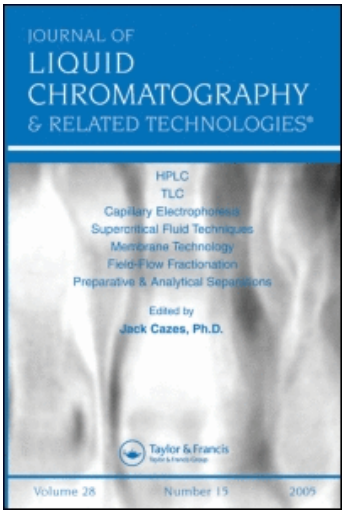

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Jiaqi Wu[a]; Janusz Pawliszyn[a]
[a] Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada

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SENSITIVITY ENHANCEMENT METHODS FOR CAPILLARY ISOELECTRIC FOCUSING/ CONCENTRATION GRADIENT IMAGING SYSTEM

JIAQI WU AND JANUSZ PAWLISZYN*
Department of Chemistry
University of Waterloo
Waterloo, Ontario, Canada N2L 3G1

ABSTRACT

Proteins in biological cells can be analyzed by the inexpensive capillary isoelectric focusing (CIEF)/concentration gradient imaging system. Proteins were focused during the focusing process by the CIEF instrument which contains a 4-cm-long, 100 μm i. d. capillary. All focused zones were detected in an on-line fashion by the universal concentration gradient imaging detector consisting of a He-Ne laser and charge-coupled device. The sensitivity of the detector was enhanced in two ways; a signal averaging method and an off-column concentration method using ultrafiltration. Sensitivity enhancement by a factor of 4.5 was achieved by the averaging method, and the detection limit was 13 μg/mL. The whole analysis time was only 2 minutes. In the concentration method, protein samples were first concentrated by more than an order of magnitude by using ultrafiltration performed in a centrifuge. Then, they were introduced into the CIEF instrument, and separated and detected. The detection limit was 1 - 5 μg/mL. The whole analysis procedure took only about 25 minutes. This speed is much faster than that of gel slab isoelectric focusing, and while similar to CIEF performed in commercial instruments. The detection limit is much lower than that of commercial CIEF instruments due to the concentration step.

* Author to whom correspondence should be sent.

INTRODUCTION

Capillary isoelectric focusing (CIEF) is the most powerful separation mode of capillary electrophoresis (CE) for resolving proteins. Small amount of proteins (nL - μL) with isoelectric point (pI) differences as small as 0.02 pH units can be separated and detected in less than 20 minutes[1]. Many applications of CIEF have been reported using commercial CE instruments, which typically use a 20 - 60 cm long capillary with an on-column UV-vis absorbance detector[2-4]. However, there are problems associated with performing isoelectric focusing in CE instruments with on-column detector. All focused protein zones must be moved through the flow cell of the on-column detector in a mobilization process[1]. In the mobilization step, distortion of the pH gradient inside the capillary is unavoidable, resulting in a loss of resolution[2,5]. The mobilization process also makes it difficult to determine pI values of proteins from their retention time[2,5]. Mobilization takes about 10 - 15 minutes of the whole 20 minute analysis process[2].

To enhance the performance of CIEF, we proposed a CIEF-universal, on-line concentration gradient imaging system which eliminates the mobilization process[6]. The separation column of the instrument is a 4 cm long capillary. All protein zones focused in the capillary can be detected in on-line fashion by the universal imaging detector without mobilization. The imaging detector consists of a low power He-Ne laser and a low cost 1024 pixel charge-coupled device (CCD) sensor. The laser beam is focused into the capillary by a cylindrical lens and the light beam intensity is monitored by the CCD after the beam passes through the capillary. The light intensity profile intercepted by the CCD is proportional to the second derivative of the concentration profile of the focused pattern of the protein sample inside the capillary[6]. The instrument is much cheaper than commercial instruments with UV-vis

absorbance detectors. pI values of proteins can be directly determined from their zone positions inside the capillary, and the reproducibility of the focused pattern is greatly improved compared to commercial instruments[6]. Since the instrument combines separation and detection into one step, the analysis time for a complex protein mixture is only 1 - 2 minutes[6]. The detection limit reaches 50 μg/mL for proteins, which corresponds to 10^{-6} M of protein samples. This detection limit is sufficient for characterization of proteins in pharmaceutical products and major components in many biochemical samples. Also, the on-line detector can be applied to isoelectric focusing performed in a capillary array[7]. Performance of CIEF in a capillary array dramatically increases the throughput of CIEF[7], making CIEF comparable to gel slab isoelectric focusing in sample throughput, but with much faster separation and quantitation.

The sensitivity of the imaging detection system, however, is not sufficient for analyzing minor protein components in biological cells which usually have concentrations lower than 10^{-6} M[8]. There are two simple and inexpensive ways for enhancing the sensitivity of the universal imaging system. One is signal averaging using a fast CCD sensor and A/D board[6]. In this method, the signal-to-noise ratio improvement is expected to be proportional to $n^{0.5}$ if the noise is random. Here, n is the number of scans.

The second method is sample concentration by fast ultrafiltration. Ultrafiltration is an existing method for protein concentration, separation and purification[9]. The purpose of the present study is to use these two methods to improve the sensitivity of our CIEF/concentration gradient imaging system and make the instrument applicable for the analysis of minor protein components in biochemical samples.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade, and solutions were prepared using deionized water. Solutions of 10 mM H_3PO_4 and 20 mM NaOH were used as anolyte and catholyte, respectively[2]. Carrier ampholytes were Pharmalyte pH 3 - 10 (Sigma). All solutions were filtered using 0.2 μm pore size cellulose acetate filters (Sartorius, Gottingen, Germany). Iron-poor bovine transferrin (apo-transferrin) was purchased from Sigma Chemical Co. Periplasmic proteins of *Escherichia coli* and microsomal membrane proteins from bean cotyledon were obtained from the Department of Biology, University of Waterloo.

Concentrating

The protein sample was first desalted by using dialysis membranes (MWCO: 5,000, purchased from Baxter, Mississauga, Ontario, Canada) against water for 2 hours. Then, the sample was concentrated by employing microfilters (microconcentrator, Microcon-3, MWCO: 3,000, AMICON, Beverly, MI) which could be inserted into a standard Eppendorf centrifuge tube. The microfilters filled with samples were centrifuged in an Eppendorf centrifuge for about 5 minutes at 12,000 rpm. Since the volume of the filter was about 0.5 mL, the filter was filled and centrifuged 3 - 4 times in order to concentrate 2 mL sample. The concentrating process was completed in about 20 minutes, and gave a final volume of about 30 μL. The filter containing the concentrated sample was placed upside down into the centrifuge tube, and the centrifuge was turned on briefly to drive the concentrated samples into the centrifuge tube. Finally, the sample was mixed with 30 μL of a solution containing 4% carrier ampholytes and 1% Triton X-100, and was transferred to the CIEF instrument. Triton was added to

prevent protein precipitation of high concentration components during the focusing process[2].

CIEF instrument

The CIEF/concentration gradient imaging system was the same as that used in previous experiments[6]. The separation column was a 4-cm-long, 100 μm i. d. square capillary. The capillary inner wall was coated with non-cross-linked acrylamide to eliminate electroosmosis[3]. The two ends of the capillary were connected to two buffer reservoirs, each 1 mm inner diameter and 1.5 cm long. The focused patterns of the protein samples were monitored on-line by the concentration gradient imaging system consisting of a He-Ne laser and a 1024 pixel CCD[6]. The data was collected by an IBM DACA board, in a PC-AT personal computer. The scanning frequency of the 1024 pixel CCD was set at 10 kHz due to the 20-kHz maximum acquisition frequency of the A/D board. An averaging method was applied to reduce the random noise, for which each measurement the CCD scanned for 30 times in 3 seconds, and signals were averaged. Separation and detection of protein samples by this instrument was completed in 1 - 2 minutes.

CIEF process

Five to 10 μL samples were injected into one of the buffer reservoirs of the CIEF instrument by a microsyringe, and the capillary was filled by pressure. The remaining sample solution in the reservoir was withdrawn by the syringe. Then a plug of a 1% agarose gel (prepared in the anolyte, 10 mM H_3PO_4) was placed in the reservoir of the capillary anodic end to avoid hydrodynamic flow inside the capillary during the focusing process[6]. The focusing voltage was 3.5 kV. The focused pattern stabilized in 2 minutes.

All other equipment and experimental conditions were the same as those used in previous experiments[6,7]. All experiments were done in duplicate or triplicate to monitor the reproducibility.

RESULTS AND DISCUSSION

Averaging method

Unlike other capillary electrophoretic techniques, in CIEF, all components in a protein sample are focused inside the capillary during the focusing process. It is a static situation, which makes it possible to do signal averaging in a longer time period for CIEF than for other electrophoretic techniques. However, there are two factors limiting the averaging time. Although the inner wall of the capillary is coated with non-cross-linked acrylamide, electroosmosis can not be completely eliminated, so all protein zones still move slowly inside the capillary during the focusing process. Also, the zone width of the protein is small, only 50 - 300 μm in the 4-cm-long capillary during the focusing process[6]. In the experiments, we found that the distortion in signal peaks was negligible when the averaging time was shorter than 3 seconds. In our experiments, a 0.3 - 3 s averaging time was used, which corresponds to 3 - 30 scans for the 1024 pixel CCD sensor.

Figure 1 shows the images of a bovine transferrin sample with and without the averaging method. The concentration of the sample is 50 μg/mL, which is almost at the detection limit of the detector as shown in Fig. 1a. However, the sample becomes detectable when the averaging method is used, in which images of 30 scans are averaged. Several peaks can be observed in Fig. 1b ranging from pH 5.3 to 5.6, which correspond to the different isoforms of transferrin[3]. As expected, the sensitivity of the detector is enhanced by a factor of about 5 (4.5) in

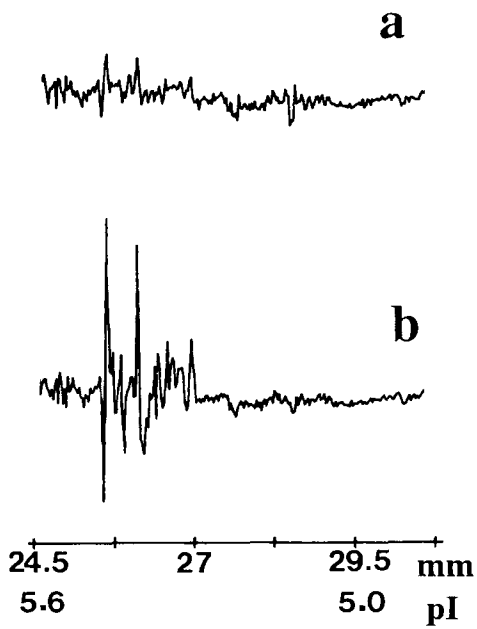

Figure 1. Electropherograms of bovine transferrin after 2 minute focusing. (a.) Without averaging and (b.) with averaging methods. Sample concentration is 50 μg/mL.

Fig. 1b compared to Fig. 1a. The detection limit of the imaging detector reaches 13 μg/mL under these conditions. Since the instrument combines separation and detection into one step, the analysis speed is very fast. The whole analysis time by this instrument is only 2 minutes.

Further enhancement for the detector is possible when more scans are averaged in 3 s. A faster A/D board is needed to perform this function. The detection limit of the instrument is expected to reach 3 μg/mL for the universal imaging detector if the present 20 kHz A/D board is replaced by a 300 kHz board.

Concentration method

The ultrafiltration procedure using the microfilter and centrifuge is a suitable method for off-column protein concentrating for the CIEF instrument since it is a simple, gentle technique; adaptable to microliter sample volumes. The concentration of a sample can be increased by a factor of 20 - 40 in 20 minutes using this concentration method. Therefore, the sensitivity of the combined ultrafiltration/CIEF method is estimated to be in sub μg/mL, which corresponds to 10^{-8} - 10^{-7} M for proteins with molecular weights of 10^4 - 10^5. This sensitivity is sufficient for analysis of many minor protein components in cells[8].

The focused pattern of microsomal membrane proteins from bean cotyledon without concentration is shown in Fig. 2a. Only 2 minutes are needed for separation and detection of this sample. The pI values of the protein zones can be directly determined from their positions in the capillary once the relationship between the position and pI is calibrated for the used carrier ampholytes[7]. Although the total concentration of the sample is about 0.4 mg/mL, since it contains about 30 proteins, and most components are present at concentrations lower than 50 μg/mL, only two to three major components of the samples can be observed in the pI range 5.3 - 5.5 as shown in Fig. 2a. Figure 2b shows the electropherogram of the same sample after concentrating. Besides the peaks corresponding to the major components, about 20 peaks can be observed around pI 4.5 - 6.5, which correspond to minor components in the sample. As expected, the peak heights of many minor components increased 20 - 30 times (for example, peaks 1 and 2 in Fig. 2b). The concentrations of these minor components are estimated to be in the range of 1 - 5 μg/mL. The sensitivity of the method is sufficient for analysis of minor protein components in the sample.

Minor protein components in cell periplasm can also be analyzed by the instrument with the off-column concentrating method. The sample

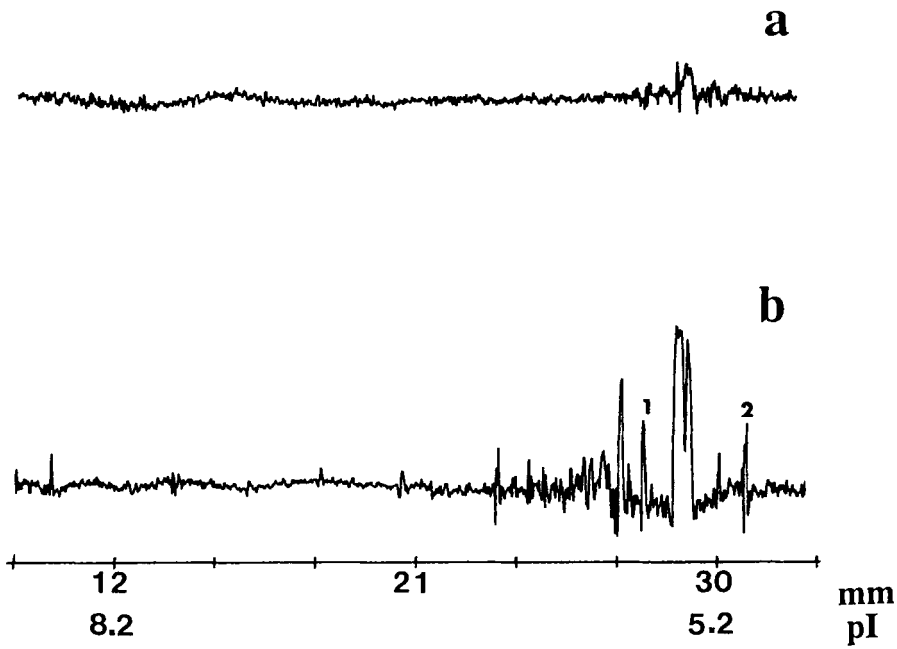

Figure 2. Electropherograms of microsomal membrane proteins from beam cotyledon (a.) without concentrating and (b.) with concentrating method.

is extracted from *Escherichia coli* cells, which contains series of cell proteins, by the reported method[10]. For the sample without concentration, as shown in Fig. 3a, only 5 - 7 major protein components can be detected by the instrument. Two major components can be observed at pI 6.1 and 6.6 in Fig. 3a, which agrees well with the results of preliminary measurement using gel slab isoelectric focusing[10]. Figure 3b shows the electropherogram of the same sample after concentration. More than 20 minor components can be observed in the electropherogram.

Figures 2 and 3 show the high resolution of the CIEF/concentration gradient imaging system. Although a short, 4 cm

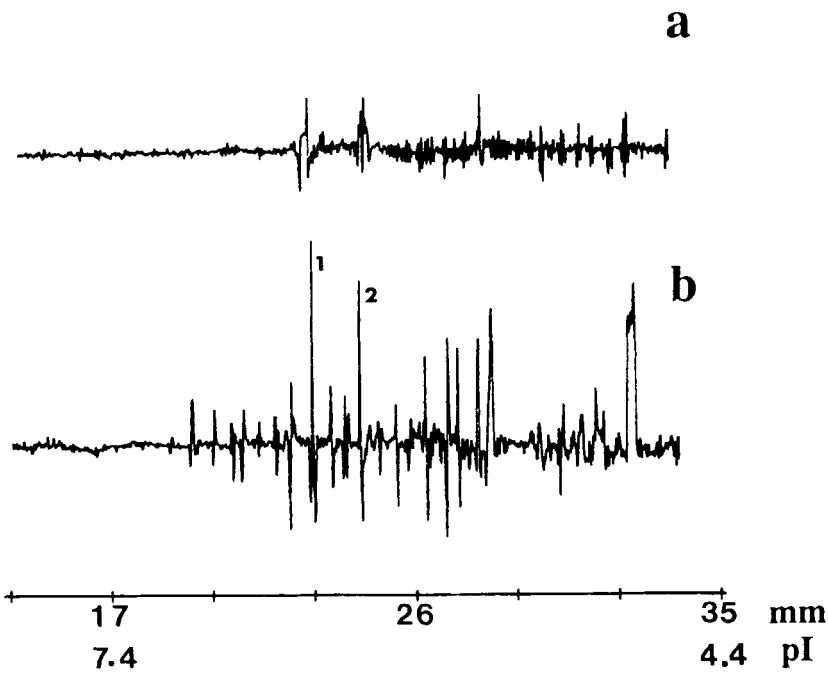

Figure 3. Electropherograms of periplasmic proteins of *Escherichia coli* (a.) without concentrating and (b.) with concentrating method.

long separation column is used, the resolution for pI is calculated to be 0.02 pH units from distance between peaks and the peak widths of peaks 1 and 2 in Fig. 3b. The high resolution of the instrument is due to the derivative nature of the detector and the use of high resolution CCD[6].

The analysis speed using the instrument with the concentrating method is still fast. It takes about 20 - 25 minutes, which includes sample concentration, separation and detection of all components. The speed is much faster than that of isoelectric focusing performed with gel slabs, and the same as that for conventional CIEF analysis of proteins

without any concentration process. However, the method has higher sensitivity (detection limit: sub μg/mL) than those of commercial CIEF instruments.

The sample consumption of the method is small. The volume of the separation column is only 400 nL for the 4 cm long, 100 μm i. d. square capillary. For the present cartridge design, a sample volume of 5 μL is needed in order to fill the bottom of the reservoir connecting to the capillary. During injection of the sample, only 400 nL of sample is introduced into the capillary. The rest of the sample solution in the reservoirs can be withdrawn by a microsyringe and used for further analysis. The sample volume needed in the introduction process can be further reduced to 1 μL by using smaller reservoirs.

As shown in Figs. 2 and 3, for some major components, the peak heights increase by less than a factor of 20 when concentration method is used. This is possibly due to concentration saturation for the major components after the concentrating process. The sample zones are broader for the high concentration components in the concentrated sample compared to the nonconcentrated samples[2]. The sensitivity of the detector is decreased for these broad zones[6]. It should be noted that although Triton X-100 is used to prevent the major components from precipitation, long focusing time can still induce precipitation of high concentration proteins[2]. Use of commercial CIEF instruments with mobilization may also increase the possibility of precipitation. During mobilization, sample precipitation causes poor reproducibility of focused patterns and prolongs migration times[2]. However, the use of the imaging on-line detector can avoid those problems. The detector eliminates the mobilization process, and all sample zones are detected at their positions during focusing. Also, the speed of separation and detection is fast, about 1 - 2 minutes. This greatly reduces the chance of precipitation.

In the above discussion, we demonstrated that minor protein components in biological cells, which have concentrations higher than 1

μg/mL, can be analyzed by the CIEF/concentration gradient imaging system instrument, when combined with the fast concentration method. The whole analysis time is less than 25 minutes. In future research, the off-column concentration method will be modified into an on-column concentrating method by using hollow fibre membranes connected to the separation column of the CIEF instrument. The use of the on-column concentration method will reduce the sample volume needed for introduction and increase the analysis speed. Combined with on-column dialysis, biochemical samples, such as blood, can be directly injected into the CIEF instrument for protein analysis.

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