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Application of capillary isoelectric focusing with universal concentration gradient detector to the analysis of protein samples

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ABSTRACT

The design of a new capillary isoelectric focusing (cIEF) instrument, composed of a rugged cartridge holding a short piece of capillary and a universal, inexpensive concentration gradient detector, was optimized and applied to the analysis of various protein samples. High-efficiency cIEF separations with sub-femtomole detection limits for absolute amounts were obtained using 10 μm I.D. capillaries with large O.D.-to-I.D. ratios. An electric field strength of 1 kV/cm applied in the focusing step resulted in a 10^{-8} *M* on-column concentration detection limit, which corresponded to 10^2 amol absolute amount of proteins. The detection volume was estimated to be 2 pl, which is among the smallest values reported to date for any optical or spectroscopic detector. When a 6-cm long capillary was used, proteins with isoelectric points ranging from 4.7 to 8.8 could be analyzed in about 5 min, the shortest analysis time ever reported for cIEF. Compared with commercial cIEF instruments with UV–visible absorbance detectors, the instrument is easier to use and has lower detection limits and better resolution. Several protein mixtures and real samples were separated with this instrument.

INTRODUCTION

Capillary isoelectric focusing (cIEF) is a newly developed, powerful capillary electrophoretic technique for separations of complex protein samples based on isoelectric point (p*I*) differences [1,2]. Because the microbore capillaries (25–200 μm I.D.) used in the cIEF offer efficient dissipation of Joule heat, and therefore, eliminate convection effects, a high separation voltage can be employed. This permits highly efficient separations as the zone width of a focused protein decreases with increasing separation voltage [3]. The technique is able to resolve proteins which differ in isoelectric point by less than 0.02 pH unit, and the separation can be completed in about 15–20 min [2,4]. The narrow capillaries also require only small amounts of sample, which is desirable for the analysis of biochemical materials, such as proteins. cIEF has great potential as an analytical tool for protein analysis.

Since 1985, there have been many reports on the applications of cIEF for the separation of protein samples, most of which were performed on commercial capillary electrophoresis (CE) instruments [2,4, 5]. However, there are three main problems with commercial CE instruments performing in the isoelectric focusing mode. First, although a narrower capillary offers a higher separation efficiency, most commercial CE instruments still use 50–75 μm I.D. capillaries, because of difficulties in performing on-column detection with narrow capillaries. For commercial CE instruments, UV–visible absorbance is the most commonly used detection method. Its performance deteriorates when used with capillaries of less than 25 μm I.D. because it is difficult to send sufficient radiation from a incoherent source across the narrow capillaries. Second, because of the presence of the UV-absorbing carrier ampholytes in cIEF, which usually have 100–1000 times higher concentrations than those of protein samples, a

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wavelength of 280 nm has to be used for the UV–visible absorbance detector instead of the wavelengths for optical absorption peaks of proteins (180–240 nm [2]). The sensitivity of the UV–visible detector for cIEF is much lower than that for other CE techniques, such as capillary zone electrophoresis. Finally, the designs of most commercial CE instruments are not suited to hold short capillaries. The best isoelectric focusing separations are reportedly achieved on short capillaries (12–14 cm) [2,4], but much longer capillaries have to be used in most commercial CE instruments because the physical size of the UV–visible absorbance detector and the design of the capillary cartridge of those instruments restrict the use of short capillaries.

To address these limitations of the current cIEF instrumentation, we proposed a new cIEF apparatus that consists of a specially designed cartridge accommodating a short piece of capillary and an inexpensive, universal concentration gradient detector [6]. We have shown the unique compatibility of the concentration gradient detector and isoelectric focusing performed on 20–100 μm I.D. capillaries [7]. The detector shows high sensitivity for cIEF owing to the high concentration gradients produced by the narrow protein zones focused inside the capillary by the isoelectric focusing process [7]. The derivative nature of the detector eliminates the high background signal generated by wide zones of the carrier ampholytes [7]. All zones can be detected during mobilization without any derivatization or decrease in resolution because of the universal nature and the small detection volume of the detector [7].

In this work, we investigated further the capability of the cIEF–concentration gradient detector method by optimizing the optical geometry of the detector so that it can be applied to narrow capillaries, and using short capillaries for fast separation of proteins with wide p*I* ranges. The potential of this technique was demonstrated by applying it to the analysis of a wide range of biological samples.

EXPERIMENTAL

Instrumental

The cIEF–concentration gradient detector system used was described in detail previously [7]. A 10 or 20 μm I.D., 350 μm O.D., 12 or 6 cm long capillary (Polymicro Technologies, Tucson, AZ, USA) was used for separation. The capillary inner wall was coated with non-cross-linked acrylamide to eliminate electroosmosis [8]. As the viscosity of the acrylamide solution used for coating the capillary increased with time, and the solution became difficult to withdraw from the narrow capillaries, the coating time for the 10 μm I.D. capillaries was only 7 min instead of 14–20 min [8] for 20 μm I.D. capillaries. The lifetime of the coating for the 10 μm I.D. capillaries is much shorter than that of 20 μm I.D. capillaries. The laser beam intensity profile after the beam had passed through the capillary was measured by a scanning photodiode with a 0.1 mm slit placed before it.

Reagents

All chemicals were of analytical-reagent grade, and solutions were prepared using deionized water and filtered through 0.2-μm pore size cellulose acetate filters (Sartorius, Gottingen, Germany) prior to use. The anolyte and catholyte were 10 m*M* H_3PO_4 and 20 m*M* NaOH, respectively [4]. All proteins were purchased from Sigma. Proteins used include carbonic anhydrase (bovine erythrocytes), carbonic anhydrase (human erythrocytes), myoglobin (horse skeletal muscle), phosphorylase *b* (rabbit muscle), α-chymotrypsin (bovine pancreas), ovalbumin (grade V), hemoglobin (human, 75% methemoglobin, balance primarily oxyhemoglobin), β-lactoglobulin B (bovine milk) and albumin (human, fraction V). Human blood serum samples were obtained from a local clinical laboratory. Monoclonal antibody to fluorescein and transferrin (bovine) were donated by HyClone Laboratories (Logan, UT, USA). Samples were mixed with carrier ampholyte (Pharmalyte, pH 3–10; Sigma) solution for a final concentration of 2% ampholyte [4]. The protein concentrations introduced into the capillary ranged from 0.1 to 0.5 mg/ml. Protein samples were desalted, if necessary, by using dialysis membranes (molecular mass cut-off 5000) purchased from Baxter (Mississauga, Ontario, Canada).

The cIEF process

First, the sample solution was introduced into the capillary by pressure, then a high d.c. voltage was applied to the two ends of the capillary. The voltage applied for focusing performed on the 20 μm I.D.

capillaries was 8 kV and on the 10 μm I.D. capillaries it was 12 kV. The current passing through the capillary was monitored to follow the focusing process. For 12 cm × 20 μm I.D. capillaries, typically, the current dropped from 1 to about 0.1 μA in 4 min. The final step was mobilization. In the present experiment, cathadic mobilization was employed, which required exchanging the catholyte with a solution containing 20 m*M* NaOH and 80 m*M* NaCl [4]. The voltages for mobilization were 10 kV for 20 μm I.D. capillaries and 12 kV for 10 μM I.D. capillaries. During the cathodic mobilization process, the proteins moved through the detector in order of decreasing p*I*. All experiments were done in triplicate to control the reproducibility.

RESULTS AND DISCUSSION

For a sample zone focused in a capillary by the isoelectric focusing process, its concentration distribution along the capillary direction, x, is assumed to be a Gaussian function [3] with a standard deviation, σ_x, which can be expressed as [7]

$$\sigma_x = \sqrt{\frac{D}{pE}} \tag{1}$$

where $E = V/l$, V is the d.c. voltage applied to two ends of the capillary, l is the overall length of the capillary, D is the diffusion coefficient of the protein sample and p is given by

$$p = \frac{du}{d(pH)} \cdot \frac{d(pH)}{dx} \tag{2}$$

where $du/d(pH)$ is the change in the mobility of the sample with respect to change in pH. As predicted by eqn. 1, better resolution is expected when a high voltage is applied. The upper limit of the applied voltage is largely decided by the capillary I.D. Narrower capillaries facilitate the application of higher voltage because of enhanced heat dissipation caused by higher surface-to-sample volume ratios [9]. Hence, narrower capillaries offer higher separation efficiency. On the other hand, the use of narrow capillaries increases the difficulties for the application of the concentration gradient detector to cIEF.

In the concentration gradient detector, a collimated laser beam is focused directly into the separation capillary. The direction of the beam is deflected when it encounters a refractive index gradient produced by a migrating sample zone [6]. This beam deflection can be created by any sample zone which has a refractive index different from that of the buffer inside the capillary. When the detector is used for cIEF, the maximum value of the deflection angle can be written as [7]

$$\theta_{max} = \pm 0.24 \cdot \frac{L}{n} \cdot \frac{dn}{dC} \cdot \frac{C_0 l}{\sigma_x^2} = \pm 0.24 \cdot \frac{dn}{dC} \cdot \frac{du}{d(pH)} \cdot \frac{d(pH)}{dx} \cdot \frac{LC_0V}{nD} \tag{3}$$

where n is the refractive index of the solution in the capillary, L is the I.D. of the capillary, C_0 is the concentration of the introduced sample and C is the concentration distribution of the sample zone along the capillary direction after focusing. As dn/dC is approximately a constant for a given solute [6], the maximum angle is proportional to the concentration of the introduced sample, C_0. The angle of deflection can be detected by a light beam position sensor which consists of two photodiodes. The photocurrent, Δi_{max}, created on the position sensor associated with the deflection angle, θ_{max}, can be written as [6]

$$\Delta i_{max} = \frac{8KiF}{\pi Z} \cdot \theta_{max} \tag{4}$$

where i is the laser beam light intensity, F is the focal length of the laser beam focusing lens, Z is the beam diameter before focusing and K is the efficiency of the conversion of light intensity into current by the light beam position sensor [6]. Δi_{max} represents the sensitivity of the whole detection system for cIEF.

As described by eqn. 3, the sensitivity of the detector decreases linearly with decreasing capillary I.D., L. For example, the sensitivity of the detector for a 10 μm I.D. capillary is only half of that for a 20 μm I.D. capillary. A linear relationship between the sensitivity of the detector and the applied voltage is also shown in eqn. 3. This means that the decrease in sensitivity due to decreasing capillary I.D. can be partly compensated for by increasing the separation voltage. As discussed earlier, high voltage can be successfully applied to narrow capillaries.

Another difficulty in applying the detector to a narrow capillary is associated with focusing the probe beam into the capillary. The use of a narrower

capillary requires a smaller laser beam spot. A shorter focal length lens should be used for focusing the laser beam into the narrower capillary, as the focused laser beam spot diameter, A, is given by

$$A = \frac{4\lambda F}{\pi Z} \quad (5)$$

where λ is the wavelength of the laser beam. In previous work [7], good results were obtained with a 30 mm focal length lens and 20–100 μm I.D. capillaries. By using this lens, the beam spot after focusing is calculated to be 24 μm by eqn. 5 from the 0.6 μm wavelength and 1 mm beam diameter of the He–Ne laser. For a 10 μm I.D. capillary, a smaller beam spot is needed. However, the use of a short focal lens will decrease the sensitivity of the detector as it is proportional to the F/Z ratio as described in eqn. 4. For example, a 13 mm focal length lens is required to focus the beam into a 10 μm I.D. capillary. This decreases the sensitivity by about 60% compared with that when a 30 mm focal length lens is used. Therefore, trade-off exists between the focal length and the capillary I.D. for obtaining good sensitivity.

Because the 30 mm focal length lens gave good sensitivity when it was used for 20–100 μm I.D. capillaries, and a longer focal length also made optical alignment adjustment easier, we decided to use this lens for 10 μm I.D. capillaries. When the 30 mm focal length lens is used to focus the laser beam for side illumination of a 20 or 10 μm I.D. capillary, the beam will be scattered over 360° in the plane perpendicular to the capillary axis since the capillary I.D. is smaller than the beam diameter [10]. The resulting fringe pattern depends on the refractive index of the capillary, the sample in the capillary and the beam polarization. There are four kinds of beam rays in the probe beam, as shown in Fig. 1: ray 1, which passes through the center of the capillary inner bore without any refraction; ray 2, which propagates through the capillary and emerges without intercepting the inner bore with two refractions at two interfaces; ray 3, which is reflected at the interface between the capillary and sample inside its inner bore; and ray 4, which propagates through both the capillary wall and the inner bore with four refractions at the interfaces. The fringe pattern is produced by the interference of all these four scattered rays, and the pattern becomes complicated

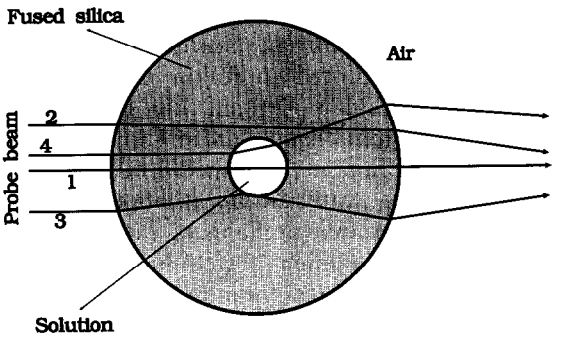

Fig. 1. Capillary cross-section and laser beam ray tracing diagram for four kinds of rays.

when narrow capillaries are used as shown in Figs. 2a and 3a, which illustrate the probe beam intensity profiles after the beam has passed through a 20 or 10 μm I.D. × 140 μm O.D. capillary. A complicated interference pattern is observed in the center of the beam. This effect is particularly noticeable for the 10 μm I.D. capillary, where only the center part of the beam passes through the inner bore of the capillary. The interference in the beam center is mainly due to the beam rays 1 and 4, which are refracted by the interface between capillary and air and interfere with each other in far field, as shown in Fig. 1. This interference pattern also changes or shifts irregularly as a consequence of changes in the refractive index of the solution inside the capillary, which can be induced by temperature inhomogeneity or a migrating sample zone. For the concentration gradient

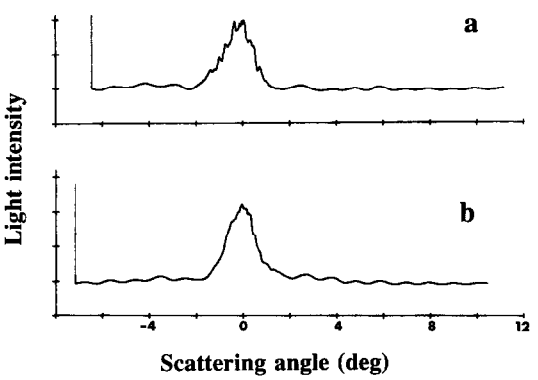

Fig. 2. Laser beam intensity profiles after the beam has passed through (a) a 20 μm I.D., 140 μm O.D. capillary and (b) a 20 μm I.D., 360 μm O.D. capillary.

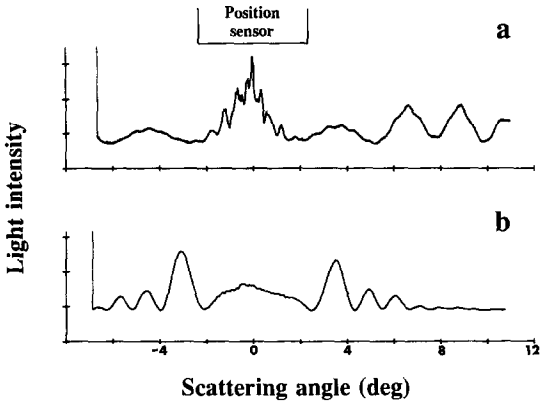

Fig. 3. Laser beam intensity profiles after the beam has passed through (a) a 10 μm I.D., 140 μm O.D. capillary and (b) a 10 μm I.D., 360 μm O.D. capillary. The light intensity coordinate in (a) is expanded twice that in (b).

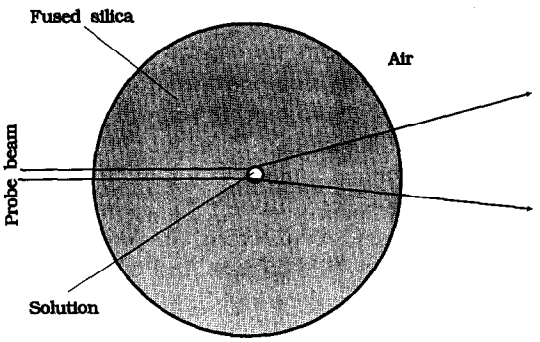

Fig. 4. Propagation directions of the light beam emitted from the center of the capillary.

detector, in which the beam deflection along the capillary axis is monitored, the shifts of the pattern along the capillary axis can cause noise, baseline fluctuation or irregular signal peak shape. This presents a major difficulty when applying the detect or to narrow capillaries.

This interference pattern can be significantly simplified by immersing the capillary in a transparent liquid having the same refractive index as that of the capillary [10]. In this way, the refraction of the beam at the interface between the capillary and air (now the liquid) is eliminated. However, in this method, the temperature should be carefully controlled, as the refractive index of the liquid and the capillary wall change with temperature, and the temperature may be different in the liquid and capillary wall owing to their different heat capacities. In our experiment, the interference pattern is simplifid by using larger O.D.-to-I.D. ratio capillaries. In Fig. 4, such a capillary is used (*e.g.*, a 360 μm O.D., 10 μm I.D. capillary). As the laser beam is focused into the center of the capillary, and the focal area of the beam is large compared with the capillary O.D., all light rays can be considered to be emitted from approximately the center of the capillary. The propagation directions of these beam rays are approximately perpendicular to the interface between the capillary and the air. After rays 1 and 4 have passed through the capillary, they will not be refracted at the capillary–air interface, and travel along their original directions. The interference between rays 1 and 4 is reduced by using larger O.D.-to-I.D. ratio capillaries as shown in Figs. 2b and 3b. As expected, the intensity profile in the beam center is of good quality, and the interference pattern is simplified even for the 10 μm I.D. capillary. In our experiment, only this part of the beam is intercepted by the photodiode light beam position sensor as shown in Fig. 3 when a 10 μm I.D. capillary is used. The above discussion suggests that it is possible to apply the concentration gradient detector to narrow capillaries without loss of sensitivity. It should be mentioned that by intercepting part of the laser beam by the light beam position sensor, we should expect a decrease in sensitivity from eqn. 4 which shows a linear relationship between the beam intensity and the sensitivity. However, for the high-intensity laser beam, the principal noise source of the measurement in the concentration gradient detector is the pointing noise [6]. The noise in signals detected by the position sensor due to the pointing noise is proportional to the beam light intensity, which means that the relative noise level due to pointing noise is a constant. This property allows the interception of part of the laser beam by the light beam position sensor for the narrow capillaries without deterioration of the signal-to-noise ratio and, therefore, the sensitivity of the detector.

Fig. 5 shows the electropherograms of two focused proteins, phosphorylase *b* (peak 1) and ovalbumin (peak 2), in a 20 and a 10 μm I.D. capillary, which are plotted in such a way that the noise levels of the two electropherograms are the same so that their sensitivities can be compared by their peak

heights. Although the capillary I.D. (L) decreases by half from 20 to 10 μm, the sensitivity loss due to this decrease is partly compensated for by an increase in the applied voltage (V) which increases from 8 to 12 kV. From eqn. 3, under these conditions, the sensitivity in concentration for the 10 μm I.D. capillary is predicted to be 75% of that for the 20 μm I.D. capillary. As expected, the peak heights in Fig. 5b are about 75% of those in Fig. 5a. Other peaks are also observed in Fig. 5, which are attributed to minor components or impurities in the sample. This result confirms that the signal-to-noise ratio does not decrease when only the center part of the laser beam is detected in the optical alignment used in the concentration gradient detector. The on-column mass detection limit for the 10 μm I.D. capillary is much lower than that for the 20 μm I.D. capillary, as the sample volume needed decreases from 38 nl for the 20 μm I.D. capillary to 9 nl for the 10 μm I.D. capillary. The detection limits in concentration are calculated from three times the baseline noise and are $3.4 \cdot 10^{-8}$ M for phosphorylase b and $6.3 \cdot 10^{-8}$ M for ovalbumin. These values correspond to 270 and 460 amol on-column mass detection limits for phosphorylase b and ovalbumin, respectively. This is the first report in which isoelectric focusing has been performed on a 10 μm I.D. capillary and attomole levels of proteins have been detected by a universal detector.

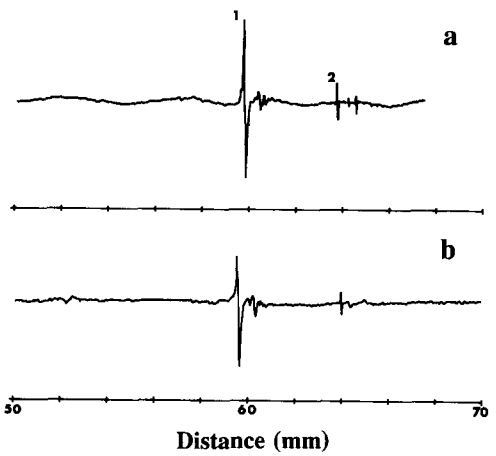

Fig. 5. Mobilization electropherograms of phosphorylase b (peak 1) and ovalbumin (peak 2) focused (a) in a 12 cm × 20 μm I.D. capillary and (b) in a 12 cm × 10 μm I.D. capillary. Concentrations of the introduced samples are 0.5 mg/ml for phosphorylase b and 0.1 mg/ml for ovalbumin, respectively, which correspond to 40 and 17 fmol, respectively, for absolute amounts of samples in the 10 μm I.D. capillary.

These experimental results confirm the feasibility of performing isoelectric focusing on a 10 μm I.D. capillary and detection in cIEF with narrow capillaries by a concentration gradient detector. Fig. 6 shows a mobilization electropherogram of five proteins focused in a 10 μm I.D. capillary, and detected by the concentration gradient detector. The concentrations of these proteins range from $1.6 \cdot 10^{-6}$ to $5.4 \cdot 10^{-6}$ M, which correspond to 15–50 fmol in absolute amount of proteins introduced into the 12 cm × 10 μm I.D. capillary. From the signal-to-noise ratio in Fig. 6, the on-column mass detection limit for each protein is again shown to be in the range of 10^2 amol.

The detection limit of the concentration gradient detector for absolute amount is lower than that of a UV–visible absorbance detector for cIEF. Although a laser-induced fluorescence detector is the most sensitive detector for CE, it is difficult to apply for many real protein samples as the detector requires derivatization for proteins, which is impossible to perform in pre- or postcolumn modes for ultramicro amounts of samples at low concentrations. Also, the reaction between protein samples and labeling reagents may be not specific, and many unwanted products will be created, increasing the difficulty in identifying and quantifying sample peaks after they are separated. However, attomole level proteins at 10^{-7} M concentrations can be separated and detected by our cIEF instrument with

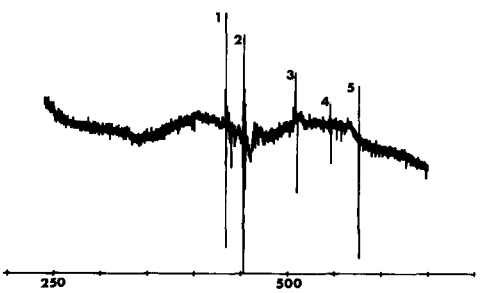

Fig. 6. Mobilization electropherogram of five proteins focused in a 12 cm × 10 μm I.D. coated capillary. 1, 2 = Human hemoglobin (absolute amount in the capillary 14 fmol) and myoglobin; 3 = human carbonic anhydrase (32 fmol); 4 = bovine carbonic anhydrase (29 fmol); 5 = β-lactoglobulin B (50 fmol). Protein concentrations, 0.1 mg/ml each.

the universal detector without any derivatization.

Although the 10 μm I.D. capillary is applicable to cIEF, its inner wall is difficult to coat by the coating method used in the experiment where a high-viscosity solution must be introduced into the capillary [8]. This problem prevents the practical use of narrow capillaries in cIEF. In our analysis of protein samples, we prefer to use a 20 μm I.D. capillary as it is easier to coat. However, it is still possible to use a 10 μm I.D. capillary or even narrower capillaries in practical cIEF analyses of protein samples because the possibility of performing isoelectric focusing on uncoated capillaries has been demonstrated recently [5].

In the mobilization process, adding salt to one of the capillary ends causes movement of sodium or chloride ions into the capillary at that end. A pH change occurs at this end, and then gradually progresses deeper into the capillary, causing sample zones to move toward the end of the capillary [4]. A long capillary prolongs the mobilization process and also causes pH gradient distortion along the capillary and, therefore, shape distortion of the sample zones, during the mobilization. Long capillaries have to be employed in the commercial instruments. Because of the size of the UV–visible absorbance detector and the design of the capillary cartridge, the detection point of the capillary has to be positioned at some distance from one capillary end toward which the focused sample zones move during the mobilization. This arrangement prevents the detection of the proteins that are focused between the detection point and the capillary end during the mobilization process. The distance ranges from 3 to 20 cm depending on the type of instrument [4,5]. For the commercial CE instruments, the pH range of the carrier ampholytes cannot be fully utilized. To recover those lost proteins between the detection point and the capillary end, two methods can be used: lengthening the separation capillary while keeping the distance from the detection point to the capillary end constant, or mixing certain reagents in the carrier ampholytes to extend the pH range in the capillary end. For example, with N,N,N′,N′-tetramethylethylenediamine the pH range can be extended from 10 to 12 at the cathode end of the capillary [4,5]. The pH range of the carrier ampholytes is still not fully utilized by the second method, and the first method increases the mobilization time and makes it difficult to mobilize proteins focused near the capillary anode end to the cathode end when cathodic mobilization is employed. We tested the application of short capillaries in the cIEF–concentration gradient detector instrument. Because of the unique design of the cartridge, theoretically there should be no minimum limitation on the distance from the detection point to the capillary end, therefore making it possible to apply short capillaries in the cIEF–concentration gradient detector instrument [7]. Fig. 7 shows a mobilization electropherogram of three proteins focused in a 6 cm × 20 μm I.D. capillary. All proteins having pI values ranging from 4.7 to 8.8 [11] can be analyzed in about 5 min, which includes 3 min for focusing and 2 min for mobilization. This rate is much faster than those of commercial CE instruments, and the highest ever reported for cIEF analysis. The use of a short capillary also increases the sensitivity of the detector as its sensitivity is proportional to d(pH)/dx, as described by eqn. 3. Owing to the small detection volume of the detector, the short capillary did not affect the resolution for the 6 cm long capillary. As shown in Fig. 7, the Gaussian derivatives generated by the concentration gradient detector can be easily converted into peaks associated with electropherograms produced by conventional concentration detection methods. The integration should be performed only on the portion of the trace around the analyte band of interest to re-

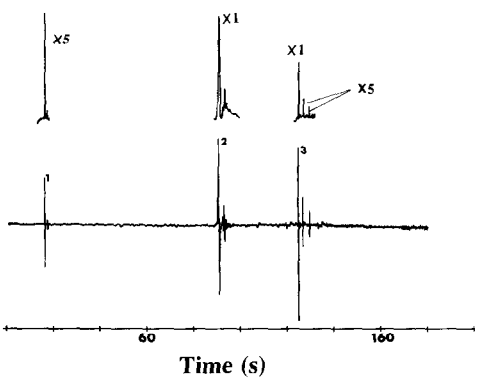

Fig. 7. Rapid analysis of proteins in wide pH range using a 6 cm × 20 μm I.D. capillary. Samples: 1 = α-chymotrypsin (major component, pI = 8.76; one minor component, pI = 8.38); 2 = phosphorylase b (major component, pI = 6.35; minor components, pI = 5.50–6.25); 3 = ovalbumin (major component, pI = 4.70). Above: integrals of portions of the electropherogram. Protein concentrations, 0.5 mg/ml each.

duce the effects of drift associated with the broad band of the carrier ampholytes present in the capillary [7]. The results confirm the feasibility of employing short capillaries in cIEF, and also suggest that the cIEF–concentration gradient detector instrument with a short capillary is suitable for the rapid screening of proteins in a wide pI range.

The resolution and reproducibility of the instrument were evaluated. Fig. 8 shows the mobilization electropherogram of human hemoglobin when carrier ampholytes of pH 3–10 were used, in which two main peaks are observed. The highest peak (peak 1) corresponds to methemoglobin (pI = 7.20) [12] which comprises about 75% of the sample, and peak 2 corresponds to oxyhemoglobin (pI = 7.00 [12] which is less than 25% in the sample. Another small peak is also observed before these two peaks, which probably corresponds to the A_2 form (pI = 7.40) of human hemoglobin [12]. The resolution can be estimated from the peak width in Fig. 8 to be 0.02 pH unit for the 12 cm long separation capillary, which is in the same order of magnitude as the best results obtained by commercial CE instruments in which longer capillaries are employed [4,5]. This high resolution is considered to be due to the small detection volume and the derivative nature of the detector [7]. Fig. 8 also shows good reproducibility of the peak shape.

The above results and discussion demonstrate the high sensitivity and reliability of the instrument. Below, some examples of applying the instrument to analyses of proteins are given.

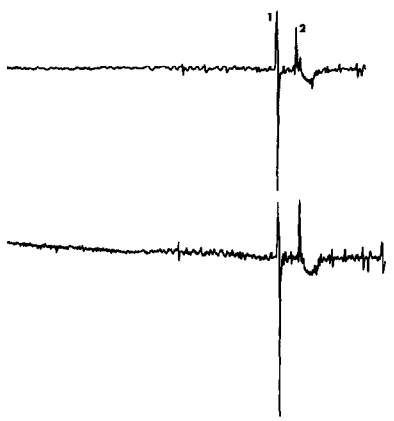

Fig. 8. Replicate separations of human hemoglobin. 1 = Methemoglobin; 2 = oxyhemoglobin. Capillary, 12 cm × 20 μm I.D.; protein concentration, 0.3 mg/ml.

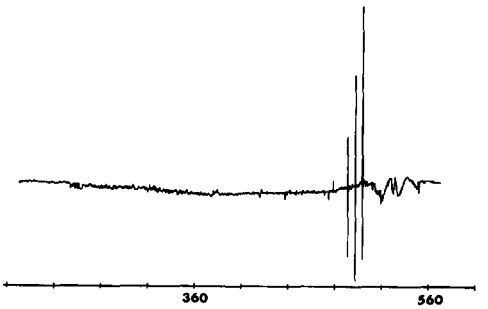

Fig. 9. Mobilization electropherogram of bovine transferrin. Capillary, 12 cm × 20 μm I.D.; protein concentrations, *ca.* 0.4 mg/ml.

Serum transferrin is an iron-transport protein with different iron-complexed or iron-free isoforms, which can be separated by isoelectric focusing. cIEF offers a fast and high-resolution separation method for studying the protein [8]. Fig. 9 shows the mobilization electropherogram of the bovine serum transferrin, which demonstrates the high resolution of the method. Each peak in Fig. 9 should represent a single isoform [8]. Because of the high resolution of the instrument, transferrin with different iron contents may be characterized by the method.

Monoclonal antibodies manufactured for clinical diagnosis and treatment may exhibit micro-heterogeneity even when they are demonstrated to be pure on the basis of their amino acid content. This mi-

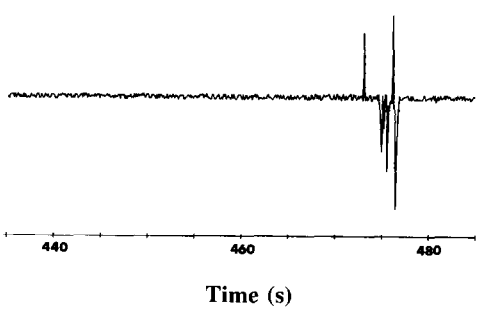

Fig. 10. Mobilization electropherogram of monoclonal antibody to fluorescein. Capillary, 12 cm × 20 μm I.D.; protein concentration, *ca.* 0.5 mg/ml.

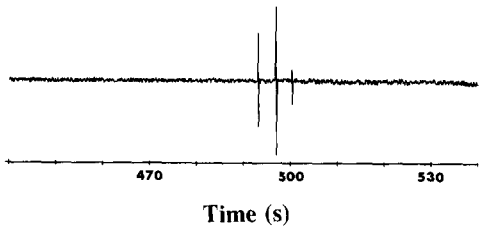

Fig. 11. Mobilization electropherogram of human serum albumin (fraction V). Capillary, 12 cm × 20 μm I.D.; protein concentration, 0.3 mg/ml.

cro-heterogeneity is usually characterized by conventional gel isoelectric focusing. cIEF also offers a high-resolution method for this purpose [2]. Fig. 10 is a high-resolution mobilization electropherogram of the monoclonal antibody to fluorescein, in which several narrow peaks can be observed, showing the micro-heterogeneity of the sample.

Human serum albumin is another important protein, which is often measured and correlated with various disease states. cIEF allows the rapid analysis of isoforms of the protein. Fig. 11 shows a mobilization electropherogram of the human serum albumin, fraction V, in which three peaks are observed around pH 5.8 [11]. Human blood serum obtained from a local medical laboratory was also analyzed by the cIEF instrument, and the results are shown in Fig. 12. Globulins and albumin are contained in the sample, which correspond to peaks around p*I* 4–6 [4]. Because of its high speed and resolution, the cIEF electropherogram pattern of the serum is possibly employed more efficiently than gel isoelectric focusing to correlate with various disease states. The analysis time for such an application can even decrease considerably if the electropherogram of the focused proteins inside the capillary is detected by an imaging system without the mobilization process [13].

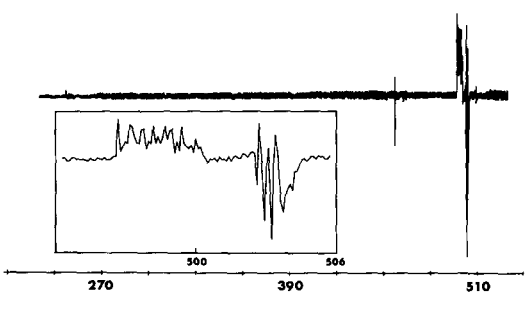

Fig. 12. Separation of human blood serum.

CONCLUSIONS

There are many advantages of the cIEF–concentration gradient detector instrument over commercial instruments. The sensitivity of the instrument is higher than those of commercial instruments. The UV–visible absorbance detector used in commercial CE instruments usually uses 280 nm as the detection wavelength instead of 180–240 nm because of the high background of the high-concentration carrier ampholytes [2,14]. At this wavelength, the sensitivity of the detector for proteins is much lower than that at 180–240 nm. For example, the absorption of bovine carbonic anhydrase at 280 nm is less than one tenth of that at 200 nm [14]. The derivative nature of the concentration gradient detector eliminates the high background generated by the wide band of carrier ampholytes, and high signal peaks are created only by narrow protein zones [7]. The detector has proved to be suitable for narrow capillaries and, because of the use of a narrow capillary which can accommodate high voltages, sharp protein zones are focused inside the capillary, resulting in high sensitivity for the concentration gradient detector. The concentration gradient detector also has a much smaller detection volume than a UV–visible absorbance detector. The detection volume of the detector can be calculated from the capillary I.D., 10 μm, and the laser beam diameter of 24 μm. It is calculated to be 2 pl, which is among the smallest values ever reported for an optical and spectroscopic detector. Because of the narrow capillary used, the instrument allows the analysis of the microenvironment of biochemical systems. Short capillaries can be used in the instrument, which facilitate the rapid analysis of protein samples. The instrument is also inexpensive and small-sized because the whole detection system consists only of a low-power He–Ne laser and a photodiode light beam position sensor, and a short capillary is used. The sample introduction and change of the buffer solution during the mobilization process can be easily performed by just operating a syringe [7]. The cost of the instrument is less than US$1000, which is much lower

than those of commercial CE instruments with a UV–visible absorbance detector that requires an expensive monochromator and photomultiplier tube. The cost and size of the instrument are expected to decrease even further if a diode laser is used in the detector. A future avenue of development of the CE instruments will be the construction of a complete CE instrument on a single silicon wafer, which includes a diode laser and a photodiode light beam position sensor as both are silicon devices.

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