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High-efficiency capillary isoelectric focusing of protein complexes from *Escherichia coli* cytosolic extracts

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Abstract

High-efficiency capillary isoelectric focusing (cIEF) separations of protein complexes obtained from soluble protein fractions are demonstrated. Size-exclusion chromatography was used as a first dimension separation to fractionate putative protein complexes with apparent molecular masses of up to 1 500 000 from an *Escherichia coli* cytosolic fraction. Non-denaturing cIEF separations using highly hydrophilic polymer-coated capillaries constituted the second dimension. The conditions developed produced reproducible and high-efficiency separations, corresponding to $\sim 2 \cdot 10^6$ theoretical plates and peak capacities of $\sim 10^3$ for pH 3–10 cIEF separations in 65 cm long capillaries. Combination of the two non-denaturing separation dimensions permitted isolation and analysis of individual protein complexes from complicated biological samples. Studies indicated that many *E. coli* complexes were stable on the time scale of the cIEF separations, but were degraded upon more extended periods of storage on ice, necessitating rapid sample processing and fast analysis techniques.

Keywords: Isoelectric focusing; *Escherichia coli*; Proteins

1. Introduction

Many, if not most, biological processes involve the participation of macromolecular complexes [1]. Mass spectrometry (MS) has been successfully used to probe individual non-covalent protein complexes using soft ionization and interface conditions [2–6]. The application of capillary isoelectric focusing (cIEF) and cIEF–MS to the analysis of samples of even greater complexity, such as cell lysates, needs to address thousands of proteins that span a broad range of ionic, hydrophilic, and hydrophobic properties [7]. Thus, appropriate high-resolution separation strategies that maintain noncovalent biomolecular associations are desirable for isolation of individual protein complexes for further characterization.

A primary consideration for the choice of separation processes is that they involve favorable physicochemical and biochemical environments that promote the stability of protein complexes. While all separation processes can perturb the stability of protein complexes, two separation methods, field-flow fraction (FFF) and size-exclusion chromatography (SEC), appear to yield the most favorable physicochemical environments for analysis of cellular protein complexes [8–12]. In FFF, interactions with separation media surfaces are not required and the minor applied forces (e.g., gravity) generally

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have negligible effects upon the stability of protein complexes. In SEC, the modern stationary phases provide minimal sample–stationary phase interactions, even under physiological salt and pH conditions desirable for the stability of protein complexes. Compared with FFF, SEC requires less complicated instrumentation, but the major shortcomings of both FFF and SEC are their low separation efficiencies.

Electrophoresis is the most widely used technique for protein separations [13], but its use for protein complexes has been relatively unexplored [14]. The matrix porosity used in gel electrophoresis imposes limitations for sieving that will have an upper (shape dependent) solute size. For example, molecular sieving in polyacrylamide gels typically limits separations to molecular masses below ~1 000 000, depending upon structure [14].

The size limitations of gels make the application of free solution electrophoresis in capillaries (e.g., 50 μm I.D.) potentially attractive for separations of protein complexes. In addition, capillary separations are particularly useful when the amount of sample is limited. Capillary zone electrophoresis (CZE) [15] and cIEF [16] are the most widely used capillary electrophoresis (CE) formats for the analysis of proteins. The application of CZE for complex mixtures of protein complexes is difficult, as extremes of pH are generally required to electrophoretically elute both acidic and basic protein complexes towards either the anode or cathode in a single separation. These extreme pH conditions are problematic for the stability of most protein complexes. In contrast, during cIEF the isoelectric pH (p*I*) of the analyte (not the pH range of ampholytes used for the separation) limits the extremes of pH to which a particular complex will be exposed [17]. Thus, in general, the average pH experienced during cIEF of such mixtures will be closer to neutral than those needed for CZE. While interactions between carrier ampholytes and protein complexes interactions can occur in cIEF, preliminary results at our laboratory using cIEF–MS for analysis of mixtures of several purified protein complexes have not revealed detectable changes resulting from interactions between protein complexes and carrier ampholytes [18]. These observations have prompted this broader evaluation of cIEF for separation of protein complexes.

In this study, we demonstrate high-efficiency cIEF separations for a wide range of putative soluble protein complexes from *Escherichia coli* cytosolic extracts. A two-dimensional SEC–cIEF approach is presented for obtaining highly enriched fractions of protein complexes under non-denaturing conditions. Some issues related to complex stability during cIEF separations are also considered.

2. Experimental

2.1. Preparation of an E. coli cytosolic fraction

E. coli (1.8 l) was grown to stationary phase (A_{600}=5.5) at 37°C in LB media (Difco, Franklin, NJ, USA), cooled on ice, and harvested by centrifugation (3000 *g*, 10 min). The resulting pellet (2.7 g wet mass) was washed with cold 0.1 *M* KCl, centrifuged, and resuspended in lysis buffer [50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 m*M* NaCl, 1 m*M* EDTA, 0.02% sodium azide, 5 m*M* dithiothreitol (DTT)] to which was added 0.1 mg/ml ribonuclease A (Pharmacia Biotech, Uppsala, Sweden). Resuspended cells were twice passed through a chilled French Press cell (MicroDisrupter, Cell Scientific, Champaign, IL, USA) equilibrated at 25 000 p.s.i. for 30 s and the lysate produced dropwise over approximately 1 min (1 p.s.i.=6894.76 Pa). The lysate was then incubated for 20 min at ambient temperature to permit RNase digestion. Following RNase treatment, agarose gel analysis (not shown) indicated that the residual nucleic acid components exceeded the exclusion limit of the Superdex 200 column. Cell debris was removed by centrifugation at 17 000 rpm in a refrigerated Eppendorf centrifuge and a cytosolic fraction was obtained by a 10-min centrifugation in a TLA120.1 rotor at 80 000 rpm (240 000 *g*). Samples for size exclusion were used directly, and additional aliquots were quick frozen in liquid nitrogen and stored at −80°C. A protein concentration of 27 mg/ml was determined using the standard Bio-Rad (Hercules, CA, USA) protein assay with bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) as a standard. Prior to cIEF separation, cytosol was desalted using Micro-Biospin 6 columns (Bio-Rad) equilibrated in 10 m*M* ammonium acetate.

2.2. Separation of E. coli cytosol by SEC, and analysis of SEC fractions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

E. coli cytosol was fractionated using a Superdex 200 HR 10/30 column (24 ml bed volume; Pharmacia Biotech) connected to a BioCad (Perseptive Biosystems, Framingham, MA, USA) chromatography system. Cytosol (0.25 ml, 6.8 mg) was injected onto the column that was pre-equilibrated in SEC buffer (10 m*M* Tris, pH 8 at 20°C, 150 m*M* NaCl, 1 m*M* $MgCl_2$) at a flow-rate of 0.3 ml/min. The column effluent (0.3-ml fractions) was collected on ice with an automated fraction collector (Waters, Milford, MA, USA), was aliquoted, quick frozen, and stored at −80°C.

For SDS–PAGE analysis, SEC fractions were diluted with 3× sample buffer and separated using a 30-well (145 mm×1 mm) 12% gel (monomer–crosslinker, 37.5:1) SDS–PAGE system using standard conditions [19]. Electrophoresis was carried out in a Dual Vertical Gel System (CBS Scientific, Del Mar, CA, USA) at 60 V for 60 min then 120 V for 240 min. Molecular mass markers were included on both outside lanes, and were prepared according to manufacturer's instructions (LMW protein standards; Bio-Rad). The separation gel was visualized using Coomassie Blue Stain, and dried onto filter paper (Whatman, Rockland, MA, USA) using a commercial gel dryer system (Bio-Rad). The dried gel was scanned at 800 dpi (UMAX Model 600 P) in 256 tone greyscale mode, and the final figure was prepared with PaintShop Pro version 4.

2.3. cIEF experiments

Untreated fused-silica capillary tubing (50 μm I.D.×190 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA) was coated with hydroxypropyl cellulose (HPC, average molecular mass of 88 000; Alldrich, Deerfield, MI, USA) as previously described [20]. The detection window was made by burning off a short (~3 mm) section of the polyimide. Ammonia hydroxide (1%, w/w, pH~10.7) and acetic acid (1%, w/w, pH~2.5) were used as catholyte and anolyte, respectively. Pharmalyte pH 3–10 (Pharmacia Biotech) was used as the carrier ampholyte to generate a pH gradient at 20 kV (Glassman High Voltage, Whitehouse Station, NJ, USA). The samples were mixed with the carrier ampholyte (final concentration of 1%, v/v) and then injected to fill the entire capillary for focusing. The focused zones were hydrodynamically mobilized by elevating the anode reservoir relative to the cathode reservoir. Both focusing and mobilization processes were monitored by UV absorbance at 280 nm (Spectra 100 UV/VIS; Spectra-Physics, San Jose, CA, USA). The p*I* calibration and the mobilization linear velocity [20] was determined using standard p*I* markers, including myoglobin (p*I* 7.2, 6.8) carbonic anhydrase (p*I* 6.6), and β-lactoglobulin A (p*I* 5.3) (Sigma)

3. Results and discussion

3.1. E. coli cytosol contains many apparent high-molecular-mass species

Greater than 98% of *E. coli* open reading frames (ORFs) encode proteins that have molecular masses (M_r) below 100 000, as illustrated in Fig. 1. However, size fractionation of *E. coli* cytosol reveals that a significant fraction of proteins elute with higher apparent M_r values. As evident from the examination of the SEC fractions by SDS–PAGE (Fig. 2) these higher M_r fractions are likely enriched in both homomeric and heteromeric protein complexes. The higher apparent M_r values observed under non-denaturing conditions is unlikely to arise from aggregation since ultracentrifugation was performed just prior to SEC to remove aggregates from the sample. The elution patterns of most protein bands in Fig. 2 appear symmetrical, indicating elution of homogeneous components by SEC. We also can dismiss the possibility that the majority of the higher M_r components are artificial due to sample–column interactions as such interactions typically result in leading or trailing of components of peaks, rather than complete shifts in the elution profile.

SEC has both benefits and limitations as a first stage separation technique. The large particle size (20 μm diameter) of the packing material used for SEC results in a limited resolution. However, the high column permeability avoids the mechanical and physical perturbations that high pressures and rapid pressure changes might exact on protein/complex

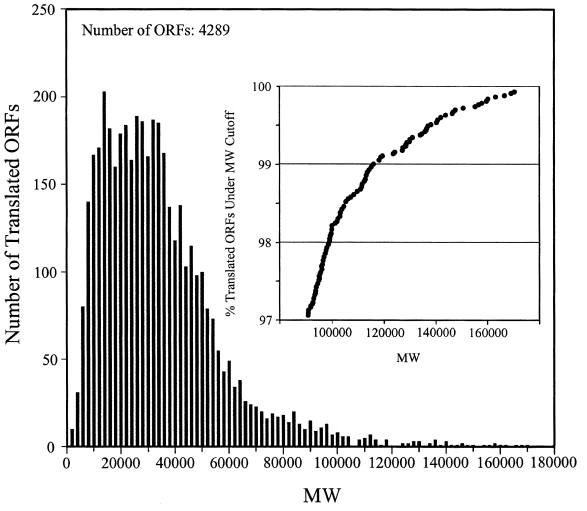

Fig. 1. Calculated M_r distribution of *E. coli* proteins predicted from the annotated *E. coli* genome sequence. [Average masses were calculated using the ORFs predicted in annotated *E. coli* genome (Genbank Accession U00096), and do not account for potential modifications] . The distribution of masses was plotted as a histogram using a bin size of 2000. Not shown are two predicted proteins of M_r 182 000 and 251 000. Inset: Scatter plot representing the cumulative percentage of predicted *E. coli* proteins (100%=4289) having M_r values less than or equal to that indicated on the horizontal axis (MW=M_r).

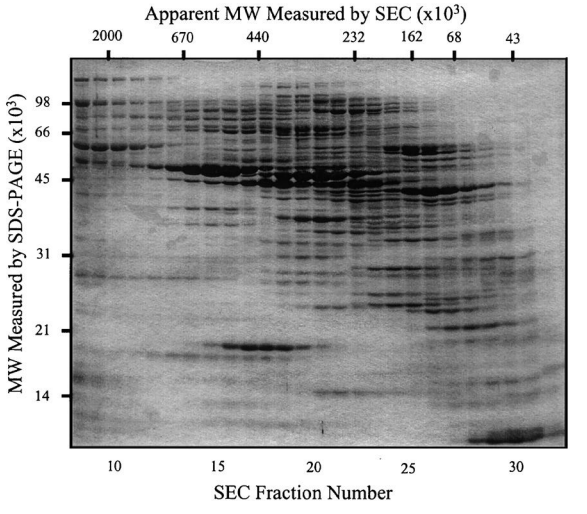

Fig. 2. SDS–PAGE separation of size fractionated *E. coli* cytosol. *E. coli* cytosol (6.8 mg) was fractionated by SEC. SDS–PAGE analysis of individual fractions (bottom scale) from SEC shows that a significant proportion of *E. coli* proteins exist as complexes during SEC with high apparent molecular masses (top scale) compared to denaturing SDS–PAGE conditions (left scale).

structure (i.e., when using smaller particle sizes with higher pressure drops).

3.2. cIEF of *E. coli* cytosol fractions

The high sensitivity of cIEF made it possible to dilute the *E. coli* cytosol (27 mg/ml) approximately 300-fold prior to cIEF analyses. Polyampholyte (pH 3–10) was added to achieve a final concentration of 0.09 mg/ml protein and 1% (v/v) ampholyte. Fig. 3

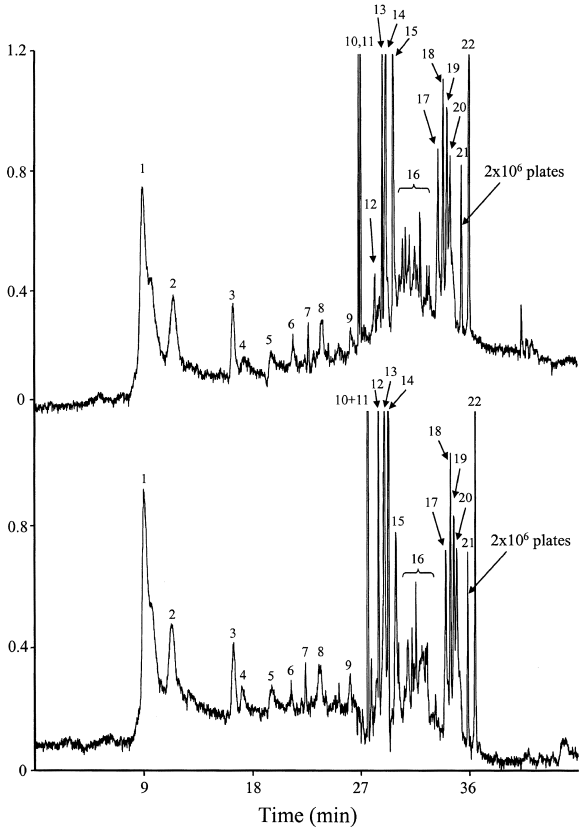

Fig. 3. Examples showing the reproducibility obtained for cIEF separations of *E. coli* cytosol. Conditions: 65 cm×50 μm I.D. HPC-coated fused-silica capillary column; sample solutions were freshly prepared prior to cIEF to contain 1% (v/v) of Pharmalyte (pH 3–10) and a total protein content of 0.09 mg/ml. Separation involved 20 min focusing followed by 8 cm elevation of the inlet for gravity mobilization. The vertical and the horizontal axes give the relative UV adsorption magnitude and elution time, respectively.

illustrates the reproducibility of cIEF analyses for the diluted *E. coli* cytosol (the peak numbers are given to aid comparison). Repeated experiments gave no indication of column plugging due to sample aggregation during these analyses.

Fig. 3 demonstrates that most peaks in the sample, including the narrowest peaks (peaks 16–22) can be reproducibly observed by cIEF. For other peaks, (e.g., peaks 10–12 and 15) some minor differences in peak resolution and intensity were evident. Using standard p*I* markers, the peaks from 1 to 22 were calibrated to have p*I* values from 4 to 8, and the largest variation is estimated to correspond to a p*I* uncertainty of ~0.05 unit. This uncertainty may arise from small changes of the capillary inner wall properties during successive runs. The narrowest peaks routinely detected for these samples have peak widths at the half height of ~3 s. With the calibrated mobilization linear velocity (~0.9 cm/min) using standard p*I* markers, this peak width corresponds to a zone length of ~0.045 cm [0.9 cm/min×(3 s/60)] in the 65 cm length capillary column and this corresponds to 0.005 pH units if a smooth gradient is assumed over the pH range of 3–10. The narrowness of these peaks corresponds to a theoretical plate number of $2 \cdot 10^6$ and peak capacity (R_s~1.0) of ~1000 [1.5×65 cm/(2×0.045 cm)] [21]. The sample concentration coefficient for the narrow zones (peaks) corresponds to ~700 [65 cm/(2×0.045 cm)].

As described above, the concentrated sample was diluted prior to cIEF separations, which may have resulted in disruption of some weaker complexes (although these complexes would likely be lost during SEC). The possible effects of dilution were examined by processing diluted cytosol diluted (10-fold) to an intermediate concentration through a single-stage continuous flow microdialysis device, previously described by our laboratory [22], using a Sialomed (Columbus, MD, USA) cellulose acetate membrane (molecular mass cutoff, MWCO= 100 000). Two fractions were obtained: a high-molecular-mass fraction significantly enriched in components above the M_r cutoff, and a low-molecular-mass fraction composed of components below the cutoff. Both fractions were prepared as above, and cIEF separations were conducted under the conditions used for the original cytosol sample. Fig. 4 shows the cIEF separations for the two dialysis

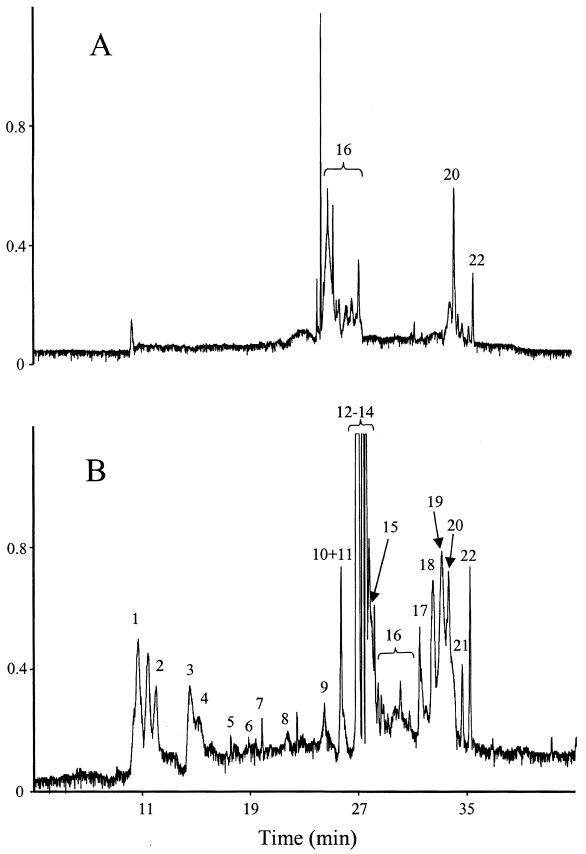

Fig. 4. cIEF of two *E. coli* cytosol fractions obtained from single-stage microdialysis separation [22] (100 000 MWCO). cIEF was used to analyze the (A) low-M_r and (B) high-M_r fractions of the dialyzed *E. coli* cytosol prepared as described in the text. Prior to cIEF runs, each fraction was mixed with 10% (v/v) ampholyte to a final ampholyte concentration of 1% (v/v). Other conditions as in Fig. 3.

fractionation samples, and the marked peaks are those observed in cIEF of cytosol (Fig. 3). For the low-molecular-mass fraction, relatively few peaks are evident (Fig. 4A). A much larger number of components observed in the cytosolic sample (Fig. 3) have a separation pattern consistent with peaks in the high-molecular-mass fraction (Fig. 4B). These results indicate that many cIEF peaks observed during analysis of *E. coli* cytosol result from higher apparent molecular mass components, and that dilution should not greatly affect the stability of complexes that survive SEC.

3.3. Non-denaturing SEC–cIEF two-dimensional separations

Both SEC and cIEF separations can be carried out under conditions that are conducive to analysis of intact protein complexes. SEC fractionates protein complexes according to their size under native conditions, while cIEF resolves intact protein complexes according to their isoelectric points with high efficiency. Therefore, non-denaturing SEC–cIEF provides orthogonal two-dimensional separation for protein complexes.

Fig. 5 demonstrates the capability of cIEF to resolve putative protein complexes from four SEC fractions encompassing the separation range of the Superdex 200 column. The p*I* scale (bottom) was calculated by separate experiments involving the addition of standard protein p*I* markers to each of the SEC solutions that were analyzed under the same experimental conditions. It became evident during cIEF analyses that the relatively low resolution of the SEC dimension can result in some peaks that are apparently present over five SEC column fractions (e.g., fraction 20 vs. fraction 15). These overlaps are consistent with the gel patterns observed in Fig. 2, although direct assignment of the overlapping peaks will require additional analysis. The resulting electropherograms are also consistent with the pattern of SEC elution observed in Fig. 2, in that fewer complexes would likely be observed in fractions containing fewer potential subunits. As also expected for non-covalent complexes, the number of peaks observed for each fraction is substantially less than the number of component proteins observed under denaturing conditions using by SDS–PAGE.

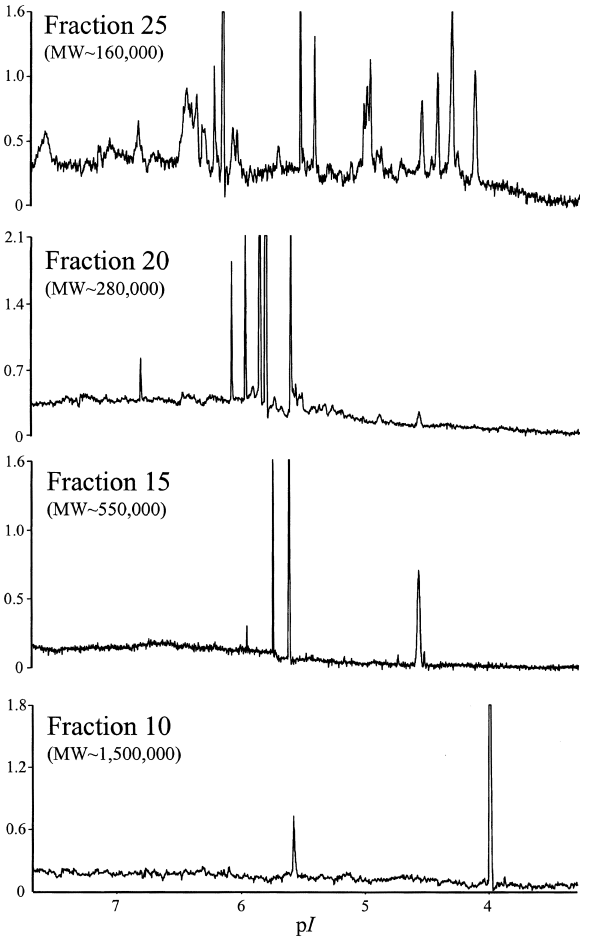

Fig. 5. cIEF separations of four SEC fractions of *E. coli* lysate proteins. Prior to cIEF each fraction was diluted by 300 times and mixed with 1% (v/v) ampholyte; p*I* scale was calibrated by adding standard p*I* markers in each sample solution. Other conditions as in Fig. 3.

Although the data obtained from both the SEC and cIEF separation dimensions provides useful information about the nature of the intact complex, our approach could yield even greater rewards when combined with a third dimension of analysis using MS. Our laboratory has recently established that mixtures of several purified homomeric and heteromeric non-covalent protein complexes can be effectively resolved by cIEF and the intact complexes or their component subunits can be observed by online mass spectrometry, depending on whether ''native'' or denaturing interface conditions are used between the capillary outlet and the mass spectrometer [18].

It should also be noted that the biological application of these approaches requires a sample representative of complexes present in the source cells or tissue, and that the sample preparation methods used can exert influence on sample composition. The use of French press for cell disruption permits the rapid production of between 0.5 and 40 ml of highly concentrated lysate depending on design of the pressure cell; however, the phenomenon of hydrostatic pressure induced oligomer dissociation [23,24] may bias observed complexes to those that are stable at high pressure, and those that reform upon restoration to atmospheric pressure. Other common lysis

techniques (e.g., sonication, bead-beating, enzymatic digestion/freeze–thaw) can reversibly or irreversibly disrupt complexes present in the sample that are sensitive to mechanical sheer forces and/or temperature variations. In the end, gentler lysis procedures may be required, and lysis efficiency sacrificed, in applications where less stable ''native'' complexes are the targets of study.

3.4. Ampholyte–protein complex interactions and sample stability

There are two major factors that may influence protein complex stability during isoelectric focusing analysis. The first is the possibility of alterations of protein complex composition upon focusing as the net charge of protein complexes approaches zero. This possibility is difficult to address experimentally; however, the cIEF approach used here is far superior to gel-IEF techniques where the focusing process takes much longer. The second complex stability issue arises from the possible effects of direct interactions between carrier ampholytes and protein complexes. These interactions have been previously observed [25] and can be investigated in our system by varying sample processing and analysis procedures during cIEF analysis.

Fig. 6 shows the protein complex stability test results using SEC fraction 15 as the test sample. Fig. 6A and B demonstrate the effect of carrier ampholyte on protein complex composition on the cIEF separation time scale. The sample was freshly prepared by adding the ampholyte solution prior to each cIEF run. Decreasing analysis time (including the focusing time) from 25 to 35 min by shorting the capillary column length from 65 to 35 cm caused no significant changes in the separation pattern.

Fig. 6C demonstrates the effect of longer-term sample exposure to carrier ampholyte. New peaks were observed for a sample solution held at 4°C for 8 h prior to cIEF. The new peaks can be attributed to protein complex denaturation and/or carrier ampholyte–protein complex interactions during 8 h of exposure of the complexes to the carrier ampholyte. By storing the sample at 4°C for 8 h without carrier ampholyte, and then adding ampholyte prior to the cIEF separation, new peaks were again observed in the electropherogram (Fig. 6D). However, the new peaks in Fig. 6D have a different p*I* distribution from

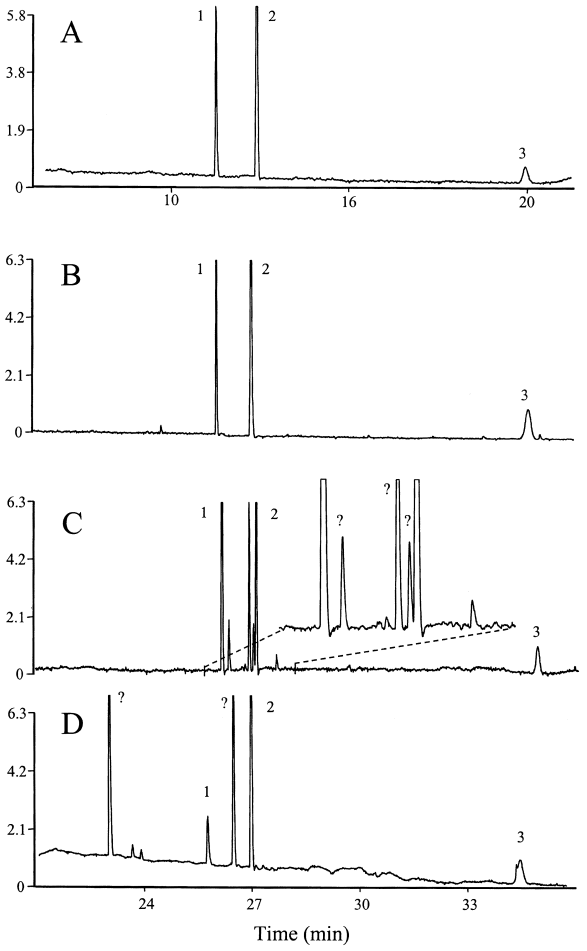

Fig. 6. Evaluation of protein complex stability using cIEF. (A) Fast separation using a 35 cm×50 μm I.D. HPC-coated capillary column, sample diluted 300 times and mixed with 1% (v/v) ampholyte prior to cIEF (6 min focusing, 6 cm gravity mobilization); (B) longer separation using a 65 cm×50 μm I.D. HPC-coated capillary column (20 min focusing, 8 cm gravity mobilization); (C) cIEF under the same conditions as (B) except for an initial 8 h storage at 4°C of the diluted sample in ampholyte; (D) cIEF under the same conditions as (B) except 8 h storage (at 4°C) of the diluted sample prior to mixing with ampholyte.

those in Fig. 6C. This suggests that the carrier ampholyte contributed to the process of protein complex instability during extended storage (8 h, on ice) prior to cIEF. From these results, it can be seen that rapid processing and analysis of samples is critical to obtaining useful information regarding intact complexes. In this respect, cIEF has a significant advantage compared with traditional gel-IEF separations, in which the slow separation process

allows a longer time (e.g., >8 h) for complex dissolution, as well as longer exposure of samples to carrier ampholyte.

4. Conclusions

High-efficiency cIEF separations of soluble protein complexes can be reproducibly achieved using highly hydrophilic polymer-coated fused-silica capillary columns. The separation efficiencies can achieve theoretical plate numbers of $\sim 2 \cdot 10^6$ and peak capacities of $\sim 10^3$. During cIEF focusing, *E. coli* cytosolic complexes appeared stable and can be effectively concentrated up to 700-fold. These results show that the combination of non-denaturing SEC and cIEF provides orthogonal two-dimensional separations that can resolve large protein complexes according to their p*I* values and apparent native molecular masses. On the time scale of cIEF (25–35 min), no detectable changes in protein complex composition were observed. However, extended storage (e.g., 8 h at 4°C) of samples in the presence of carrier ampholyte resulted in significant changes to the separations. Thus, rapid sample processing and analysis appear crucial for obtaining reliable cIEF results.

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