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Protein analysis by isoelectric focusing in a capillary array with an absorption imaging detector

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Abstract

Isoelectric focusing (IEF) was successfully performed in capillary arrays with up to four capillaries. Separated proteins in the capillary array were detected by an UV absorption imaging detector. The whole analysis time for all samples in the capillary array was only 3 min due to the real-time imaging detector. The instrument was applied to analyse several protein samples including different human hemoglobin variants, myoglobin, transferrin, carbonic anhydrase and a monoclonal antibody to fluorescein. Because of good reproducibility of the focused pattern, unknown samples can be run simultaneously with a standard in the multichannel instrument and the components of unknown samples can be identified by comparing their zone positions to those of the standard. Minor components can be determined by the instrument in the presence of major components with 100 times higher concentrations in human hemoglobin samples. This instrument could be a powerful analytical tool for clinical analysis and for quality control in pharmaceutical companies.

1. Introduction

Performing isoelectric focusing (IEF) in narrow capillaries (capillary isoelectric focusing, cIEF) improved the IEF technique in many aspects [1,2]. The efficient dissipation of Joule heat by the narrow capillary allows a high separation voltage, resulting in highly efficient separation. Also, analysis speed of cIEF (usually, 10–30 min) is much faster than slab-gel IEF (h). The sample consumption of cIEF is small, in the nanoliter range. Quantitation for protein samples is much simpler in cIEF than in slab-gel IEF due to the use of an on-column detector in cIEF [2]. Although there are thus many advantages of cIEF over slab-gel IEF, and cIEF has been developed for nearly 10 years, until now the cIEF technique still could not replace slab-gel IEF for protein separations. This might be attributed to the time delay between the appearance of a new technique and its acceptance by the potential users. However, the many problems encountered with the current commercial cIEF instruments also prevent cIEF from replacing slab-gel IEF for protein analysis in biochemical and clinical laboratories, and quality control in pharmaceutical companies.

Currently, cIEF is usually performed in commercial capillary zone electrophoresis (CZE) instruments which have a 20–60 cm long capillary and an on-column UV absorption detector. In these instruments, after the IEF separation

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process (focusing process), all separated protein zones have to be moved past the detection point of the on-column detector located at one end of the capillary column. This mobilization can be produced by hydrodynamic force [1], electroosmotic flow [3], or adding salts to one end of the capillary [1]. Usually, the conditions and speed of the mobilization process have to be optimized for different samples in order to achieve the highest resolution [3,4]. In all these methods, however, the mobilization speed is uneven [3,4] and reproducibility is poor [5], causing deterioration of resolution and making isoelectric point (p*I*) measurement difficult. The mobilization process takes 10–30 min while the focusing only takes 4–6 min. A long mobilization process increases the risk of protein precipitation in the narrow capillary [5]. Although the runtime of cIEF for a single sample is much faster than that of slab-gel IEF, the number of samples that can be run in a given time is much smaller than that with slab-gel IEF since cIEF is a single channel separation technique while slab-gel IEF can simultaneously separate several samples in different lanes.

Over the past 3 years, research has been conducted at our laboratory to overcome the above problems with current cIEF methods. Our solution is to use imaging detectors [6–8] since after the focusing process all protein zones are stationary inside the capillary column. The mobilization process is eliminated by using the imaging detectors, so they eliminate all problems associated with the mobilization process. To accommodate the imaging detectors, short capillaries (4 cm long) are used, which also increases the speed of separation. Imaging detectors using a charge-coupled device (CCD) camera can monitor cIEF separations performed in a capillary array and measure the spectra of protein zones focused inside the capillary column [9]. Performing cIEF in a capillary array increases the sample throughput. In this paper we report the application of a multicapillary cIEF-UV absorption imaging detector system to protein analysis. A capillary array of up to four capillaries was used in the study.

2. Experimental

2.1. Apparatus

The multicapillary cartridge in the cIEF instrument is similar to that used in our previous experiments [9]. Separation columns were 100 μm I.D., 200 μm O.D., 4 cm long square silica capillaries (Vitro Dynamics, Rockaway, NJ, USA). The capillary inner walls were coated with non-crosslinked acrylamide to eliminate electroosmotic flow [1]. As shown in Fig. 1, the two ends of each capillary were connected to buffer reservoirs.

The light source of the absorption imaging detector was a 150 W tungsten or D_2 lamp. Two wavelengths were used in the experiment, 410 nm and 280 nm. Monochromatic light was obtained by passing the beam through bandpass filters. The light beam was directed to the capillary cartridge by a bundle of 300 optical fibers, having a 200 μm O.D., as shown in Fig. 1. A 5-cm focal length quartz cylindrical lens was used to collimate the light beam and project it onto the capillaries fixed in the capillary cartridge. The images of all capillaries in the cartridge were recorded simultaneously by a CCD camera (TEA/CCD-1752PF, Princeton Instruments, Trenton, NJ, USA). The camera was cooled to −38°C by a built-in electric cooler. The recorded images were collected and processed by an A/D board and CSMA software provided

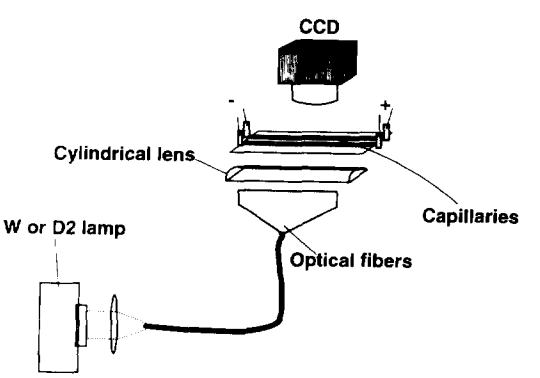

Fig. 1. Multicapillary cIEF with UV absorption imaging detector.

with the CCD camera by Princeton Instruments, in an IBM compatible personal computer.

First, images recorded before the separation voltage was turned on were used as the background. All images were normalized by the light intensity recorded simultaneously to correct low frequency intensity fluctuations in the light source [9]. The images recorded after completion of the focusing process were divided by the background image, and the resulting images correspond to the relative changes in light intensity, which were converted into absorption units by the computer.

2.2. Reagents

All solutions were prepared using deionized water, and filtered through 0.2-μm pore size cellulose acetate filters (Sartorius, Göttingen, Germany) prior to use. All chemicals were reagent grade, and solutions of 10 m*M* H_3PO_4 and 20 m*M* NaOH were used as the anolyte and catholyte, respectively. Samples included human hemoglobin A, F, S, C control, hemoglobin A, S control, hemoglobin A, A_2 control (Helena Laboratories, Beaumont, TX, USA), horse myoglobin, bovine transferrin (iron-saturated), human carbonic anhydrase (Sigma), and a monoclonal antibody to fluorescein (donated by A.R. Torres of HyClone Laboratories). Protein samples were mixed with the carrier ampholytes (Pharmalyte pH 3–10, Sigma) to a final ampholyte concentration of 2%. The concentrations of the protein samples were in the 50–200 μg/ml range.

2.3. Isoelectric focusing process

The mixture of protein samples and carrier ampholytes was injected into the capillaries by pressure using a syringe. Then, a plug of 1% agarose gel (prepared in the anolyte, 10 m*M* H_3PO_4) was placed in the reservoirs at the anodic ends of the capillaries to avoid hydrodynamic flow inside the capillaries. Finally, reservoirs at both ends of the capillaries were filled with their respective electrolytes, and the separation voltage was turned on. The separation voltage was 2 kV. The focusing process was completed when the current passing through the capillaries stabilized, which took about 3 min. The images of the capillaries were recorded by the CCD camera.

3. Results and discussion

The cIEF absorption imaging detector system is a multichannel instrument, and has a high sample throughput. Several samples can be separated and detected simultaneously, similar to slab-gel IEF. However, the analysis speed of our system is much faster than that of slab-gel IEF, and is simpler for quantitative determination since quantitation with slab-gel IEF requires a staining procedure after separation. Fig. 2 shows the image of two replicate hemoglobin samples (hemoglobin A, S control) separated in two capillaries simultaneously, and recorded by the absorption imaging detector after completion of the focusing process. The image looks like the image of a stained slab gel after IEF separation, but it was obtained only 3 min after separation had started. The concentration of each component can be determined directly from its peak area in the image. The sensitivity of the instrument was reported in our previous paper [10]. The image shows good reproducibility of the

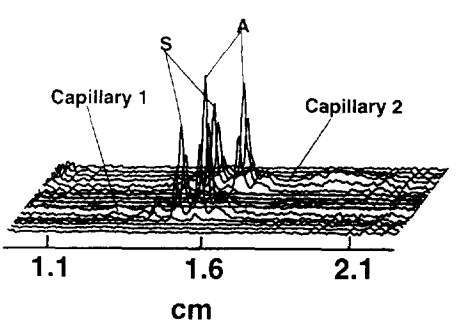

Fig. 2. Absorption image of two hemoglobin A, S control samples separated by IEF simultaneously in two capillaries. The image was recorded 3 min after start of the focusing process. Two major zones can be observed in each capillary. The detector was operated at 410 nm.

focused patterns in the two capillaries for the two replicate samples. The standard deviation in zone positions under the conditions used is less than 0.06 pH units, which is much better than that obtained for cIEF performed in commercial instruments.

Because of the good reproducibility an unknown sample can be run with a standard simultaneously in different capillaries, and each component in the unknown sample can be identified by directly comparing its zone position to that of the standard. Fig. 3 shows one example. In Fig. 3, four protein samples are separated in a four-capillary array. In the first capillary, the sample is a human hemoglobin standard which includes hemoglobin variants A, F, S, and C. A hemoglobin sample from a patient with sickle cell trait is separated in the second capillary. Two major components in the sample, hemoglobins A and S, can be identified by their zone positions against the zone positions of the standard separated in the first capillary. In the third capillary, the sample is bovine transferrin (iron saturated form). Since the absorption detection is performed at 410 nm in this experiment and the iron-saturated transferrin only has weak absorption at 410 nm, the electropherogram of transferrin only shows a small peak at pH 6. In the fourth capillary the sample is horse myoglobin. Two peaks can be observed at about pH 6.9 and 7.3. All four electropherograms were obtained 3 min after start of the focusing process. The sample throughput is much higher than that of

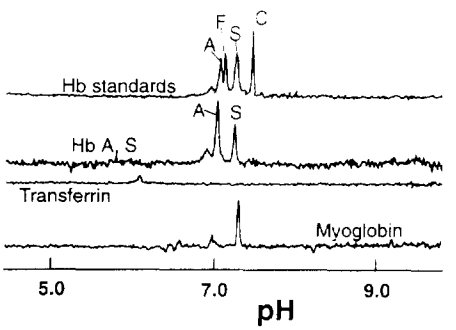

Fig. 3. Electropherograms of four protein samples separated simultaneously by IEF in a capillary array with four capillaries, and detected by the absorption imaging detector at 410 nm. Hb is hemoglobin.

cIEF performed in commercial instruments. Theoretically, the instrument can accommodate more than four capillaries. However, the manual sample injection method used in the study will be a problem for cIEF performed in a larger capillary array. This problem can be solved by using an automated sampler in the instrument.

Although capillary zone electrophoresis has been reported in a capillary array with more than 100 capillaries and detection by fluorescence detectors [11], this is the first report on whole column detection for cIEF performed in a capillary array.

Because of the short analysis time of the instrument and the wide dynamic range (for light intensity measurement) of the absorption imaging detector [9], it could be a powerful tool for clinical analysis. One possible clinical application is the identification and determination of human hemoglobin variants. Determination of some minor hemoglobin variants can provide important diagnostic information for doctors. Hemoglobin A_2, which only comprises 1–2% of total hemoglobin in normal human blood, is frequently altered in patients with thalassemia syndromes; however, its concentration is stable in normal persons. Hemoglobin A_{1c}, which constitutes 4–7% of the total hemoglobin, is a glycosylated form of hemoglobin A which increases in diabetes and is used to monitor long-term glycemic control in diabetic patients [12]. Determining percentages of these minor variants requires a high dynamic range in detector since they have to be detected simultaneously with other major hemoglobin variants. This can be done with the cIEF–absorption imaging detector system. Fig. 4 shows an electropherogram of hemoglobins from a normal adult. Peaks of hemoglobin A_2 and A_{1c} can be observed although the concentration of variant A_2 is only 1% of the total hemoglobin in this sample. The concentrations of all components can be determined by measuring their peak areas [10]. The concentration of variant A_2 could be determined with a relative standard deviation of less than 10% using the present method. Four hemoglobin samples can be run simultaneously, and the analysis time for the four samples is only 3 min.

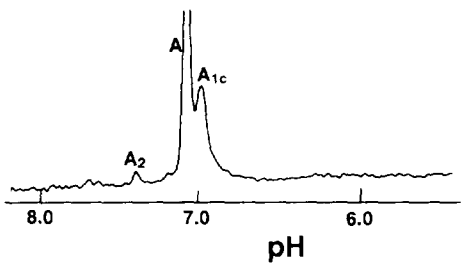

Fig. 4. Electropherogram of hemoglobin from a normal adult separated by cIEF and detected by the absorption imaging detector at 410 nm.

In the above sections, only images taken at 410 nm were discussed. The detector can also be operated in the lower UV range. For cIEF, absorption detection is usually performed at 280 nm due to the strong UV absorption of carrier ampholytes [1]. Fig. 5 shows electropherograms of two samples which have no absorption at 410 nm. Carbonic anhydrase gave two peaks around pH 6.4–6.8. For the monoclonal antibody to fluorescein, which is stored in a high-concentration buffer solution, a desalting process is usually necessary before performing cIEF separation due to the low salt tolerance of the cIEF method [4]. Conventional desalting processes take hours, during which the protein may become denatured. A fast and convenient method is simply diluting the sample to decrease the salt concentration in the buffer. In this experiment, the antibody sample is diluted by 100 times. After dilution the protein still can be detected by the absorption imaging detector operated at 280 nm. The split in its peak at pH 5.5 may be due to microheterogeneity of the sample.

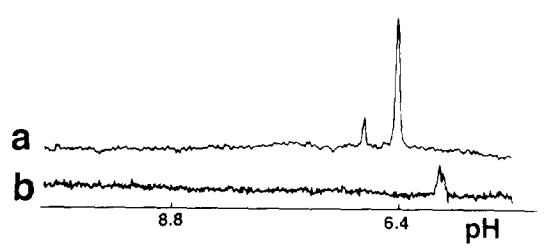

Fig. 5. (a) Electropherogram of human carbonic anhydrase. (b) Electropherogram of a monoclonal antibody to fluorescein. Detection by the absorption imaging detector was performed at 280 nm.

This fast and multichannel cIEF instrument is expected to be a useful analytical tool in pharmaceutical companies for quality control.

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References

[1] S. Hjerten and M. Zhu, *J. Chromatogr.*, 346 (1985) 265.
[2] T. Wehr, M. Zhu, T. Rodriguez, D. Burke and K. Duncan, *Am. Biotechnol. Lab.*, 8 (1990) 22.
[3] J.R. Mazzeo and I.S. Krull, *Anal. Chem.*, 63 (1991) 2852.
[4] M. Zhu, R. Rodriguez and T. Wehr, *J. Chromatogr.*, 559 (1991) 479.
[5] P.G. Righetti and C. Gelfi, *J. Cap. Elec.*, 1 (1994) 27.
[6] J. Wu and J. Pawliszyn, *Anal. Chem.*, 66 (1994) 867.
[7] J. Wu and J. Pawliszyn, *J. Liq. Chromatogr.*, 16 (1993) 1891.
[8] J. Wu and J. Pawliszyn, *Am. Lab.*, October (1994) 48.
[9] J. Wu and J. Pawliszyn, *Analyst*, (1995) in press.
[10] J. Wu and J. Pawliszyn, *Electrophoresis*, (1995) in press.
[11] K. Ueno and E.S. Yeung, *Anal. Chem.*, 66 (1994) 1424.
[12] G. Cossu, M. Manca, M.G. Pirastru, R. Bullita, A.B. Bosiso and P.G. Righetti, *J. Chromatogr.*, 307 (1984) 103.