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Capillary isoelectric focusing: the problem of protein solubility

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Abstract

A whole family of protein solubilizers, compatible with native structure and maintenance of enzyme activity, is reported for preventing protein precipitation and aggregation at the p*I* value under conditions of very low ionic strength, as typical of isoelectric focusing methodologies. In addition to mild solubilizers proposed in the past, such as glycols (glycerol, ethylene and propylene glycols) non-detergent sulphobetaines, in concentrations up to 1 *M*, are found to be quite effective in a number of cases. Other common zwitterions, such as taurine and a few of the Good's buffers (e.g., Bicine, CAPS) are also quite useful in acidic pH gradients and up to pH 8. Addition of sugars, notably saccharose, sorbitol and, to a lesser extent, sorbose (20% in capillary IEF and in the 30 to 40% concentration range in gel-slab IPGs), greatly improved protein solubility in the proximity of the p*I*. The improvement was dramatic if these sugars were mixed with 0.2 *M* taurine. In the case of hydrophobic peptide antibiotics, mixtures of 6 *M* urea and 25% trifluoroethanol were found to markedly improve solubility. All these additives, unlike non-ionic or zwitterionic surfactant, have the advantage of remaining monomeric, i.e., of being unable to form micelles, even at concentrations >1 *M*. Thus, their elimination from the protein zone can be easily accomplished by gel filtration or by centrifugation through dialysis membranes. Using these additives, capillary IEF of proteins should be now applicable to a number of difficult cases, such as the separation of mildly hydrophobic macroions.

Keywords: Isoelectric focusing; Protein solubilizers; Additives; Sulfobetaines; Proteins

1. Introduction

Both isoelectric focusing (IEF) techniques, in soluble amphoteric buffers (carrier ampholytes, CA) and in insoluble, non-amphoteric buffers (immobilized pH gradients, IPG), are now well-standardized and widely-used methods in all fields of life sciences, especially the highly popular gel-slab version [1,2]. CA–IEF is now gaining momentum also in the capillary format, due to obvious advantages of on-line data acquisition and miniaturization of reagent and sample requirements (for reviews see [3–9]).

CA–IEF, nevertheless, suffers from at least two major drawbacks: (a) the low resolution limit, at present set at around 0.01 to 0.02 Δp*I* and (b) the phenomenon of protein precipitation at the p*I*. The first problem is mainly linked to the fact that, with present technology, narrow pH intervals, of less than 1 pH unit, can hardly be engendered, due to lack of suitable narrow cuts of carrier ampholytes. In contrast, in IPGs, pH intervals of only 0.1 pH unit can be easily arranged, which increments the resolving power to as low as 0.001 Δp*I* [2]. We have addressed this problem when trying to separate different classes of haemoglobin (Hb) and have been able to achieve

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ample base-line resolution between closely-spaced pairs of bands by resorting to non-linear pH gradients. There are several ways to generate such gradients, the simplest one consisting of adding a "separator" (i.e., an amphoteric molecule with a relatively high buffering power) focusing on a given part of the carrier ampholyte pH gradient and thus flattening the pH region in which it is isoelectric [10]. In a first instance, in the screening of umbilical cord Hbs, we were confronted with the problem, for quantitation purposes, of fully separating the fetal (F) vs. the adult (A) and acetylated fetal (F_{ac}) Hbs. In this case non-linear pH gradients were generated by adding 50 m*M* β-alanine to the standard pH 6–8 carrier ampholyte interval [11]. In a second instance, for proper quantitation of glycated Hbs (Hb A_{1c}), we were faced with the problem of separating two species (A vs. A_{1c}) having minute Δp*I*s (of the order of 0.02) with the added burden of having one of the two species present in large quantities (Hb A, typically >90%). In this last case, successful separations and proper densitometric evaluations could be achieved by adding an equimolar mixture of "separators", β-alanine and 6-amino caproic acid, at concentrations of 0.33 *M* [12].

There still remains the severe problem of protein solubility at the p*I* value. This problem is aggravated by increasing sample concentrations (overloading is often necessary in order to reveal minor components) and by decreasing the ionic strength (*I*) of the background electrolyte. In this last case, it has been calculated that a 1% carrier ampholyte solution, once focused, would exhibit a remarkably low *I* value, of the order of 0.5 milli-equiv. L^{-1} [13]. As demonstrated by Grönwall [14], the solubility of an isoionic protein, plotted against pH near the isoionic point, is a parabola, with a fairly narrow minimum at relatively high *I*, but with progressively wider minima, on the pH axis, at decreasing *I* values. This means that, in unfavourable conditions, protein precipitation will not simply occur at a precise point of the pH scale (the p*I*), but will occur in the form of smears covering as much as 0.5 pH units.

In the present report we have addressed the problem of protein solubility at the p*I* and we report a whole series of additives capable of completely preventing or largely alleviating this noxious phenomenon. We have excluded drastic solubilizers, such as detergents and urea, which denature the macroions. In most cases, we have been able to obtain full solubilization with mild agents, able to fully preserve the protein three-dimensional structure and compatibility with full enzymatic activity.

2. Experimental

2.1. Chemicals

Ampholine carrier ampholytes, pH 4–6.5, pH 6–8, pH 6.5–9.0, pH 8–10.5, pH 3–10, dextrans 10 and 40 kDa and the following immobilines: p*K*s 3.6, 4.6, 6.2, 7.0, 8.5 and 9.3, Repel-Silane and Gel Bond PAG film were from Pharmacia LKB (Uppsala, Sweden). Acrylamide, TEMED and persulphate were from Bio Rad Labs. (Hercules, CA, USA). The novel monomer, N-acryloyl amino propanol (AAP), was synthesized and characterized according to Simò-Alfonso et al. [15,16]. The following solubilizers were used: non-detergent sulphobetaines (NDSB), as synthesized by Vuillard et al. [17–19] [in particular the two compounds 3-(N,N-dimethyl-N-ethyl) propyl sulphonic acid, NDSB-195, and 3-(N,N-dimethyl-N-phenyl)propyl sulphonic acid, NDSB-256]; bicine, urea, CHAPS, sorbitol, taurine, glycerol, ethylene glycol, propylene glycol, sucrose, sorbose, CAPS (from Sigma, St. Louis, MO, USA). Poly(vinyl alcohol) was from Polysciences (Warrington, PA, USA).

2.2. Biological samples

The following proteins were analysed: L-aspartate oxydase (LASPO) (a kind gift from Prof. S. Ronchi, University of Milano); thermamylase (an α-amylase from *Bacillus licheniformis*, a kind gift from Novo Nordisk, Bagsvaerd, DK); alcalase (an alcaline protease of the subtilisin family, prepared from a selected strain of a *Bacillus licheniformis*, a kind gift from Novo Nordisk) and a glycopeptide antibiotic of the teicoplanin family (having a hepta-tyrosine as a skeleton, here called Hepta-Tyr; a kind gift from Dr. Zerilli, Lepetit Research Center, Gerenzano, Italy). Except for thermamylase and alcalase, which were only 60% pure (but devoid of exogenous enzyme activities), the other samples were extensively

purified. LASPO gave a single band in SDS gels; the five sharp bands visible in the IPG pattern of Fig. 1D are isoforms since, by zymogramming, they all exhibit the same enzyme activity (zymogram not shown).

2.3. Capillary isoelectric focusing

Capillary IEF was performed on a Bio Focus 2000 (Bio Rad) instrument, equipped with fused-silica capillaries of 75 μm ID and 24/19.4 cm total/effective length (Polymicro Technologies, Phoenix, AZ, USA). The capillary inner wall was deactivated by a poly(AAP) coating, as described [15]. Isoelectric Lys (50 m*M*, pH 9.7) and acetic acid (50 m*M*, pH 3.5) were used as catholyte and anolyte, respectively. The carrier ampholyte consisted of 2.5% Ampholine pH 4–6.5 (for LASPO) or pH 8–10.5 (for Hepta-Tyr) or pH 6–8 (for thermamylase) or pH 6.5–9.0 (for alcalase), all added with 0.5% pH 3–10 range. Samples were injected already premixed to the focusing solution, containing all the ingredients for solubilization. No TEMED was added to the cathodic gradient extremity, since this was found to considerably lengthen the mobilization time. Focusing was typically performed at 10 kV constant, with initial current values ranging from 7 to 9 μA (2 μA at steady state) and variable focusing times from 150 to 400 s, according to the different experimental protocols. One type of mobilization protocol was used, consisting of adding 20 m*M* Na phosphate to the cathodic compartment, coupled to a syphoning effect, obtained by having a higher anolyte level (650 μl) and a lower catholyte level (450 μl) (however, for alcalase mobilization, due to the high viscosity of the medium, 60 m*M* Na-phosphate was utilized). During mobilization, 10 kV (constant voltage) were applied. In all experiments, the capillary was thermostated at 15°C and the sample vial at 6°C. All samples were detected at 280 nm. Every fourth

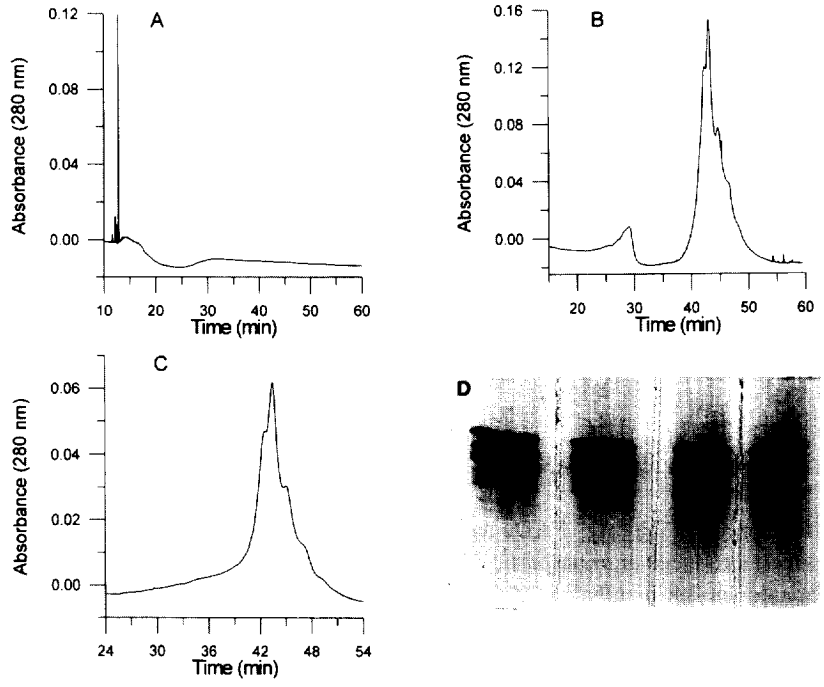

Fig. 1. Isoelectric focusing of L-aspartate oxidase in capillaries in carrier ampholyte buffers (panels A–C) and in immobilized pH gradients (IPG, panel D). (A) Control run in the absence of additives, pH 4.0–6.5 interval; (B) added with an equimolar mixture of 0.5 *M* NDSB-195 and 0.5 *M* NDSB-256 (non-detergent sulphobetaines); (C) added with 1 *M* Bicine; (D) corresponding IPG run in a pH 5.1–5.5 interval, in the presence of an equimolar mixture of 0.5 *M* NDSB-195 and 0.5 *M* NDSB-256 and 10% glycerol. Here and in Figs. 2–5, in the IPG gels, the cathode is at the high end.

run, the capillary was washed with 0.1% CHAPS in water for 120 s, followed by a rinse with distilled water for 120 s.

2.4. Analytical IPGs

All IPG experiments were performed in the LKB 2117 Multiphor II horizontal electrophoresis system together with the LKB Macrodrive 5 power supply and Multitemp II thermostat. IPG gel casting was carried out by using the LKB 2117-903 2-D gradient and Immobiline gel kit. The following IPG intervals were prepared: pH 4.0–6.5 and pH 5.1–5.5 (with a pH 6.5 plateau) for LASPO; pH 6–8 (for thermamylase); pH 7–10 (for Hepta-Tyr) and pH 4–10 (for alcalase), grafted onto 5%T, 4%C polyacrylamide gels. The respective recipes can be found in [2]. After casting and polymerization, the gels were extensively washed in distilled water, dried and then reconstituted to their original weight by reswelling in a cassette in presence of the various additives. Forty-μl protein samples (in general 1 mg/ml) were usually loaded in plastic troughs at the anodic gel side (except for LASPO, where cathodic application had to be adopted), after a long pre-focusing (6 h) at 1500 V due to the high viscosity of some additives. Electrophoresis was then continued overnight (18 h) at a voltage of 2000 V. Staining was in Coomassie Blue R-250 in 10% acetic acid, 30% ethanol in presence of 0.1% copper sulphate except for Hepta-Tyr, where a colloidal suspension of Coomassie G-250 had to be utilized) [36].

3. Results

3.1. Focusing of L-aspartate oxidase (LASPO)

LASPO is a flavoprotein, having FAD as a cofactor [20,21], which produces iminoaspartate by oxidation of its substrate and ultimately, by condensing it with dihydroxyacetone, converts it into quinolinate under catalysis of quinolinate synthase A. The FAD moiety is quickly lost upon focusing, which results in high instability of the protein followed by aggregation and irreversible precipitation. The instability is highly enhanced in acidic milieus, so the sample, in an IPG gel, is typically seeded in a cathodic pH plateau set at pH 6.5. LASPO binds anions preferably and is usually stabilized in a high ionic strength milieu. As shown in Fig. 1A, in a control capillary IEF experiment, in the absence of any additive, the protein precipitates and only sharp spikes (probably representing aggregated particles) are carried past the detector. Attempts at solubilization with a variety of additives (compatible with enzyme activity) such as glycerol, ethylene glycol, propylene glycol, failed. The only mixture that could restore full solubility was a combination of non-detergent sulphobetaines, 0.5 *M* of the M_r 195 and 0.5 *M* of the M_r 256 compounds (Fig. 1B). As shown in this figures, LASPO is resolved into 3 major and a number of minor isoforms, all eluting between 40 and 50 min. Interestingly, a very similar pattern was obtained by using, 1 *M* bicine, as additive, i.e., one of the Good's buffers [22] (Fig. 1C). Fig. 1D shows the pattern which can be obtained in a very narrow IPG gradient, spanning a pH 5.1–5.5 interval. The gel is re-swollen with the same additives of Fig. 1B (an equimolar mixture of the two non-detergent sulphobetaines). Due to the very shallow pH gradient utilized, the three major isoforms are now fully resolved and 4 to 5 minor components, with lower p*I* values, are also visible. It is thus seen that, although with quite different resolutions, the data in capillary IEF and in gel-slab IPGs fully agree.

3.2. Focusing of thermamylase

Thermamylase is an α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) belonging to a family of endoamylases that catalyse the hydrolysis of α-D-(1,4) glycosidic linkages in starch components, releasing malto-oligosaccharides and glucose. The one we have analysed has been purified from *Bacillus licheniformis*, is a single polypeptide chain of 483 amino acids and is called thermamylase because it exhibits a temperature optimum as high as 90°C [23,24]. These α-amylases have important industrial applications, since they are used in desizing of fabrics, in the baking industry, in the production of adhesives, pharmaceuticals and detergents, in sewage treatment and in animal feed. Attempts at focusing this protein in IPG pH 6–8 intervals failed completely. Only precipitation at the application point and

smears and streaking were obtained. Use of standard additives (glycerol, ethylene and propylene glycols), including detergents, did not improve much the pattern. Curiously, even addition of non-detergent sulphobetaines, quite successful with LASPO, although improving the pattern, did not produce well resolved bands. Excellent resolution and focusing patterns were finally obtained in mixtures of neutral additives (typically 20% sucrose, but also sorbitol and, to a lesser extent, sorbose) and zwitterions, in particular 0.1 *M* taurine. As shown in Fig. 2, capillary IEF resolves a major peak and a number of minor components, with a pattern very similar to that obtained in IPGs, in the same pH 6–8 interval (insert; note however that in IPGs the solubilizers were 30% sucrose and 0.2 *M* taurine).

3.3. Focusing of alcalase

Alcalase is a protease of the class of subtilisins, in fact it is a mutant of savinase, with a lower p*I* value. Subtilisins are alkaline serine proteases produced by a wide variety of *Bacillus* species. In view of their industrial application for detergent and food processing, subtilisins have been extensively investigated as promising targets for protein engineering. Since the market for subtilisins, as additives for household laundry detergents, commands the largest share of the world-wide market of industrial enzymes (estimated at $200 million in 1991), it is easy to understand the pressure in the industry to produce newer mutants with improved performance. One of these mutants, savinase (produced at Novo Nordisk) is a serine endopeptidase with an extended binding cleft comprising at least eight binding subsites [25]. It is a relatively small protease, composed of 269 amino acids. Its main commercial use is also as an additive in washing powders. As native savinase has a very high p*I* value (11.2) [26], a novel mutant, called alcalase, with a much reduced p*I* (8.4) and higher enzymatic activity has been engineered at Novo Nordisk. Attempts at focusing this enzyme in IPGs also met with limited success. A mixture which finally gave us excellent patterns consisted of 20% glycerol, 20% sorbitol and 1 *M* of the M_r 195 zwitterion. As shown in Fig. 3, in this solubilizing cocktail, alcalase gave two major peaks and a number of sharply focused minor components. The insert (left lane) shows the corresponding gel patterns obtained in IPGs in the same pH 4–10 range (the right lane being the p*I* marker kit). Considering

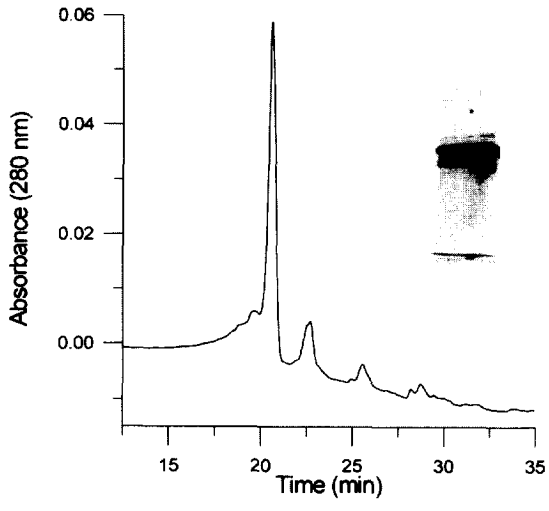

Fig. 2. Isoelectric focusing of thermamylase in capillaries in carrier ampholyte buffers and in immobilized pH gradients (insert), both in the same pH 6–8 interval. Additives: a mixture of 20% sorbitol and 0.1 *M* taurine in capillary IEF and 30% sorbitol and 0.2 *M* taurine in IPGs.

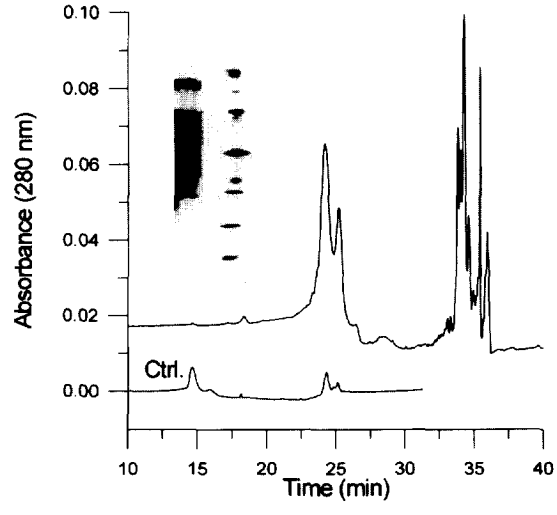

Fig. 3. Capillary IEF in the pH 6.5–9.0 interval and gel slab IPGs (insert) in the pH 4–10 range of alcalase. In both cases, the solubilization cocktail consisted of 1 *M* NDSB-195 (non-detergent sulphobetaine), 20% sorbitol and 10% glycerol. In the insert, the left lane is alcalase, the right lane is a p*I* marker kit. Ctrl.: control capillary IEF, in the absence of solubilizers.

that sugars and polyol derivatives gave consistently good results, we also tested polysaccharides as additives, in the hope that low levels of these polymers could offer the same solubilizing power. However dextrans (both the 40 KDa and 10 kDa sizes) and poly(ethylene glycol) (10 kDa) failed to keep the protein in solution at the p*I*. Interestingly, and quite unexpectedly, poly(vinyl alcohol) (133 kDa) at a concentration of 2.5% for capillary IEF and 5% for gel-slab IPG, gave a remarkably good focusing pattern (see Fig. 4 for capillary IEF and the insert for the corresponding IPG gel).

3.4. Focusing of peptide antibiotics

We were next confronted with the problem of focusing a semisynthetic antibiotic, a small glycopeptide of the teicoplanin family, exhibiting broad activity against highly glycopeptide-resistant enterococci. This glycopeptide consists of a backbone of seven tyrosine residues and is extremely insoluble at its p*I* value (assessed as 8.60) [27]. None of the above solubilizers was successful in keeping it in solution. The only solubilizing cocktail was a mixture of 3.5% zwitterionic detergent (CHAPS) and 8 *M* urea. However, for preparative purification purposes CHAPS was highly undesirable, since it could hardly be eliminated from the glycopeptide fraction. In an attempt at finding small M_r solubilizers, we finally tested a mixture of 6 *M* urea and 10% trifluoroethanol (TFE), which gave us excellent patterns in both capillary IEF (Fig. 5A) and IPGs (Fig. 5B; in this last case, however, the concentration of TFE was raised to 25%).

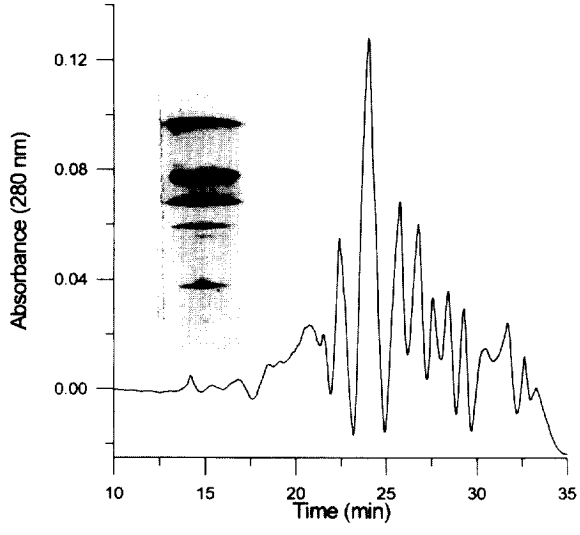

Fig. 4. Capillary IEF in the pH 6.5–9.0 interval and gel slab IPGs (insert) in the pH 4–10 range of alcalase. The solubilizer consisted of 2.5% (for capillary IEF) and 5% (for IPGs) poly(vinyl alcohol).

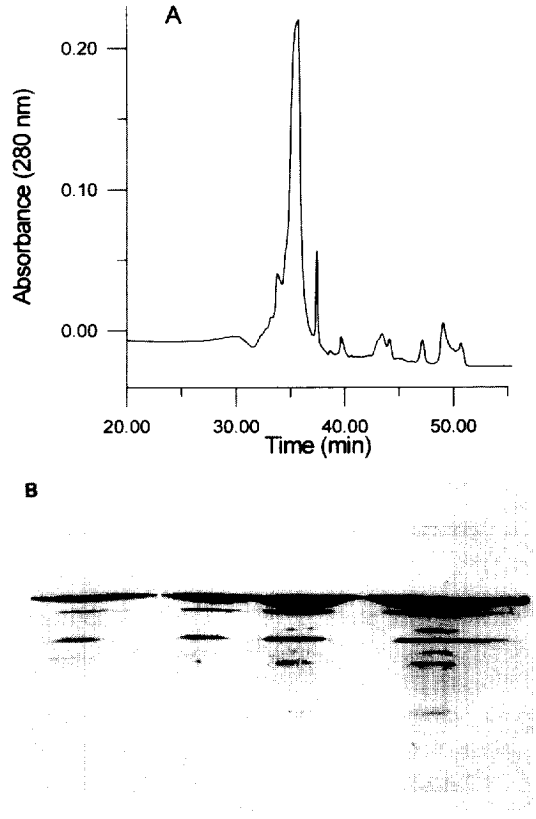

Fig. 5. (A) Capillary IEF in the pH 7.0–10.0 interval and (B) gel slab IPGs in the same pH range of a glycopeptide antibiotic (Hepta-Tyr). The solubilizer consisted of a mixture of 6 *M* urea and 10% trifluoroethanol for capillary IEF and 6 *M* urea and 25% TFE for IPGs. In (B) the sample load is (from left to right): 100, 200, 350 and 500 μg/lane.

4. Discussion

In the last year, our group has been extensively engaged in the purification of gram quantities of recombinant DNA proteins and of novel antibiotic

peptides for both clinical trials and attempts at crystal growth. Although most of these protein samples which were brought to our laboratory had been extensively purified by chromatographic techniques, they were found, by focusing methods (especially by the IPG technique) to still contain a family of contaminants (or isoforms) exhibiting often both higher and lower p*I* components. It was thus necessary to purify them to charge homogeneity, and this was attempted with the aid of our multicompartment electrolyzer with isoelectric membranes [28,29]. This technique has been used, up to the present, highly successfully for purifying a dozen different proteins [30]. However, the set of proteins and peptides reported here failed to stay in solution with known solubilizers adopted by us in the past (notably glycerol, ethylene and propylene glycols), quite successfully, for instance, in the case of recombinant human growth hormone [31]. When purifying proteins for clinical trials, two types of additives had to be carefully avoided: detergents (both neutral and zwitterionic) and urea. Yet, mixtures of surfactants and urea belong to the classical solubilizers as largely adopted, e.g., in two dimensional maps of whole cell lysates, as originally proposed by O'Farrell [32]. The reasons why this family of solubilizers had to be rejected in our protocols are quite obvious: detergents, due to their large micellar size, are quite difficult to completely eliminate from a protein solution; urea denatures and unfolds proteins (this does not apply, of course, to Hepta-Tyr, which does not have a tertiary structure). Thus we focused on a whole family of potential solubilizers, which had to be of a relative small size and had to keep protein integrity.

Particularly encouraging was the use of zwitterions, especially at high concentrations (ca. 1 *M*). It is noteworthy that their use had been advocated long ago by Alper et al. [33]. Although their use had declined, recent reports by Vuillard et al. [17–19] suggest that this was indeed an avenue worth exploring, as their results with a novel class of zwitterions (called non-detergent sulfobetaines) have been encouraging not only in focusing of mildly hydrophobic membrane proteins, but also in using such zwitterions as additives for crystal growth. To this class of compounds, we can now add other zwitterions, such as taurine, Bicine and CAPS. It must be emphasized that these zwitterions must be "poor carrier ampholytes", according to the classical definition of Rilbe [34], i.e., they must have a wide p*I*–p*K* value, so as to be isoelectric over an ample pH interval. The class of compounds synthesized by Vuillard et al. [17–19] meets these requirements, since they contain a strong acid (sulphate) and a quaternary amino group. The other compounds we have mentioned (e.g., taurine, CAPS, bicine) are useful only up to pH 8: above this, they begin to have a net charge and migrate in the electric field (from this point of view, CAPS, with $pK_1 = 1.0$, $pK_2 = 10.4$ is the best species, since it has a ΔpK value of 9.4, thus it is isoelectric over a wider pH region when compared to all other Good's buffers). The class of glycols we explored in the past (glycerol, ethylene and propylene glycols) turned out to be quite ineffective in all applications reported here. What did not seem to have been explored so far was the idea of mixing different solubilizers, an ideal strategy in the present case. As shown in Figs. 2 and 3, mixtures of 30–40% sugars and 0.2 *M* taurine were highly effective in maintaining sample solubility at the p*I* value even under relatively high protein loads. A nice explanation for this solubilizing power could come from the work of Timasheff and Arakawa [35] on stabilization of protein structure by solvents. They explored the physical basis of the stabilization of native protein structures in aqueous solution by the addition of co-solvents at high (ca. 1 *M*) concentrations. According to them, class I stabilizers (such as sucrose and zwitterions, e.g., amino acids, taurine) act by increasing the surface tension of water and by being preferentially excluded from the hydration shell of the protein. In fact, all these chemicals show a negative value of the binding parameters, signifying that there is an excess of water in the domain of the protein, i.e., that the macromolecule is preferentially hydrated. It is of interest to note that all these phenomena occur at high concentrations of the co-solvents, typically above 1 *M*, as found in the present report. As a result, the protein is in a state of "superhydration", which might prevent binding to Immobilines in the gel matrix and might markedly improve solubility at the p*I* value. It goes without saying that these additives fully maintain enzyme activity throughout the purification process (data not shown).

As a final remark, we want to highlight the fact that the interplay between IPG and capillary IEF has been fundamental to the success of this project. Gel-slab IPGs allowed quick visualization of gel patterns and a quick test of many different solubilizing conditions, since gel strips could be cut and reswollen separately in a wide variety of solubilizers. The conditions optimized in IPGs could then be quickly transferred to capillary IEF. As a general trend, it appears that the total concentration of solubilizing cocktails could be substantially lowered in capillary IEF as compared with those adopted in IPGs. This might simply be due to the fact that, due to the higher sensitivity of on-line detection, as compared to Coomassie blue staining, there is less sample focused in a single zone in the capillary as compared with the gel-slab. Moreover, the concentration of sugars had to be lowered to 20% due to their rather high viscosity, hampering an easy filling of the capillary. Gel-slabs, in comparison, are fully compatible with sugar concentrations of up to 40%, since the slab is dried and allowed to reswell in such solubilizers by simple capillary action and passive diffusion. Finally, we want to emphasize that, per se, the idea of using zwitterions in capillary electrophoresis is not new: a number of authors have already reported them. E.g., Petersen and Merion [37] proposed a number of such compounds: trimethyl ammonium propyl sulphonate, triethyl ammonium propyl sulphonate and tripropyl ammonium propyl sulphonate (the first one was later on commercialized by Millipore-Waters under the trade name of AccuPure Z1-methyl). Curiously, however, such zwitterions have been mostly adopted for quenching or modulating the electroosmotic flow of naked silica walls and no one ever reported their use as protein solubilizers. This might also be due to the fact that the best strategy, as shown by our data, is indeed a mixture of zwitterions and sugars, no single solubilizer alone having a dramatic impact on the protein pattern.

5. Conclusions

The present report demonstrates the possibility of finding suitable experimental conditions capable of maintaining proteins in solution at their pI values during analytical and preparative IEF. An array of such solubilizers have been proposed up to the present for accomplishing this task: glycerol, ethylene and propylene glycols, non-detergent sulfobetaines, zwitterionic (such as CHAPS) and neutral (such as Nonidet) detergents. In the case of hydrophobic peptides, addition of 20–25% trifluoroethanol was found to be highly beneficial. We can now add other categories, such as different sugars (saccharose, sorbitol, sorbose), the zwitterion taurine (and all non-detergent sulphobetains proposed by Vuillard et al. [17–19]) and especially mixtures of these two classes of compounds. The idea of exploring mixtures of additives, in the hope of a cooperative effect, is certainly worth exploring in a number of difficult cases.

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