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Optimizing separation conditions for proteins and peptides using imaged capillary isoelectric focusing

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Abstract

Separation conditions for antibodies, glycoproteins and peptides were optimized to fully realize the potential of automated imaged capillary isoelectric focusing (imaged cIEF) for protein analysis. Two commercially available capillary coatings, polyacrylamide and fluorocarbon, were found to provide reproducible results for cIEF separations. Both coatings could last more than 100 runs under normal cIEF conditions. Up to 30 m*M* salts (Na^+) could be added to samples to prevent protein precipitation before and during isoelectric focusing performed under imaged cIEF. Short analysis time of the imaged cIEF also aided in the prevention of protein precipitation. High current at the beginning of the focusing for samples in salt could be avoided by applying a voltage gradient. Additions of up to 6 *M* urea and 20% glycerol could enhance solubility of proteins and peptide. Imaged cIEF was applied to the quantitation of monoclonal antibodies.

Keywords: Capillary isoelectric focusing; Detection, electrophoresis; Whole-column imaging detection; Proteins; Antibodies; Peptides; Glycoproteins

1. Introduction

Capillary isoelectric focusing (cIEF) [1] offers many advantages over gel-based isoelectric focusing (IEF), such as ease of automation, quantitation and faster analysis speed. At present, most commercially available capillary electrophoresis (CE) instruments offer IEF analysis using cIEF kits [2]. Commercial CE instrument perform cIEF as follows [3]: (1) protein sample and carrier ampholyte plugs are introduced into the capillary column between the catholyte and anolyte (the catholyte is usually NaOH, and the anolyte, H_3PO_4); (2) a d.c. voltage (0.5–1 kV per centimeter column) is applied across the catholyte and anolyte to perform IEF. Under the voltage, the carrier ampholytes establish a pH gradient within the capillary column from its anodic end to its cathodic end. At the same time, proteins within the column are separated and focused at the positions where their isoelectric point (p*I*) values are the same as the pH values [4].

Since commercial CE instruments are equipped with a single-point, on-column UV absorption detector, all focused protein bands within the capillary must be moved past the point detector. Thus, a mobilization step is necessary following the focusing process [3]. Alternatively, mobilization can commence during the IEF in what is called ''one-step'' cIEF method [2]. There are many ways to perform the mobilization, such as adding salts to one of the electrolytes, using electroosmotic flow or pressure [2]. Many problems are associated with the mobilization process, such as increased analysis time and

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uneven resolution due to uneven mobilization speed [3].

In the past few years, we have been working on new ways to perform cIEF. A new technique we applied to cIEF is whole-column imaging detector [5–7]. Since in cIEF, all protein bands within the capillary column are stationary or near stationary at the end of the focusing process, whole-column imaging detectors appear to be ideal for their detection. Use of a whole column imaging detector eliminates the mobilization process, thus, avoiding problems associated with the process.

To accommodate the whole-column imaging detector in cIEF, a column cartridge holding a 5 cm long capillary was designed [8]. The sample introduction procedure for the imaged cIEF instrument with the cartridge is automated using any commercial liquid chromatography (LC) autosampler, and controlled by a personal computer [9]. The sample throughput of the automated imaged cIEF method for most proteins is up to 10 samples per hour [9].

To fully realize the potential of the automated imaged cIEF, several difficulties associated with cIEF method have to be addressed, such as unreliable capillary coatings, and precipitation of unstable proteins during focusing. To stabilize the proteins during IEF, usually, additives, such as salts, organic solvents and detergents are mixed with the proteins [10]. Tolerance of these additives in the imaged cIEF method needs to be determined. Since long IEF time also increases the chance of protein precipitation, IEF time should be optimized for different protein samples. In this paper, we will show the results of using two stable coatings for columns of the imaged cIEF. We will also discuss the optimization of cIEF separation conditions for proteins using the whole column imaging detector and additives. The imaged cIEF is shown to be a useful tool for fast quantitation of monoclonal antibodies.

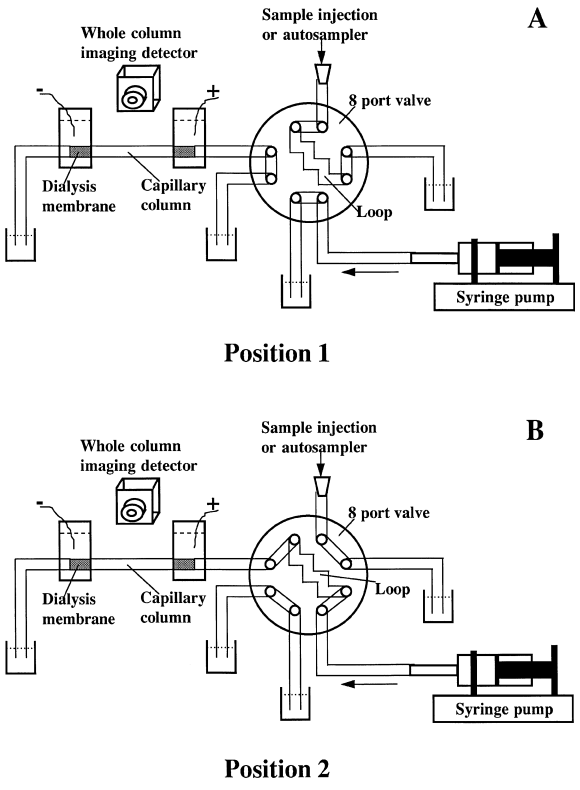

Fig. 1. Imaged cIEF instrument with automated sampling system. (A) The injection valve is at position 1. (B) The valve is at position 2.

2. Experimental

2.1. Apparatus

The cIEF column and sample introduction system are shown in Fig. 1. The structure of the cartridge was reported in our previous paper [8]. The separation column is a 50 mm×100 μm I.D.×200 μm O.D. silica capillary (Polymicro Technologies, Tucson, AZ, USA). The outside coatings of the capillary are removed for whole column detection. The inner wall of the capillary is coated with either polyacrylamide (Supelco, Bellefonte, PA, USA) or fluorocarbon (J&W Scientific, Folsom, CA, USA) to eliminate or reduce electroosmotic flow. The two ends of the column, as shown in Fig. 1, are connected to two pieces of 3 mm long dialysis hollow fiber membrane (rel. molecular mass cut-off: 18 000, Spectrum Medical Industries, Los Angeles, CA, USA). The two sections of the fiber are inserted into the electrolyte reservoirs.

Interface between the column cartridge and an eight-port, two-position HPLC injection valve (Valco, Houston, TX, USA) is illustrated in Fig. 1. The injection port of the valve is linked to either a needle port for manual sample injection using a

micro syringe, or a HPLC autosampler (Model ISS-100, Perkin-Elmer, Norwall, CT, USA). The sample loop volume in the injection valve is 2.5 μl. The infusion pump is a syringe pump (Model A-99, Razel Scientific, Stamford, CT, USA). The syringe used with the pump can be 1 ml–3 ml syringes. The flow-rate of the pump is set at 6 μl/min. The syringe pump operates during the entire experiment. For each sample injection, 15 μl of sample is injected manually or by the autosampler to ensure the sample loop is filled with the sample. The sample injection system and the imaged cIEF instrument are fully controlled by a personal computer.

The detection system of the instrument is a whole-column optical absorption imaging detector operated at 280 nm, which is similar to our previous experiments [11].

2.2. Reagents

All solutions are prepared using deionized water. Solutions of 100 m*M* H_3PO_4 and 40 m*M* NaOH are used as the anolyte and catholyte, respectively. Samples include human hemoglobin AFSC control (Helena Labs., Beaumont, TX, USA), monoclonal anti-α_1-acid glycoprotein, monoclonal anti-α_1-antitrypsin (both antibodies were obtained from Calbiochem-Novabiochem, La Jolla, CA, USA), human IgG (Sigma) and human α_1-acid glycoprotein (Sigma). A peptide prepared by ourselves is also used as the sample. Methylcellulose, urea, phosphate-buffered saline (PBS), glycerol and all carrier ampholytes are purchased from Sigma. All chemicals are reagent grade. The final concentrations of carrier ampholytes in samples are 4%. The concentrations of proteins are 0.05–2 mg/ml.

2.3. Sample introduction and isoelectric focusing processes

As shown in Fig. 1A, when the switch valve is at position 1, sample solution of 15 μl is injected into the sample loop manually or by the autosampler. The valve is then switched to the position 2 (Fig. 1B). At this position, the sample solution in the loop will be flushed into the separation column. When the column is filled with the sample, which can be calculated from the volume of the inlet tubing and the flow-rate of the syringe pump, the valve is switched back to the position 1. At the position 1, the capillary column is isolated from the sample loop and the autosampler. A 3 kV d.c. voltage is applied to the two electrolyte reservoirs to start IEF, as shown in Fig. 1A. At the end of the focusing process, all protein bands within the capillary column are recorded by the whole column absorption imaging detector without mobilization. The d.c. voltage is turned off. The sample loop is washed by injecting wash solvent into it while the valve is at the position 1. Again, the valve is switched to position 2 to wash the column for 30 s. This makes the injection system ready for next sample.

3. Results and discussion

Two commercially available coatings for capillary column were evaluated for our cIEF method. First one is Supelco polyacrylamide coating, and the second is J&W Scientific fluorocarbon coating. When the fluorocarbon coating is used, as suggested by J&W Scientific, methylcellulose should be used to condition the capillary column and added to samples to further reduce the electroosmotic flow. In our experiments, 0.15% methylcellulose was added to all samples. More than 100 runs of hemoglobin sample were performed on the column coated with either polyacrylamide or fluorocarbon. Both coatings showed reproducibility under the IEF conditions for hemoglobin samples. Fig. 2 shows the electropherograms of run number 1 and number 150 on a fluorocarbon coated capillary column. Both coatings are expected to last for more than 100 runs under the conditions.

Many large proteins are very hydrophobic. For these proteins, loss of salt during either dilution or IEF may cause precipitation. The electropherograms of monoclonal anti-α_1-antitrypsin is shown in Fig. 3. The protein was directly diluted 10-times from its stock solution by carrier ampholyte solution, and analyzed by the imaged cIEF. The signal intensity is low and the focused patterns are not reproducible. This is due to the low concentration of salt in the final sample solution. This condition may cause the protein to precipitate even before it is injected into the instrument. Hydrophobic proteins require a mini-

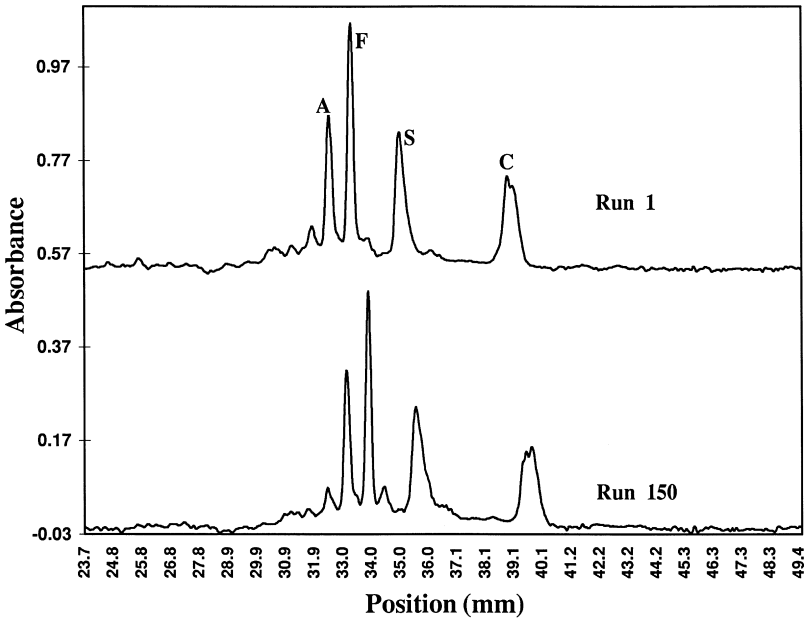

Fig. 2. Comparison of run 1 and run 150 on a fluorocarbon-coated capillary column. The sample is 1/200 dilution of human hemoglobin AFSC control. IEF conditions: 4% pH 3–10 and 4% pH 6–8 carrier ampholytes, 5 min focusing.

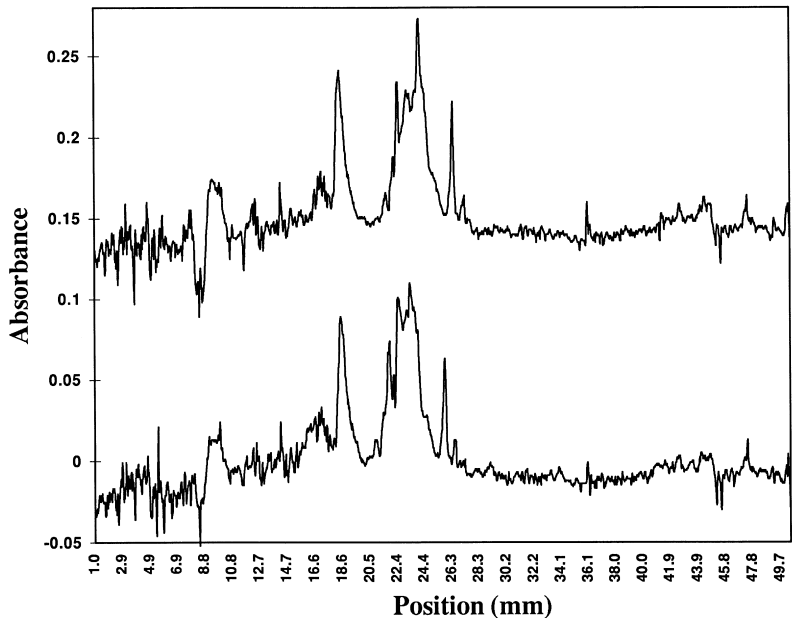

Fig. 3. IEF of monoclonal anti-α_1-antitrypsin (0.75 mg/ml) without salt buffer. Conditions: 2% pH 3–10 and 2% pH 6–8 carrier ampholytes, 3.5 min focusing.

mum salt concentration in their sample solutions in order to maintain solubility.

Before analyzing protein samples prepared in salt buffers, the salt tolerance of the imaged cIEF has to be determined. We used current within the capillary column recorded during IEF to estimate the tolerance. Sigma's PBS buffer (stock solution contains 150 m*M* NaCl and 150 m*M* sodium phosphate, total 300 Na^+) was added to the sample as the salt source. IEF of hemoglobin, which is a protein with good solubility, in 7.5 m*M* (diluted 40-times from Sigma's PBS stock solution), 15 m*M* and 30 m*M* PBS solutions, and solution without PBS was performed. The concentration of pH 3–10 carrier ampholytes in the sample was 8%. The current changes within the capillary column during the focusing under all these conditions were recorded (Fig. 4). For the sample without PBS, the current is 48 μA at the beginning of the focusing. It drops fast along the time once the focusing process starts and stabilizes when it drops to 5 μA in 3.5 min. For samples in PBS solutions, the current also drops along the time because salt molecules are driven out of the capillary column during the focusing under the separation voltage. The current also stabilizes after 3.5–5 min.

The major difference between samples in salt solutions and in solution without salt is the current at their start points of focusing. For samples in salt solutions, the current at the start point increases linearly with increasing salt concentration until the concentration reaches 30 m*M* PBS. Salt concentrations higher than 30 m*M* PBS produce almost the same start current. For this reason, the high start current within the column will be maintained for a longer period when the salt concentration is higher than 30 m*M* PBS even the focusing process starts to mobilize the salt molecules out of the column. For example, this period is 50 s at 120 μA current if the salt concentration is 100 m*M* PBS. This ''plateau'' in the current curve at the beginning of the focusing, as indicated by the arrow in Fig. 4, will tend to overheat the solution within the column, and may distort and even destroy the pH gradient established by the carrier ampholytes [12]. To avoid the over heating, the salt concentration should be kept lower than 30 m*M* PBS. Under this condition, as shown in Fig. 4, the ''plateau'' is narrow (less than 5 s). The current drops quickly along the time and will be close to the value of the sample without salt when the current stabilizes. The focusing process takes place normally for the hemoglobin sample when salt concentration is lower than 30 m*M* PBS. From these experiments, the salt tolerance of the imaged cIEF is estimated to be 30 m*M*.

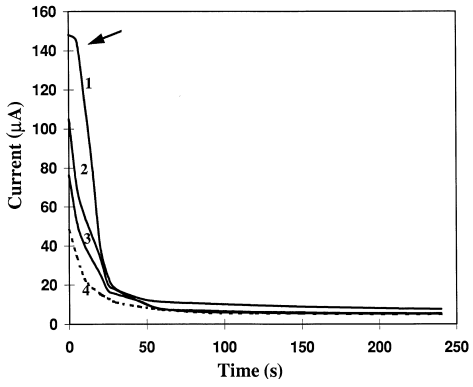

Fig. 4. Current within the capillary column during IEF. IEF conditions: 8% pH 3–10 carrier ampholytes. Sample: hemoglobin AFSC control, 1/200 dilution. 1=In 30 m*M* PBS; 2=in 15 m*M* PBS; 3=in 7.5 m*M* PBS; 4=without PBS.

To prevent proteins from precipitation, all sample solutions in our experiments contained 15–30 m*M* PBS. However, for samples containing 30 m*M* PBS, the current under the 3 kV at the beginning of the focusing reaches 110 μA under which the temperature within the capillary is estimated theoretically [13,14] to be as high as 80°C. To reduce the current at the beginning of the focusing process, a voltage gradient starting from 0.5 kV was employed in the first 15 s of the focusing process. The voltage was raised to 3 kV in 15 s at which the current had already dropped to about 40 μA.

As mentioned above, during IEF process, salt molecules will be mobilized out of the capillary column under the separation voltage. Only non-ionic and ampholytes can stay within the column [10]. The salt concentration within the capillary column at the end of focusing process is almost zero even if salt buffer is added to the sample. Proteins at their zero net charge status and under low salt concentration will tend to precipitate quickly at the end of the focusing. Short focusing time is critical for these proteins. The analysis time of the imaged cIEF can be as short as 4 min for most proteins [5]. The

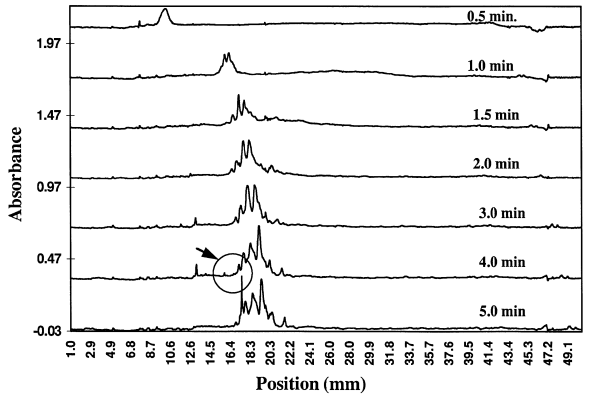

Fig. 5. IEF process of monoclonal anti-α_1-antitrypsin (0.75 mg/ml). Conditions: 2% pH 3–10 and 2% pH 6–8 carrier ampholytes, 30 m*M* PBS.

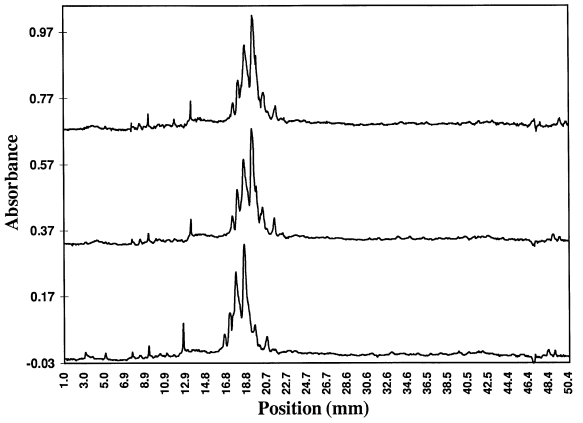

Fig. 6. IEF of monoclonal anti-α_1-antitrypsin (0.75 mg/ml). Conditions are as in Fig. 5. 3 min 40 s focusing.

optimal focusing time for a sample should be the time that allows for completion of the focusing before precipitation takes place. Fig. 5 shows an example which is the images recorded by the whole column imaging detector during the IEF of monoclonal anti-α_1-antitrypsin. In this experiment, the sample was prepared in 30 m*M* PBS buffer instead of simply by diluting the sample as shown in Fig. 3. The best resolution is obtained at 4 min as shown in Fig. 5. There is no sign of protein precipitation before 4 min due to the salt in the sample. However, as indicated by the arrow in Fig. 5, the protein starts to precipitate after 4 min because the salt molecules are driven out of the column in the 4 min focusing, creating sharp spikes in the images. No reproducible focused pattern can be obtained after the sample precipitation [12]. To avoid the precipitation, the focusing time was set just less than 4 min in the experiment. When the focusing time is 3 min 40 s, reproducible focused pattern can be obtained as shown in Fig. 6.

This example shows that the ''whole-column display'' ability of the imaging detector simplifies the experimental procedure needed to find optimal IEF conditions. The optimal focusing time can be determined by the imaged cIEF from a single run. In conventional cIEF with mobilization process, a series of runs with different mobilization speeds have to be performed to determine the optimal focusing time.

Peptides can also be analysed by cIEF [15,16]. Due to the high UV absorption of the carrier ampholytes used in cIEF in the wavelength range shorter than 280 nm, UV absorption detectors have to operate at 280 nm. At this wavelength, only tyrosine and tryptophan have absorption. Sensitivity of UV absorption detector is low for many peptides. Fig. 7 shows the electropherogram of a peptide which contains no tryptophan and only one tyrosine. In order to detect it, the peptide at high concentration (2 mg/ml) was injected to the instrument. Since the peptide is concentrated in the focusing, it soon precipitates in less than 2 min after the focusing starts, as show in Fig. 7. The precipitation makes further focusing impossible. This problem can be

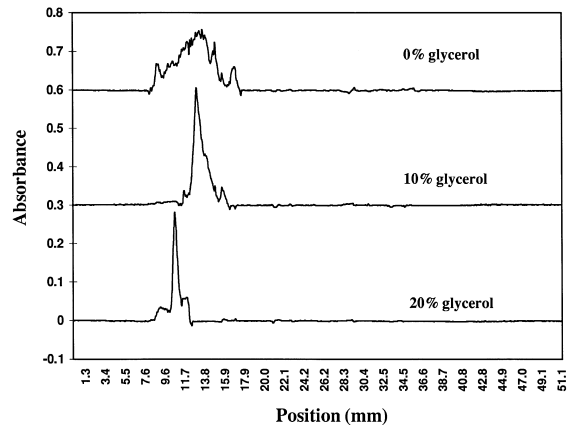

Fig. 7. IEF of a peptide: Ac–Asp–Asn–Asp–pTyr–Ile–Ile–Pro–Leu–Pro–Asp–Pro–Gly–OH, 2 mg/ml. Conditions: 4% pH 3–10 carrier ampholytes, 2 min focusing.

solved by adding glycerol to the sample to enhance its solubility as shown in Fig. 7. Reproducible results can be achieved when 20% glycerol is added to the sample.

In cIEF, the focusing time depends on the mobility of sample molecule. The mobility of a molecule is related to its molecular mass. For the small-molecular-mass peptide, such as that shown in Fig. 7, the focusing time is shorter than that for a protein. The focusing process is complete in only 2 min. At the end of the 2 min, the peptide zone is detected by the whole column detector, and its p*I* value can be determined by its position within the capillary column [17]. The sample throughput of the imaged cIEF for this peptide is 15 samples per hour. The method may be an useful tool for the fast measurement of p*I* values of peptides.

Urea is widely used in both slab gel IEF and cIEF to enhance the solubility of hydrophobic proteins [18]. But urea denatures proteins, which causes shifts in their p*I* values. In our experiments, up to 6 *M* urea was used as the matrix for some protein samples. One example is monoclonal anti-α_1-acid glycoprotein. Without urea it is difficult to achieve reproducibility in its focused pattern even the sample is prepared in salt buffer. In the experiment, the protein with 4 *M* urea matrix was injected consecutively for 20 times. As shown in Fig. 8, the focused pattern is reproducible. Also, the result in Fig. 8 demonstrates reproducibility in peak position. For the 20 injections, the standard deviation in peak position is 0.33 mm for the 50 mm long capillary column.

Human IgG is a protein with a 148 000 molecular mass. Its electropherogram is shown in Fig. 9A. It is quite heterogeneous as its peaks spread in a wide range of p*I*. The sample was prepared in 4 *M* urea. Even in the urea matrix, the protein starts to precipitate if the focusing time is longer than 5 min. In our experiment, the optimal focusing time was 3.5 min. At 3.5 min, all peaks within the column were detected simultaneously by the whole column imaging detector, and the focusing voltage was then turned off. In this way, the focusing times for all bands of the protein are the same. This is different from the conventional cIEF with the mobilization

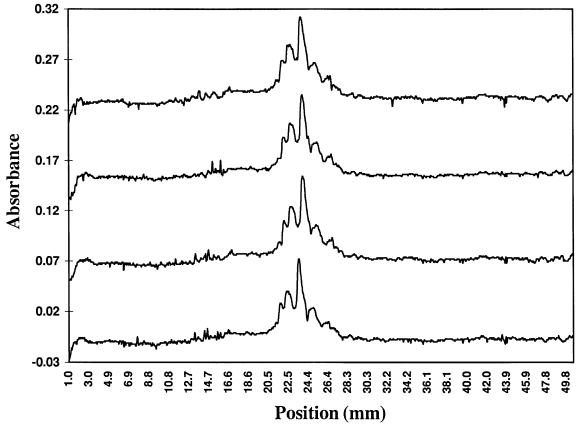

Fig. 8. IEF of monoclonal anti-α_1-acid glycoprotein (0.33 mg/ml). Conditions: 2% pH 3–10 and 2% pH 6–8 carrier ampholytes, 4 *M* urea, 15 m*M* PBS, 4 min focusing.

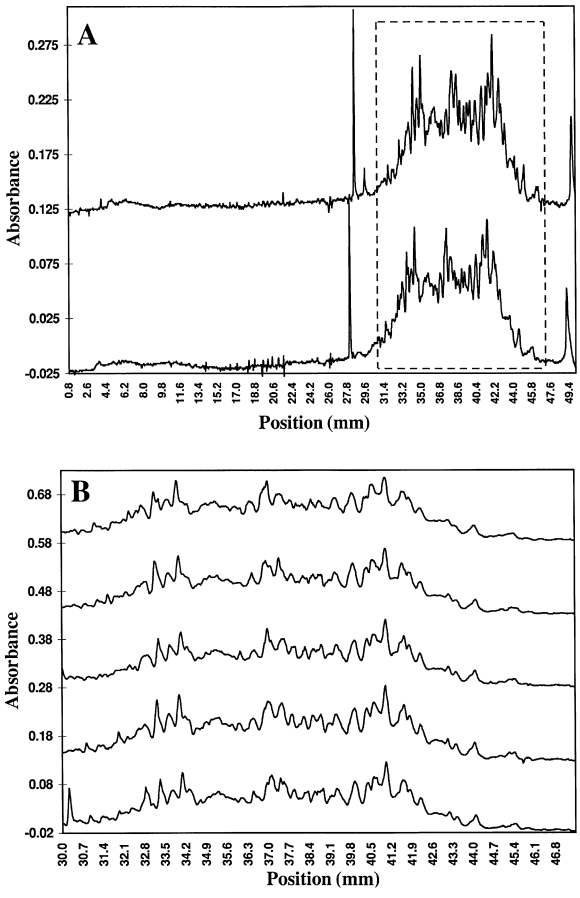

Fig. 9. (A) IEF of human IgG (2 mg/ml). Conditions: 4% pH 2–11 carrier ampholytes, 4 *M* urea, 15 m*M* PBS, 3.5 min focusing. (B) Electropherograms of five consecutive runs of human IgG. Conditions are as in (A).

process using commercial CE instrument. When a commercial CE instrument with a point detector is used, during the mobilization, if the first band arrives at the detection point at 3 min, the last one may arrive at 5 min. The bands between them will experience different focusing times since during the mobilization the focusing voltage is always on [4]. This may not be a problem for stable proteins or proteins with good solubility, such as hemoglobin. However, it may be a problem for some proteins, such as IgG. At 3 min, the protein may not be focused well while at 5 min, the protein may have precipitated. The imaged cIEF may avoid the problem. There are many spikes in Fig. 9A. Details can be seen by expanding the area indicated in Fig. 9A. The results of five consecutive injections are shown in Fig. 9B. More than 20 peaks can be observed in the figure. They are reproducible.

All those multi-peak patterns in the electropherograms of the antibodies shown above reveal their intrinsic microheterogeneity which is due to the presence of a series of different glycoforms of the proteins. Now, analytical chemists pay increasing attention to the glycoproteins because of their medical and biotechnological implications. A model glycoprotein is α_1-acid glycoprotein (AGP) since it has a high molecular mass (40 000), a high percentage of glycosylation (45% carbohydrate content) and sialyl residues [19]. The protein was analysed by the imaged cIEF with and without urea matrix. The focused pattern of the protein without urea is shown in Fig. 10. The heterogeneity of the protein is revealed by six unresolved shoulders observed in the pattern (indicated by arrows in Fig. 10). The focused pattern is changed when 6 *M* urea is added to the sample and it is denatured. The high resolution result is shown in Fig. 11. More than six peaks are observed in the electropherograms. The analysis time for the protein is only 4 min. This speed is much faster than that of capillary zone electrophoresis method which needs 60 min [20]. Compared to Fig. 10, the p*I* values of the denatured protein in Fig. 11 are shifted, and peaks spread in a wider p*I* range.

Since the whole column imaging detector is based on the absorption of protein samples, it is a quantitative detection method. The imaged cIEF was applied to quantitation of monoclonal antibodies. The focused pattern of monoclonal anti-α_1-antitrypsin reveals six isoforms, as shown in Fig. 12. Table 1 lists percentage of each isoform in the protein measured by the peak area. The relative standard deviation (R.S.D.) is in the range from 4% for major isoforms and 13% for minor isoforms. The relationship between the area of each peak and the concentration of the sample is linear in the concentration range from 0.05 mg/ml to 1 mg/ml.

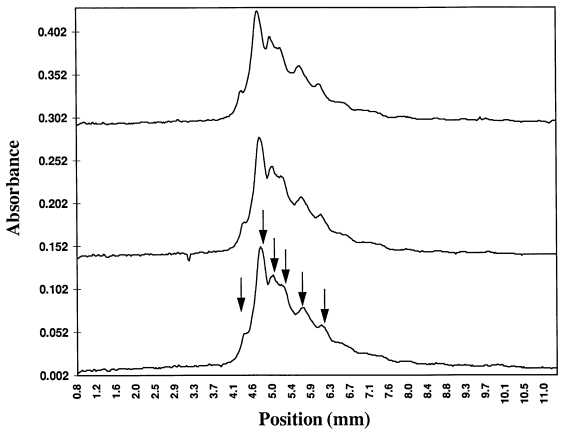

Fig. 10. IEF of human α_1-acid glycoprotein (0.5 mg/ml). Conditions: 4% pH 4–5 carrier ampholytes, 7 min focusing.

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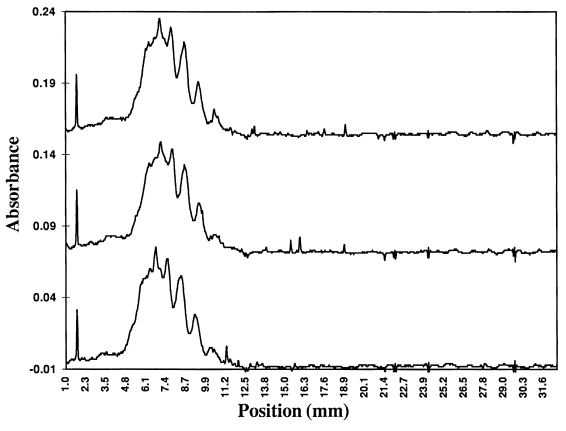

Fig. 11. IEF of human α_1-acid glycoprotein (1 mg/ml). Conditions: 4% pH 4–5 carrier ampholytes, 6 *M* urea 4 min focusing.

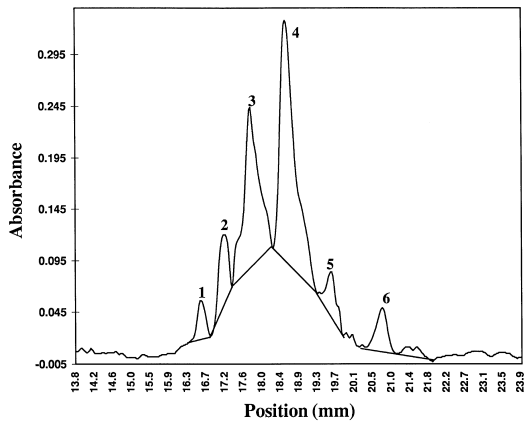

Fig. 12. Electropherogram of monoclonal anti-α_1-antitrypsin (0.75 mg/ml). It shows six isoforms of the protein. Conditions are as in Fig. 6.

autosampler. We also thank Dr. David Mao of J&W Scientific and Dr. Ming X. Huang of Supelco for free capillary coatings.

Table 1
Percentage of isoforms of monoclonal anti-α_1-antitrypsin

Peak	Area (%)	R.S.D. (%)
1	5.1	13
2	6.5	13
3	26.0	8
4	44.2	4
5	6.1	10
6	5.6	12

References

[1] S. Hjertén, M. Zhu, J. Chromatogr. 346 (1995) 265.
[2] X. Liu, Z. Sosic, I.S. Krull, J. Chromatogr. A 735 (1996) 165.
[3] T.J. Pritchett, Electrophoresis 17 (1996) 1195.
[4] T. Wehr, M. Zhu, R. Rodriguez, D. Burke, K. Duncan, Am. Biotechnol. Lab. 8 (1990) 22.
[5] J. Wu, J. Pawliszyn, Anal. Chem. 64 (1992) 224.
[6] X.-Z. Wu, J. Wu, J. Pawliszyn, Electrophoresis 16 (1995) 1474.
[7] J. Wu, J. Pawliszyn, Anal. Chem. 66 (1994) 867.
[8] J. Wu, J. Pawliszyn, Anal. Chem. 67 (1995) 2010.
[9] J. Wu, A. Watson, J. Chromatogr. B, (1998) in press.
[10] P.G. Righetti, Isoelectric Focusing – Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[11] J. Wu, J. Pawliszyn, Analyst 120 (1995) 1567.
[12] M. Zhu, R. Rodriguez, T. Wehr, J. Chromatogr. 559 (1991) 479.
[13] E. Gruhka, R.M. McCormik, J.J. Kirkland, Anal. Chem. 61 (1989) 241.
[14] M. Mikheyev, Fundamentals of Heat Transfer, Mir Publishers, Moscow, 1968.
[15] J. Mazzeo, J.A. Martineau, I.S. Krull, Anal. Biochem. 208 (1993) 323.
[16] L. Vonguyen, J. Wu, J. Pawliszyn, J. Chromatogr. B 657 (1994) 333.
[17] J. Wu, J. Pawliszyn, Am. Lab. Oct. (1994) 48.
[18] K.M. Yim, J. Chromatogr. 559 (1991) 401.
[19] S. Kishino, K. Miyazaki, J. Chromatogr. B 699 (1997) 371.
[20] R. Bonfichi, J. Chromatogr. A 741 (1996) 139.