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Application of capillary isoelectric focusing with absorption imaging detection to the analysis of proteins

Jiaqi Wu, Janusz Pawliszyn *

Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Abstract

A capillary isoelectric focusing instrument with an on-line optical absorption imaging detector was used to analyse protein samples. The separation column was a 4 cm × 100 μm I.D. capillary. The light source of the imaging detector was a argon ion laser. The light beam from the laser was focused into the capillary by a cylindrical lens. An 1024-pixel charge-coupled device (CCD) measured intensity of light beam passing through the capillary. The optical alignment of the detector was optimized to eliminate interference produced by the refractive index gradient created by sample zones inside the capillary. The signal-to-noise ratio of the detector was enhanced by averaging 30 scans of the CCD every 3 s. The on-line imaging detector allows simultaneous separation and detection so that the analysis time for a sample is only 2–4 min. Several protein samples were analyzed by the instrument, including human hemoglobin variants, cytochrome *c*, myoglobin and transferrin.

1. Introduction

Capillary isoelectric focusing (cIEF) [1] is a powerful capillary electrophoretic technique for protein separations. Protein samples are separated by cIEF based on their isoelectric point (p*I*) differences. Samples in nl volume can be analyzed by cIEF in 20 min [2]. Its resolution reaches 0.01 pH units [2]. Since 1985, cIEF has been studied, optimized, and commercialized [2–4].

The current cIEF method is complicated by the mobilization process for detection. Salt mobilization [1] distorts the linear pH gradient inside the capillary [2], making it difficult to determine the p*I* values of the protein samples from their retention times. The resolution of the zones with longer retention times also deteriorates [2,5]. The resolution of the focused zones may also deteriorate because of the laminar flow induced by the hydrodynamic mobilization process. All mobilization methods take 10–30 min [1,5,6]. These problems can be solved by using an on-line imaging detection system to replace the on-column detectors used for cIEF. Since in cIEF, proteins are focused at the position where their p*I* values are equivalent to pH values, this is a stationary situation.

Several optical absorption imaging schemes have been developed for cIEF. A photographic method was used to monitor protein zones focused inside the capillaries [7]. However, it is difficult to perform quantitative determination using photographic films. Another method measures protein zones focused inside a capillary by scanning the capillary across a single-point, on-column, UV–Vis absorption detector [8]. Because of the slow scanning speed, the detector can not monitor real-time electrophoretic pro-

* Corresponding author.

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cess inside the capillary, especially when an averaging method is used [8].

In our laboratory, a universal concentration gradient imaging detector using a charge-coupled device (CCD) has been developed for cIEF, which detects refractive index gradient generated by the concentration gradients of protein zones [9,10]. This imaging detector can be modified into an UV–Vis absorption imaging detector by employing a halogen lamp as the light source [11]. Compared to the concentration gradient imaging detector, the absorption imaging detector has a higher dynamic range, and higher sensitivity for samples having strong absorption [11]. However, the light beam from a lamp is difficult to focus into a narrow-I.D. capillary (<200 μm I.D.) [11]. Also, the optical alignment of the detector was not optimized in our previous work, resulting in poor performance [11]. A laser beam is a good light source for the imaging detector due to its coherent beam. The feasibility of using a argon ion laser for the optical absorption imaging detector for cIEF was shown in our previous paper [12]. In this paper, we will report results of applications of the cIEF–absorption imaging detection system to protein analysis.

2. Experimental

2.1. Apparatus

The capillary cartridge and high-voltage d.c. power supply were similar to those of our previous experiments [10,11]. A 4 cm × 100 μm I.D. square glass capillary (Vitro Dynamics, Rockaway, NJ, USA) was used for the separation. The capillary inner wall was coated with non-cross-linked acrylamide to eliminate electroosmotic flow [3].

Fig. 1 shows the optical configuration for the experiment. A 5-mW light beam from a argon ion laser (Model 165; Spectra-Physics, Cranbury, NJ, USA) was employed as the light source. For transferrin sample, 496.5 nm lasing line was used, and for other samples, the wavelength of

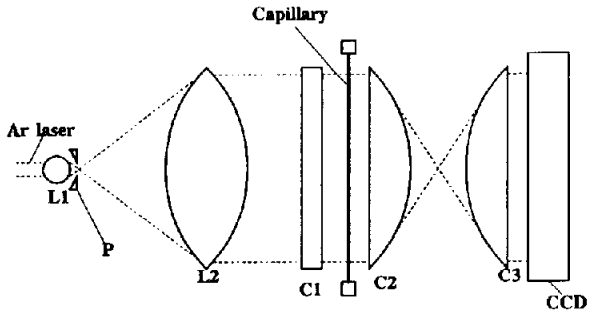

Fig. 1. Experimental setup of the cIEF–absorption imaging detection system. L1 = Magnification (20 ×) microscopic objective lens; P = pinhole (25 μm diameter); L2 = lens (25 cm focal length); C1 = cylindrical lens (5 cm focal length); C2, C3 = cylindrical lenses (8 cm focal length).

the light source was 514.5 nm. The laser beam was first expanded to a 5-cm-diameter beam spot by a beam expander consisting of a 20 × microscopic objective lens (L1), a 25-μm pinhole (P) and a 25-cm focal length lens (L2). The expended beam was passed through a 27 mm × 10 mm slit in order to obtain a light beam with uniform intensity in its cross section. Then, the beam was focused into the capillary by a 5-cm focal length cylindrical lens (C1) mounted on a three-axis stage. The size of the beam spot at the focal point of the cylindrical lens was about 27 mm × 3 μm. After the probe beam passed through the capillary, it was focused by an 8-cm focal length cylindrical lens (C2) perpendicular to the first cylindrical lens (C1). The distance between the capillary and the C2 lens is 5 mm. Finally, the beam was projected by another 8-cm focal length cylindrical lens (C3) onto a 1024-pixel, linear CCD sensor (S3903-1024Q; Hamamatsu, Hamamatsu City, Japan) which has a 25 mm × 0.5 mm sensing area. The distance between the C3 lens and the CCD sensor was also 5 mm. This alignment monitors 25 mm of the 4-cm long capillary.

The data was collected by an IBM DACA board attached to a PC-AT personal computer, using ASYST software (ASYST Software Technology, Rochester. NY, USA). The scanning speed of the 1024-pixel CCD was set at 10 Hz. For each measurement, the CCD scanned 30

times in 3 s, and the images were averaged to reduce the random noise. The light intensity profile measured before the separation voltage was turned on was recorded as a background image. The light intensity signal detected by the CCD was converted into absorption units by the computer.

2.2. Reagents

All chemicals were reagent grade, and solutions were prepared using deionized water. Solutions of 10 m*M* H_3PO_4 and 20 m*M* NaOH were used as the anolyte and catholyte, respectively. Protein samples included myoglobin (from horse skeletal muscle, Sigma Chemical), cytochrome *c* (from horse heart, Sigma), human hemoglobin standard (hemo control AFSC, Helena Labs., Beaumont, TX, USA), Fe-saturated transferrin (bovine, Sigma) and bovine transferrin sample (donated by HyClone Labs., Logan, UT, USA). Samples were mixed with carrier ampholytes solution (Pharmalyte pH 3–10, Sigma) to a final concentration of 2% ampholytes. The protein concentrations introduced into the capillary ranged from 100 to 400 μg/ml. Solutions were filtered using 0.2-μm pore size, cellulose acetate filters (Sartorius, Göttingen, Germany) prior to use.

2.3. Isoelectric focusing process

First, the sample solution was introduced into the capillary by pressure. A plug of 1% agarose gel prepared in the anolyte, 10 m*M* H_3PO_4, was placed in the reservoir at the anodic end of the capillary to avoid hydrodynamic flow inside the capillary. Then, reservoirs at both ends of the capillary were filled with their respective electrolytes, and a 3.5 kV d.c. voltage was applied. The current which passed through the capillary was monitored to follow the focusing process. The focusing process usually ended in 2–4 min when the current dropped to 3 μA and stabilized. All experiments were done in triplicate to ensure reproducibility.

3. Results and discussion

The refractive index gradients inside the capillary created by sample zones may interfere with the absorption measurement. These interference can be minimized by placing the CCD at the imaging point of the capillary where the beam rays deflected by the sample zones return to their original positions [12]. For the optical alignment used in the experiment, as shown in Fig. 1, the distance between C3 lens and the CCD is the same as the distance between C2 lens and the capillary. In this alignment, the CCD's position is the imaging point of the capillary.

The noise of a argon laser beam in intensity is higher than that of a halogen lamp. Because the sensitivity of an absorption detector is dominated by the noise level in the light source, the detection limit of the laser-based imaging detector is expected to be higher than that of a single-point absorption detector with a lamp as the light source. However, for this imaging detector for cIEF, the detection limit can be improved by using an averaging method. Because all protein zones are stationary inside the capillary after focusing, a long averaging time can be used for the CCD sensor, which enhances the signal-to-noise ratio (S/N) of the detector. In the detector, because a high intensity light beam is used (5 mW), a short exposure time for CCD has to be used (0.1 s) to avoid saturation. Under this condition, the averaging is performed by the computer. The CCD operated at 10 Hz scans 30 images in 3 s, and these images are averaged by the computer. Fig. 2 shows the same electropherograms of the 20 μg/ml myoglobin sample with and without averaging. The S/N improvement is about four times. Increasing the number of scanning may further increase the S/N of the imaging detector. However, because the electroosmotic flow can not be totally eliminated, under the present conditions the averaging time can not be longer than 3 s due to the movement of the sample zones inside the capillary. For this reason, the scanning number is limited by the speed of the A/D board and the computer. The optical defects in the capillary wall also affects the S/N of the detection [12].

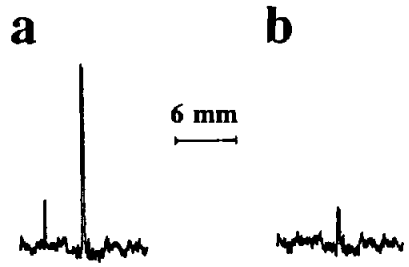

Fig. 2. Electropherograms of 20 μg/ml of myoglobin. (a) Averaged image of 30 scans in 3 s, (b) image of 1 scan. In order to compare signal-to-noise ratio, electropherograms are plotted in such a way that they have the same noise levels.

Further improvement in detection limit of the imaging detector can be made by using a capillary or narrow channel with good optical quality, and high speed A/D board and fast scanning CCD.

The present cIEF–absorption imaging detection system is very useful for analysis of many proteins, including myoglobin, cytochrome *c*, hemoglobin and transferrin. The detector shows good sensitivity for proteins having absorption at the wavelengths of the argon lasing lines. For the detection of other proteins, a UV laser has to be employed as the light source. This will increase the cost of the detector. However, since a high-power laser beam is not required for the absorption detector, cheaper laser devices are applicable, such as a laser beam from the second or third harmonic emission of a diode laser (sub-mW) [13], which has sufficient intensity for the absorption imaging detection system.

Myoglobin is usually used as a p*I* marker in IEF. It has weak absorption at 514.5 nm, and can be detected by the imaging detector. Fig. 3 is the electropherogram of the 100 μg/ml myoglobin sample. Its p*I* value can be directly measured from its position inside the capillary. The p*I* value of the major component was measured to be 7.51, and the value of the minor component was measured to be 6.85. These values agree with reported data [14]. The separation and detection processes took less than 2 min in this experiment.

Another example is cytochrome *c*, which has weak absorption at 514 nm. The p*I* value of the protein is 9.6. For the capillary cartridge used in

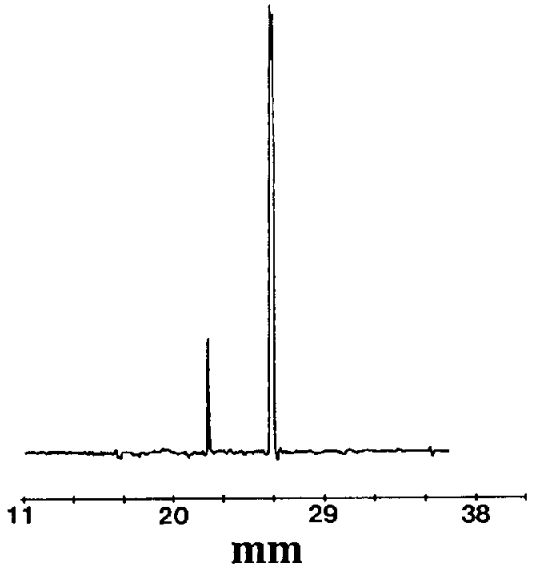

Fig. 3. Electropherogram of 100 μg/ml of myoglobin. The image was obtained 2 min after separation voltage was turned on.

the experiment, the capillary was glued to the buffer reservoirs. The glued connection points are 1–2 mm in length, and they are out of range of the imaging detector. This length corresponds to 0.2–0.4 pH units. It is difficult to detect cytochrome *c* by this instrument when the carrier ampholytes have a pH range from 3 to 10. However, the pH range of the carrier ampholytes at the cathodic end of the capillary can be extended to pH 12 by adding 0.5% of N,N,N′,N′-tetramethylethylenediamine (TEMED) into the carrier ampholytes–sample mixture [2]. Fig. 4 shows a electropherogram of 200 μg/ml of cytochrome *c* separated and detected under these conditions.

Fast screening of the hemoglobin variant in human blood is an important method for diagnosing abnormal blood in patients. Current techniques used for the screening are high-performance liquid chromatography (HPLC) [15] and cIEF [4]. The cIEF method has higher resolution than the HPLC method, but slower speed. cIEF–absorption imaging detection, however, has both higher resolution and higher speed than the HPLC method. Fig. 5 shows the electropherogram of four standard human hemoglo-

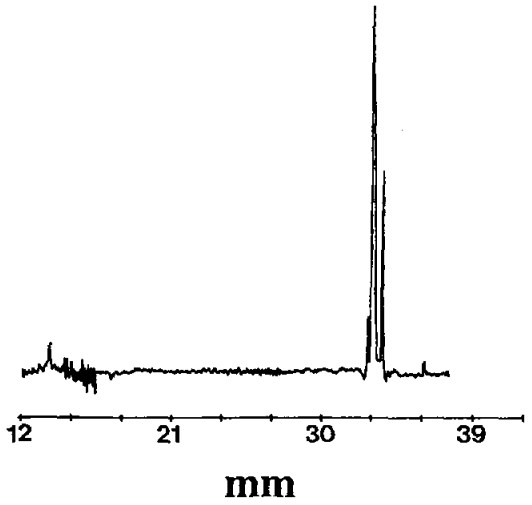

Fig. 4. Electropherogram of 200 μg/ml of horse cytochrome *c*.

bin variants obtained within 2 min of turning on the separation voltage. The blood sample is diluted by 300 times. The sensitivity of the detector is good, and so it makes practical the direct analysis of blood samples without a desalting process.

The resolution of the cIEF instrument is high even for the 4-cm-long capillary used in the instrument. The p*I* difference between variants A (p*I* 7.10) and F (p*I* 7.15) is 0.05 pH units. The resolution of the instrument is estimated to be 0.01 pH units from the distance between the A and F peaks. This resolution is the same as that of a conventional cIEF instrument using 20-cm-long capillaries [2].

An interesting sample for cIEF–imaging detection is transferrin. Transferrin is an Fe transportation protein in blood serum. It has two forms: an Fe-complexed form and Fe-free form. Each form also has several isoforms [3]. The Fe-free form has no absorption in the 400–500 nm region; however, the Fe-complexed form has a low absorption peak at 495 nm. For the transferrin samples, a argon lasing line of 496.5 nm was used in the absorption imaging detector. Fig. 6 shows electropherograms of Fe-saturated bovine transferrin and bovine transferrin sample without treatment. In this experiment, both absorption imaging and universal concentration gradient imaging detectors [12] were employed,

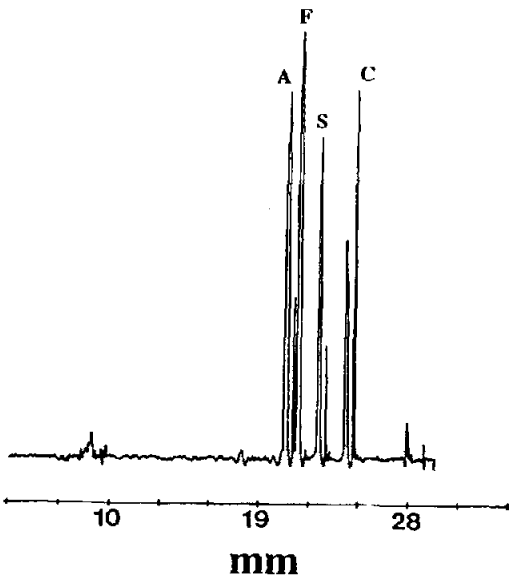

Fig. 5. Electropherogram of human hemoglobin variant standard (AFSC). The standard is diluted by 300 times.

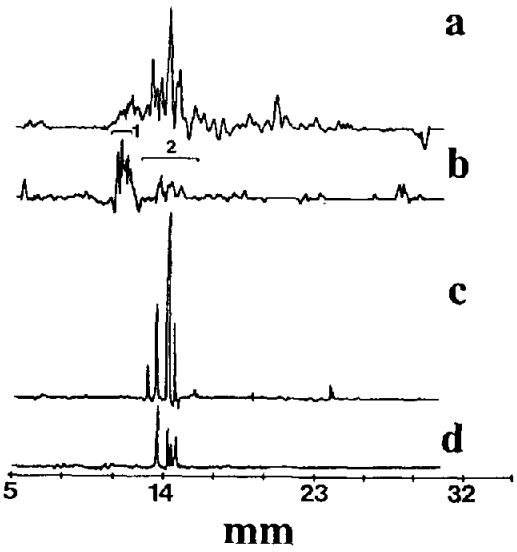

Fig. 6. Electropherograms of bovine transferrin. (a) Fe-saturated transferrin detected with concentration gradient imaging detector, (b) transferrin sample without treatment detected by concentration gradient imaging detector, (c) Fe-saturated transferrin detected with absorption imaging detector, (d) transferrin sample detected with absorption imaging detector. Concentrations of proteins are 400 μg/ml.

and both detections were performed simultaneously [12]. All the isoforms of transferrin can be detected by the universal concentration gradient imaging detector. However, only Fe-complexed isoforms can be detected by the absorption imaging detector operated at 496.5 nm. By using the both imaging detectors, Fe-free and Fe-complexed isoforms can be distinguished.

In Fig. 6, peaks in area 1 should correspond to Fe-free isoforms, and most of peaks in area 2 should correspond to Fe-complexed isoforms. Several peaks are observed for both the Fe-free and Fe-complexed forms of transferrin, which correspond to different isoforms. In the electropherograms of the untreated bovine transferrin sample (Fig. 6b and d), the peak heights are lower than those in Fig. 6a and c, even though the concentrations of the two samples are the same, and also one peak disappears in Fig. 6b and d. This is because in Fig. 6a and c, the protein consists only of Fe-complexed forms, and for sample in Fig. 6b and d, part of the protein is in Fe-free forms. Also, some isoforms in area 2 can only be formed under Fe-saturated condition, so these isoforms can not be observed in Fig. 6b and d.

4. Acknowledgements

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