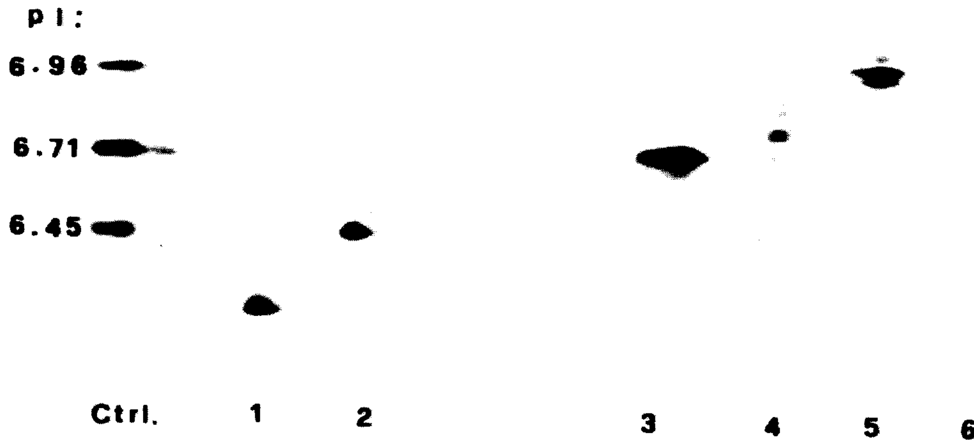

Fig. 4. Analytical IPG run of the content of the multicompartment electrolyzer in the purification of sEGFR. The gel contained an immobilized pH gradient (IPG, pH 5–8) grafted on a 5% T, 4% C matrix. The protein samples were applied in surface wells at the cathode. Focusing was at 10°C for 6 h at 5000 V. Staining with Coomassie Brilliant Blue in presence of Cu^{2+}. 1–6: content of each of the six chambers of the electrolyzer assembled with seven isoelectric membranes with p*I* values: 5.90, 6.34, 6.63, 6.76, 6.92, 7.05 and 7.35. Ctrl.: control, unfractionated sEGFR. The p*I* values of the three major isoforms are given on the left side. Note the high purity of each isoform (from Weber et al., see Ref. [42], with permission).

trolyzer, it was difficult to explain, in many cases, the origin of such band multiplicity. Assuming that the minor components originated from a parental species, via some in vivo or in vitro modification mechanisms, while it is quite easy to hypothesize a number of mechanisms leading to generation of lower p*I* species (e.g., deamidation, glycation, sulfation, phosphorylation and the like) it was quite difficult to understand modifications leading to higher p*I* bands. The problem was particularly vexing with a preparation of an interleukin-6 (IL-6) mutein we had obtained [44]. In addition to a few minor components of lower p*I*, we also had an extra band at p*I* 6.70 (p*I* of native IL-6 mutein: 6.56) (see Fig. 5) which defied attempts at characterization. Finally, after purification in the IsoPRIME apparatus, mass spectra of the p*I* 6.70 form gave an additional mass of +32 u, suggesting the addition of two oxygen atoms (a potential oxidation of Met to a sulphoxide). However, the five Met residues in this higher p*I* form were found to be reduced. In addition, in vitro oxidation of the native p*I* 6.56 form with H_2O_2 (known to oxidize Met residues) gave a form with different p*I* values than the p*I* 6.70 isoform. Upon digestion and fingerprinting, the peptide from residue 50 to 65 of the p*I* 6.70 species exhibited an anomalous elution behaviour in RP-HPLC and retained a +32 u mass shift with respect to the calculated value (Fig. 6). This fragment contains the only two Cys residues, but oxidation to sulphones was excluded. Finally, by combining the data of Figs. 5 and 6, the puzzle was solved: it was noted that, by incubation in excess dithiothreitol, the higher p*I* band was converted to the main p*I* 6.56 band, and

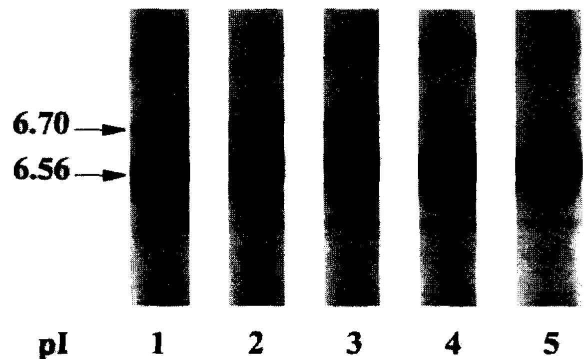

Fig. 5. Reduction of the IL-6 mutein more basic (p*I* 6.70) component. Analytical IPG pH 6.2–7.1 interval of a partially purified mutein not incubated (lane 1) and incubated for 5 min with 0.5 m*M* (lane 2), 1 m*M* (lane 3), 5 m*M* (lane 4) and 10 m*M* (lane 5) dithiothreitol (DTT). The p*I* values are indicated on the left. Note the conversion of the higher p*I* form into the control p*I* 6.56 IL-6 mutein at 5 m*M* DTT (from Breton et al., see Ref. [44], by permission).

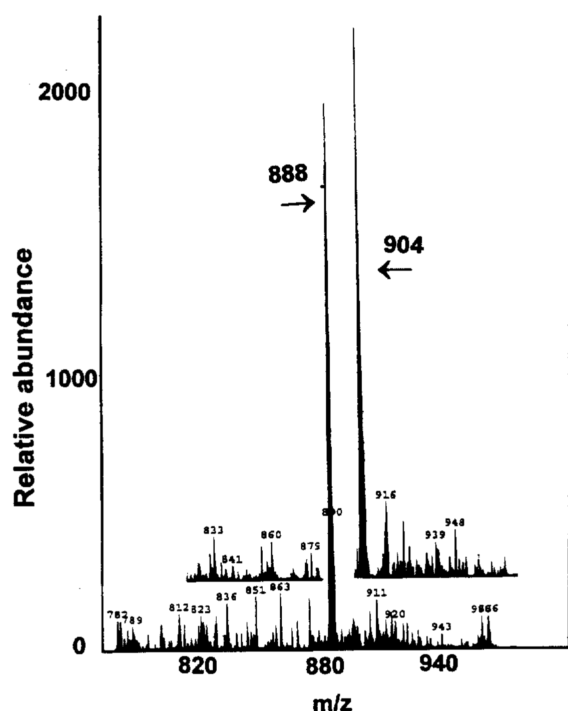

Fig. 6. Overlay mass spectra of doubly charged peptide 50–65 from control IL-6 mutein [$(m+2H)^{2+}=888$] and from the higher p*I* derivative (p*I* 6.70) [$(m+2H)^{2+}=904$] allowed to calculate, respectively, a molecular mass value of 1774 u (corresponding to the theoretical value) and of 1806 (+32 u) (from Breton et al., see Ref. [44], by permission).

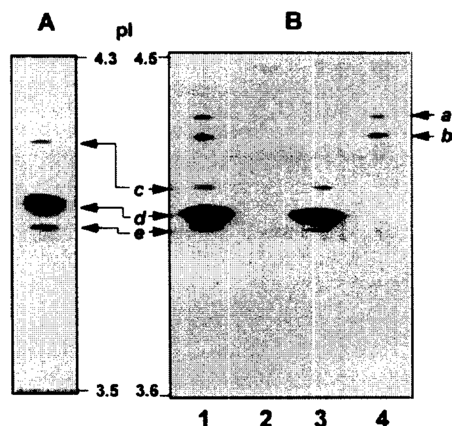

Fig. 7. Isoelectric focusing on immobilized pH gradients of recombinant hirudin variant HM2. Analytical separation of HM2 (p*I*=4.03) in the p*I* range 3.60–4.50, showed four minor contaminants (B, lane 1). Control of preparative separation on the multicompartment electrolyzer (B, lanes 2, 3, 4, respectively, corresponding to chambers 1, 2 and 3) allowed to separate the two more basic compounds a (p*I*=4.31) and b (p*I*=4.25) while micro-preparative slab gel (A), in the p*I* range 3.50–4.30, allowed to separate the other two contaminants c (p*I*=4.10) and e (p*I*=3.98) (from Bossi et al., see Ref. [46], by permission).

this was accompanied by a loss of 32 u. Thus, this higher p*I* form was attributed to the addition of an extra sulphur atom to the only disulphide bridge, to give an unusual trisulphide molecule [44].

Another interesting example in the analysis of minor forms of a pure protein preparation came from our work on hirudins [46]. A preparation of rDNA hirudin (variant HM2) from *Hirudinaria manillensis*, purified to homogeneity, was found, by IEF in IPGs, to still contain a total of 5% of minor components, three with higher p*I* values (4.10, 4.25 and 4.31), one with lower p*I* value (3.98) as compared with the main form (p*I* 4.03) (see Fig. 7). The two more basic minor components were recovered by separation on the multicompartment electrolyzer with isoelectric membranes, whereas the other two minor contaminants were eluted from an IPG gel slab. The use of IPGs in the gel slab format could be a useful complementary, small scale approach to recover proteins for studying minor heterogeneity [50]. The isolated four minor isoforms of HM2 were further characterized by electro-spray mass spectra, limited proteolysis and sequence analysis and were found to be cleavage products of the parent, full length hirudin molecule (M_r 6797 u), as follows: the p*I* 4.31 (M_r 5032 u) had lost 16 amino acids from the N-terminus; the p*I* 4.25 (M_r 6212 u) lacked 5 amino acids from the C-terminus; the p*I* 4.10 (M_r 2980 u) was a cleavage product at the residue Cys^{37}; the p*I* 3.98 (M_r 6610 u) lacked the dipeptide Val--Ser at the N-terminus (Fig. 8). In conclusion, combining the extreme resolving power of IPGs with the high accuracy of mass spectra was found to be a winning strategy in decoding post-synthetic modifications so often encountered in rDNA proteins.

6. The electrolyzer as an immobilized enzyme reactor

Even if we were to end this review here we are confident that the readers would be amply satisfied

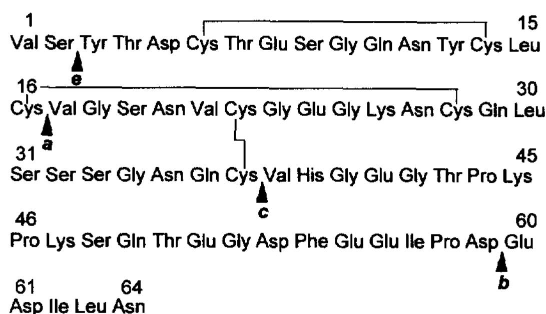

Fig. 8. Primary sequence of HM2. Recombinant hirudin variant HM2 is constituted by a 64 amino acid polypeptide chain with disulfide bonds between Cys^{6}–Cys^{14}, Cys^{16}–Cys^{28} and Cys^{22}–Cys^{37}. The positions of proteolytic cleavages are indicated with black arrows and the resulting fragments are reported under the sequence; these fragments correspond to bands a, b, c and d, as labelled in Fig. 7 (from Bossi et al., see Ref. [46], by permission).

and would not ask for the money for this issue of J. Chromatogr. back. Yet, there is a last, unique development which we want to annotate here as a grand finale. It occurred to us that the multicompartment electrolyzer (ME) could be used as an enzyme reactor: the enzyme could be trapped between two isoelectric membranes and charged reaction products could be removed by the electric field. As the ME is an open system, it offers the opportunity of developing continuous processes. The first example of an enzyme reactor based on the ME was proposed by Chiari et al. [48]: in this preliminary report, a coenzyme-dependent enzymatic transformation was studied. The enzyme β-hydroxysteroid dehydrogenase was trapped isoelectrically into a chamber delimited by isoelectric membranes encompassing its isoelectric point (p*I*). An amphoteric buffer was co-immobilized into the reactor chamber. The substrate (dehydrocholic acid) and reduced cofactor (NADH) were continuously fed into the enzyme chamber, while the product (3β-hydroxy-7,12-dioxo-5β-cholanoic acid) and the oxidized cofactor (NAD^{+}) were transported electrophoretically and collected, separately, into two neighbouring chambers at the anodic side. This novel reactor has been nicknamed, presently, multicompartment immobilized enzyme reactor (MIER). In a second example, urease (p*I* 4.6) was trapped between a p*I* 4.0 and a p*I* 8.0 membranes, thus permitting operation (via suitable amphoteric ions buffering at pH 7.5) at the pH of optimum of activity (pH 7.5) [49]. The charged product (ammonium ions) quickly left the enzyme chamber under the influence of the electric field, thus allowing sustained activity for much longer time periods than in conventional reactors. As an example, while in a batch reactor 90% enzyme activity was lost in 200 min, only 2% activity was lost in the same time period in the MIER reactor. As an additional bonus, the MIER reactor allows conversion rates of >95% in a wide range of substrate concentrations, whereas batch-type reactors rarely achieve better than 50% conversion under comparable experimental conditions. The experimental set-up of this unique reactor is shown in Fig. 9. The reaction can also be followed by monitoring the current as the product is transported from the reactor into a neighbouring chamber: as shown in Fig. 10, a

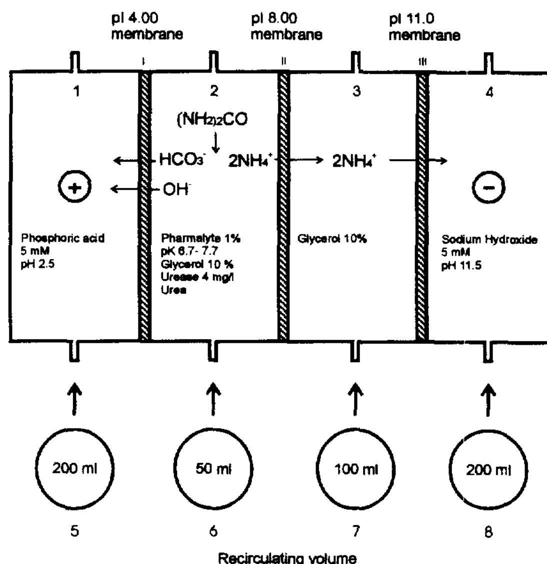

Fig. 9. Experimental set-up of the MIER apparatus. Chambers: 1, anodic compartment; 2, enzyme chamber; 3, product (ammonia) collection chamber; 4, cathodic compartment. I–III: isoelectric membranes, with pIs 4.0, 8.0 and 11.0, respectively. The power supply, peristaltic pump and stirring unit are not shown. The MIER is based on two cross-flows: a hydraulic flow, recycling all contents of each chamber into a reservoir, and a perpendicular electric current flow moving charged, non-isoelectric ions across the various chambers (from Nembri et al., see Ref. [49], by permission).

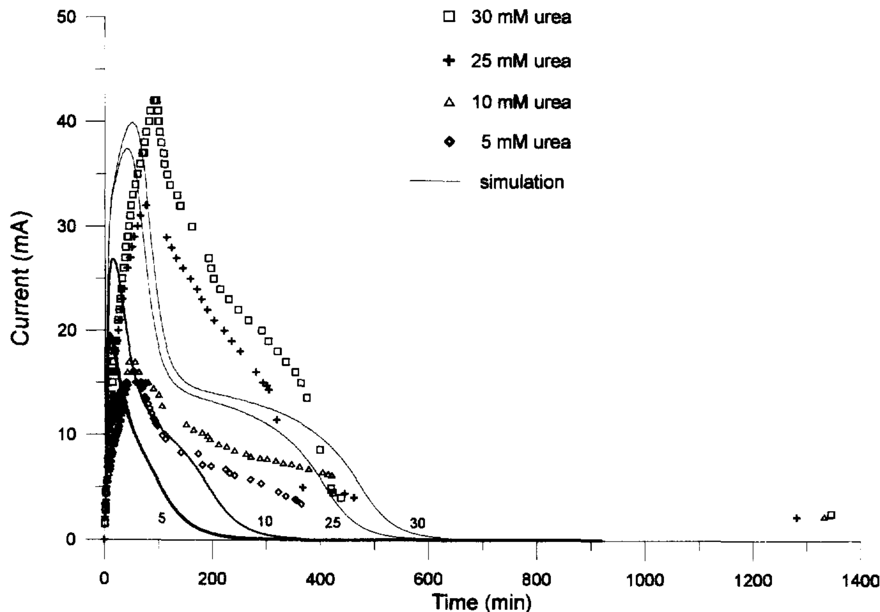

Fig. 10. Profile of current vs. time during operation of the MIER apparatus. The urease chamber (No. 2) has been infused with different substrate levels, ranging from 5 m*M* up to 30 m*M*. The production of ammonia and its transport in the nearest cathodic chamber can be seen as bursts of current, followed by a slow decline. The solid lines represent computer simulations of the process (from Nembri et al., see Ref. [49], by permission).

current spike, of higher magnitude at higher substrate levels, is followed by a slow current decline. Computer simulation of the process, by an approximate (at the moment) theory, showed a reasonable correlation with experimental data. Among some other, outstanding advantages: the enzyme, although immobilized, is kept in solution, thus its kinetic parameters (K_m, V_{max}) are essentially identical to those of a free, native enzyme form.

7. Conclusions

We hope we have proven to the readers, beyond any reasonable doubt, the unique quality and outstanding performance of our multicompartment electrolyzer with isoelectric membranes. This equipment has proven very valuable in purifying rDNA products for human consumption and in collecting reasonable amounts of 'pure' impurities accompanying the desired product so as to allow for their chemical characterization. Here too (as also discussed in the companion review on IPGs) interfacing the ME apparatus with mass spectrometry has proven very valuable in assessing the origin of some minor fractions, with particular regard to higher p*I* components, which up to the present had defied any attempt at decoding. At present, two main mechanisms have been found for generation of higher p*I* species from a parental macromolecule: (a) proteolytic cleavage with removal of peptides containing acidic amino acids; (b) formation of trisulphide bridges. It is hoped that additional mechanisms will be found as this research progresses. Finally, a unique application has been found for the ME equipment: the isoelectric trapping of soluble enzymes in between two membranes and its use as an enzyme reactor coupled to an electric field. It is anticipated that this unusual reactor will find main applications in biotechnology and bioengineering.

8. List of abbreviations

CA Carrier ampholytes
EGFR Epidermal growth factor receptor

IEF	Isoelectric focusing
IL-6	Interleukin-6
IPG	Immobilized pH gradients
ME	Multicompartment electrolyzer
MIER	Multicompartment immobilized enzyme reactor
p*I*	Isoelectric point

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References

[1] K. Jones, Liq. Chromatogr. Gas Chromatogr. Int. 4 (1991) 32–37.
[2] E. Meehan, S. Oakley, Liq. Chromatogr. Gas Chromatogr. Int. 5 (1992) 32–38.
[3] L. Szepesy, G. Rippel, Liq. Chromatogr. Gas Chromatogr. Int. 5 (1992) 24–29.
[4] R.E. Majors, Liq. Chromatogr. Gas Chromatogr. Int. 4 (1991) 14–19.
[5] H. Svensson, I. Brattsten, Arkiv. Kemi 1 (1949) 401–411.
[6] I. Brattsten, Arkiv. Kemi 4 (1951) 503–522.
[7] I. Brattsten, A. Nilsson, Arkiv. Kemi 3 (1951) 337–345.
[8] I. Brattsten, Arkiv. Kemi 8 (1954) 227–244.
[9] I. Brattsten, Arkiv. Kemi 8 (1955) 347–360.
[10] W. Grassmann, Angew. Chem. 62 (1950) 170–176.
[11] W. Grassmann, Ber. Physiol. 139 (1950) 220–227.
[12] J. Porath, Biochim. Biophys. Acta 22 (1956) 151–175.
[13] J. Porath, Arkiv. Kemi 10 (1956) 523–527.
[14] J.S.L. Philpot, Trans. Faraday Soc. 36 (1940) 38–48.
[15] W. Pauli, Biochem. Z. 152 (1924) 355–366.
[16] J. Barrollier, E. Watzke, H. Gibian, Z. Naturforsch. 13(b) (1958) 754–762.
[17] K. Hannig, Z. Anal. Chem. 181 (1961) 244–250.
[18] K. Hannig, in M. Bier (Editor), Electrophoresis, Vol. 2, Academic Press, New York, 1967, pp. 423–471.
[19] O. Vesterberg, T. Wadström, K. Vesterberg, H. Svensson, B. Malmgren, Biochim. Biophys. Acta 133 (1967) 435–445.
[20] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[21] M. Bier, G.E. Twitty, J.E. Sloan, J. Chromatogr. 470 (1989) 369–376.
[22] M. Bier, T. Long, J. Chromatogr. 604 (1992) 73–83.
[23] N.B. Egen, M. Bliss, M. Mayersohn, S.M. Owens, L. Arnold, M. Bier, Anal. Biochem. 172 (1988) 488–494.
[24] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990.
[25] B. Bjellqvist, K. Ek, P.G. Righetti, E. Gianazza, A. Görg, R. Westermeier, W. Postel, J. Biochem. Biophys. Methods 6 (1982) 317–339.
[26] E. Giaffreda, C. Tonani, P.G. Righetti, J. Chromatogr. 630 (1993) 313–327.
[27] M. Chiari, P.G. Righetti, A. Negri, F. Ceciliani, S. Ronchi, Electrophoresis 13 (1992) 882–884.
[28] K. Ek, B. Bjellqvist, P.G. Righetti, J. Biochem. Biophys. Methods 8 (1983) 134–155.
[29] C. Gelfi, P.G. Righetti, J. Biochem. Biophys. Methods 8 (1983) 156–171.
[30] P.G. Righetti, C. Gelfi, J. Biochem. Biophys. Methods 9 (1984) 103–119.
[31] M. Faupel, B. Barzaghi, C. Gelfi, P.G. Righetti, J. Biochem. Biophys. Methods 15 (1987) 163–176.
[32] P.G. Righetti, E. Wenisch, M. Faupel, J. Chromatogr. 475 (1989) 293–309.
[33] P.G. Righetti, E. Wenisch, A. Jungbauer, H. Katinger, M. Faupel, J. Chromatogr. 500 (1990) 681–696.
[34] V.R. Anicetti, B.A. Keyt, S.W. Hancock, Trends Biotechnol. 7 (1989) 342–349.
[35] D. Naveh, Biopharm. 3 (1990) 28–36.
[36] P.A. Maurides, G.R. Akkaraju, R. Jagus, Anal. Biochem. 183 (1989) 144–151.
[37] L. Anderson, N. Anderson, Clin. Chem. 30 (1984) 1898–1905.
[38] A.R. Williamson, M.R. Salaman, H.W. Kreth, Ann. N.Y. Acad. Sci. 209 (1973) 210–224.
[39] P.G. Righetti, M. Faupel, E. Wenisch, Adv. Electrophoresis 5 (1992) 159–200.
[40] C. Ettori, P.G. Righetti, C. Chiesa, F. Frigerio, G. Galli, G. Grandi, J. Biotechnol. 25 (1992) 307–318.
[41] E. Wenisch, P.G. Righetti, W. Weber, Electrophoresis 13 (1992) 668–673.
[42] W. Weber, E. Wenisch, N. Gunther, U. Marnitz, C. Betzels, P.G. Righetti, J. Chromatogr. A 679 (1994) 181–189.
[43] E. Wenisch, K. Vorauer, A. Jungbauer, H. Katinger, P.G. Righetti, Electrophoresis 15 (1994) 647–653.
[44] J. Breton, N. Avanzi, B. Valsasina, L. Sgarella, A. La Fiura, U. Breme, G. Orsini, E. Wenisch, P.G. Righetti, J. Chromatogr. A 709 (1995) 135–146.
[45] E. Wenisch, P. Schneider, S.A. Hansen, R. Rezzonico, P.G. Righetti, J. Biochem. Biophys. Methods 27 (1993) 199–213.
[46] A. Bossi, P.G. Righetti, C. Visco, U. Breme, M. Mauriello, B. Valsasina, G. Orsini, E. Wenisch, Electrophoresis 17 (1996) 932–937.
[47] A. Bossi, P.G. Righetti, E. Riva, L. Zerilli, Electrophoresis 17 (1996) 1234–1241.
[48] M. Chiari, N. Dell'Orto, M. Mendozza, G. Carrea, P.G. Righetti, J. Biochem. Biophys. Methods 31 (1996) 93–104.
[49] F. Nembri, A. Bossi, S. Ermakov, P.G. Righetti, Biotechnol. Bioengineer. 53 (1997) 110–119.
[50] U. Breme, J. Breton, C. Visco, G. Orsini, P.G. Righetti, Electrophoresis 16 (1995) 1381–1384.