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Extension of separation range in capillary isoelectric focusing for resolving highly basic biomolecules

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Abstract

The non-availability of commercial carrier ampholytes in the pH range greater than 11 has contributed to difficulties in focusing and resolving highly basic proteins/peptides using capillary isoelectric focusing (cIEF). Two different approaches, involving the use of *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) and ampholyte 9–11, are investigated for their effects on the extension of separation range in cIEF. The addition of TEMED into pharmalyte 3–10 not only prevents the peptides/proteins from focusing in sections of the capillary beyond the detection point, but also extends the separation range to at least isoelectric point (p*I*) 12. The combination of ampholyte 9–11 with pharmalyte 3–10 surprisingly provides baseline resolution between bradykinin (p*I* 12) and cytochrome *c* (p*I* 10.3). The sample mixture, containing bradykinin, the high-p*I* protein calibration kit (p*I* 5.2–10.3), and cytochrome *c* digest, is employed to demonstrate the cIEF separation of proteins and peptides over a wide pH range of 3.7–12.
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1. Introduction

Traditionally, proteins have been characterized by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). All the sample proteins are separated first by isoelectric point (p*I*) and then by size in a two-dimensional gel. Despite the selectivity and sensitivity of 2D-PAGE, this technique as practised today is the collection of manually intensive procedures. Furthermore, there are well-known restrictions, including the limited solubility of hydrophobic and membrane proteins and the difficulty in focusing highly basic and acidic proteins, which contribute to incomplete display of the proteome using 2D-PAGE. However, new detergents have been reported to enhance membrane solubility for 2D-PAGE protein analysis [1–3].

It should be emphasized that the extremely high resolution of 2D-PAGE for protein separation is mostly contributed by isoelectric focusing in the first separation dimension. By transferring isoelectric focusing separation from gel to capillary format, it has been demonstrated that the focusing effect in capillary isoelectric focusing (cIEF) not only contributes to a high-resolution protein separation with a p*I* difference as small as 0.005 pH units [4,5], but also permits the analysis of low abundance proteins with a typical concentration factor of 50–100 times. Additionally, the preparative capabilities of cIEF are much larger than most of the capillary-based electro-

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kinetic separation techniques since the entire capillary is initially filled with a solution containing proteins/peptides and carrier ampholytes for the creation of a pH gradient inside the capillary.

Protein mixtures of biological origin often pose tremendous analytical challenges due to their complexity, low abundance, and extremes in p*I* and other physicochemical properties. For example, the eukaryotic 80S ribosome, comprising two subunits of 40S and 60S, is one of the large macromolecular complexes in the cell. For *Saccharomyces cerevisiae*, it is calculated that a large section of the ribosomal proteins lie in the basic p*I* region (see Fig. 1). Despite the tendency of the basic proteins to migrate into the catholyte by either diffusion or gradient shift, it has been possible to some extent to perform separations up to p*I* 12 in 2D-PAGE using immobilized pH gradients [6,7]. The non-availability of commercial carrier ampholytes in the pH range greater than 11 has limited the cIEF separation of basic analytes up to a p*I* of 10.17 [8].

High-resolution cIEF separations are demonstrated in this report for analyzing peptides and proteins ranging from p*I* 3.7 to 12. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) is usually added into the carrier ampholytes to improve the resolution of basic proteins in cIEF [9] and also to prevent the basic proteins from focusing in the distal region of the capillary [10]. In our studies, the use of TEMED together with pharmalyte 3–10 enables baseline resolution between bradykinin (p*I* 12) and standard proteins from a high-p*I* calibration kit (p*I* 5.2–10.3). Instead of using TEMED, the extension of the p*I* separation range in cIEF can also be achieved by simply combining ampholyte 9–11 with pharmalyte 3–10.

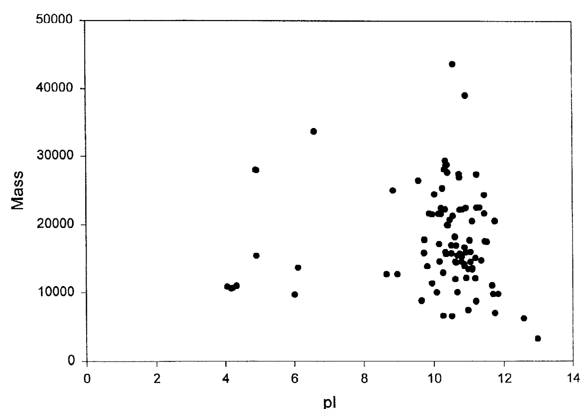

Fig. 1. Predicted relative molecular mass and isoelectric point for cytosolic ribosomal proteins of *Saccharomyces cerevisiae* (calculated from http://www.biology.wustl.edu/gcg/isoelectric.html).

2. Experimental

2.1. Materials and chemicals

Fused-silica capillaries with the dimensions of 50 μm I.D.×192 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). High-p*I* protein calibration kit (5.2–10.3) and pharmalyte 3–10 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Ampholyte 9–11, bovine pancreatic trypsin, bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg), horse heart cytochrome *c*, phosphoric acid, sodium hydroxide, and TEMED were acquired from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) and ultrapure urea were obtained from Bio-Rad (Hercules, CA, USA) and ICN (Aurora, OH, USA), respectively. All solutions were prepared using water purified by a Nanopure II system (Dubuque, IA, USA) and further filtered with a 0.22 μm membrane (Costar, Cambridge, MA, USA).

2.2. Preparation of cytochrome c digest

Horse heart cytochrome *c* was denatured in 8 *M* urea overnight. The denatured proteins were desalted using a PD-10 column (Amersham Pharmacia Biotech) equilibrated in 10 m*M* Tris at pH 8. Proteins were proteolytically digested using bovine pancreatic trypsin (10 m*M* Tris at pH 8; trypsin/protein, 1:50, w/w) at 37 °C for 20 h.

2.3. Preparation of polymer-coated capillaries

Fused-silica capillaries were coated with hydroxypropylcellulose (average M_r 100,000) (Aldrich, Milwaukee, WI, USA) for the elimination of electroosmotic flow and protein adsorption onto the capillary wall by following the procedures described previously [11,12]. Briefly, a 100-cm-long capillary

was initially filled with 5% hydroxypropylcellulose solution. The solution was then purged using nitrogen at 0.14 MPa. To immobilize the polymer layer onto the capillary inner wall, the capillary was heated from 60 to 140 °C at 5 °C/min and then at 140 °C for 20 min in a GC oven (HP 5890A, Hewlett-Packard, Avondale, PA, USA).

2.4. Capillary isoelectric focusing–UV measurements

The cIEF apparatus was constructed in-house using a CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY, USA). A 37-cm-long coated capillary (30 cm to UV detector) was rinsed with deionized water and then filled with a solution containing carrier ampholytes and peptide/protein samples. Focusing was performed at 18.5 kV constant voltage for 15 min with the use of 20 m*M* phosphoric acid and 40 m*M* sodium hydroxide as the anolyte and the catholyte, respectively.

To induce the gravity mobilization of focused analyte bands, the inlet reservoir containing the anolyte solution was raised 5 cm above the outlet reservoir. A constant voltage of 18.5 kV was again applied during the mobilization. The peptide/protein zones were monitored by a Spectra 100 UV–Vis detector (Thermo Separation Products, San Jose, CA, USA) at 280 nm. For the creation of a UV detection window, a 2-mm section of polyimide coating on the exterior surface of the capillary was removed using hot sulfuric acid.

3. Results and discussion

In cIEF, the analyte separation and concentration occur simultaneously during the focusing process. Such concentration effect may facilitate the analysis of low abundant proteins using cIEF. The electropherograms shown in Fig. 2 illustrate high-resolution separation of high-p*I* protein calibration kit (p*I* 5.2–10.3, see Table 1) in the absence and presence of bradykinin (p*I* 12). The concentration of each protein present in the sample mixture containing carrier ampholytes was only around 25 μg/ml. Together with the use of cytochrome *c* digest (see Table 2), the results clearly demonstrate the cIEF separation of proteins and peptides over a wide pH range of 3.7–12.

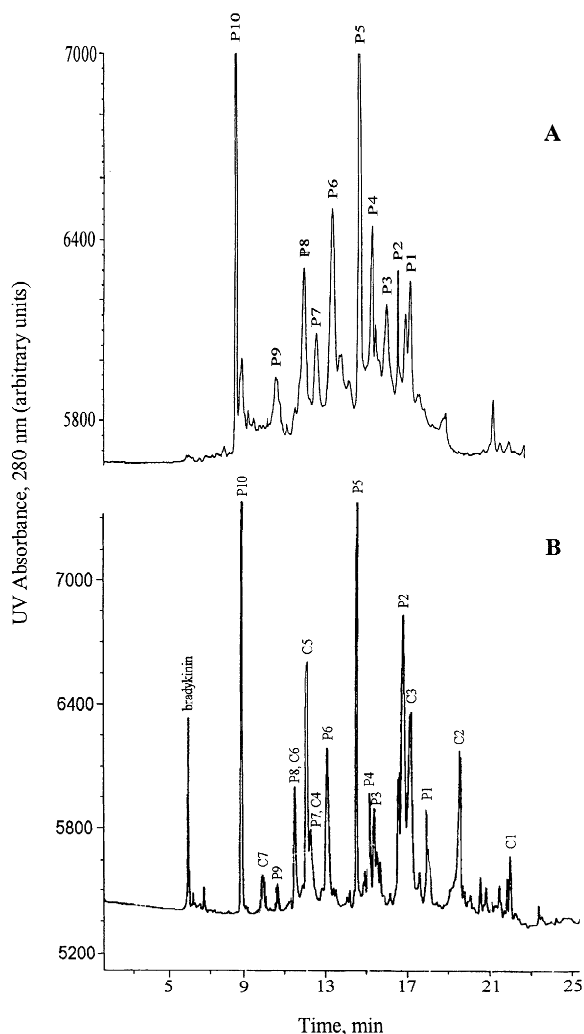

Fig. 2. Extension of cIEF separation range using TEMED. Capillary, hydroxypropylcellulose coating, 37 cm×50 μm I.D.×192 μm O.D.; sample mixtures, 1% pharmalyte 3–10, 0.43% TEMED, and (A) 25 μg/ml for each protein in high-p*I* calibration kit (see Table 1) or (B) mixture of high-p*I* protein calibration kit with 0.5 mg/ml bradykinin and 0.25 mg/ml cytochrome *c* digest (see Table 2); anolyte, 20 m*M* phosphoric acid; catholyte, 40 m*M* sodium hydroxide; electric field strength, 500 V/cm; hydrodynamic mobilization; detection, UV absorbance at 280 nm, 7 cm from cathodic end.

Table 1
List of proteins in high-p*I* calibration kit

Labeling used in Fig. 2	Protein	p*I*
P1	β-Lactoglobulin	5.20
P2	Carbonic anhydrase B (bovine)	5.85
P3	Carbonic anhydrase B (human)	6.55
P4	Myoglobin, acidic band	6.85
P5	Myoglobin, basic band	7.35
P6	Lentil lectin, acidic	8.15
P7	Lentil lectin, middle	8.45
P8	Lentil lectin, basic	8.65
P9	Trypsinogen	9.30
P10	Cytochrome *c*	10.25

3.1. Extension of separation range using TEMED

To prevent the peptides/proteins from focusing in sections of the capillary passing the detection point, TEMED, which is a highly basic organic compound, is typically employed in cIEF to block the distal region of the capillary. As a rule of thumb, the ratio of TEMED concentration (%, v/v) to ampholyte concentration should be approximately equal to the ratio of the "non-effective" capillary length to total length [10]. However, the p*I* of bradykinin is much higher than the pH range covered by pharmalyte 3–10. Thus, a solution mixture containing 0.43% TEMED and 1% pharmalyte 3–10 was used to obtain an effective separation length of 21 cm in a 37-cm-long capillary. It should be emphasized that the UV detector was placed at 7 cm from the outlet reservoir. In this study, TEMED actually blocked an additional 9 cm ahead of the detection point.

A mobilization velocity of ~1 cm/min was achieved by raising the inlet reservoir 5 cm above the outlet reservoir. The migration time of cytochrome *c* (p*I* 10.3) was around 8.8 min (see Fig. 2A) and provided evidence for the blockage of capillary section ahead of the detection point using TEMED. A protein peak width of ~0.2 min at base (Fig. 2A) corresponded to a bandwidth of ~0.2 cm at a migration speed of ~1 cm/min. This yields a sample concentration factor of ~185 in a 37-cm-long capillary column. As demonstrated by the work of Shen et al. [12], further enhancement in sample loading and concentration can be achieved by employing longer capillary columns and greater electric voltages.

The electropherogram shown in Fig. 2B demonstrates the separation of highly basic bradykinin (p*I* 12) from the high-p*I* protein calibration kit and cytochrome *c* digest. The sample mixture, containing bradykinin, the high-p*I* protein calibration kit, and cytochrome *c* digest, provides a model system which covers a wide p*I* range of 3.7–12. An excellent linear correlation ($R=0.991$) between the p*I* of peptide or protein and the migration time was obtained and is shown in Fig. 3. The presence of TEMED in pharmalyte 3–10 not only prevented the peptides/proteins from focusing in sections of the capillary beyond the detection point, but also extends the separation range to at least pH 12.

By comparing the results shown in Fig. 2A and B, there were some small changes in the migration time and the peak intensity for the proteins in the high-p*I*

Table 2
List of tryptic digest of horse heart cytochrome *c* containing aromatic amino acids

Labeling used in Fig. 2	Cytochrome *c* tryptic peptides containing aromatic amino acids	p*I*
C1	EETLME**Y**LENPK	3.67
C2	EDLIA**Y**LK	4.37
C3	TGQAPGFT**Y**TDANK	5.50
C4	MI**F**AGIK	8.50
C5	**Y**IPGTK	8.59
C5	GIT**Y**K	8.59
C6	I**F**VQK	8.75
C7	TGPNLHGL**F**GR	9.44

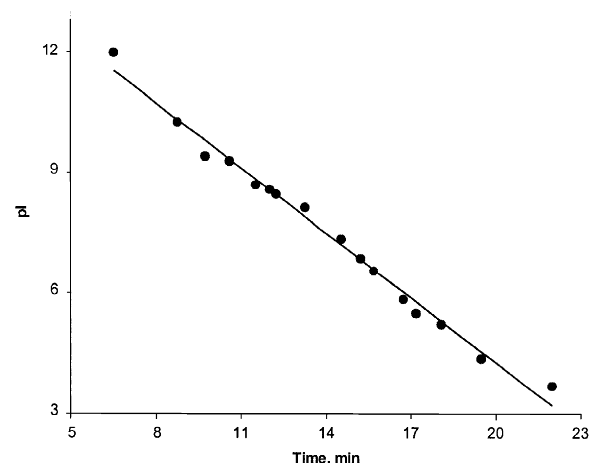

Fig. 3. Calibration curve of migration time (from Fig. 2B) versus p*I* of protein markers (from Table 1) and cytochrome *c* peptides (from Table 2).

calibration kit in the absence and presence of bradykinin and cytochrome *c* digest. As reported by Shimura et al. [8], similar variations were also observed for the cIEF separations of peptide p*I* markers in the absence and presence of test protein samples. For UV detection in cIEF, wavelengths are limited to 280 nm and above due to the background absorbance of carrier ampholytes. Thus, a typical cIEF separation of protein digest only reveals the peptides containing aromatic amino acids including phenylalanine (F), tryptophan (W) and tyrosine (Y) (see Fig. 2B and Table 2).

By assuming a Gaussian distribution for analyte concentration, the bandwidth of a focused analyte peak in cIEF can be represented by:

$$4\sigma = 4\{(D/V)[L(\mathrm{d}x/\mathrm{dpH})/(-\mathrm{d}\mu/\mathrm{dpH})]\}^{1/2}$$

where D is the diffusion coefficient of the species; V is the applied electric potential; dx/dpH is the reciprocal pH gradient; and dμ/dpH is the change of analyte mobility against solution pH [10]. Thus, one may expect limited application of cIEF for the peptide separations because the effective charge of peptides approaches zero over a broader pH range than those of most proteins. However, the results obtained in this study (see Fig. 2B) together with the work of Shen et al. [12] prove the use of cIEF for achieving at least comparable separation efficiency and resolution for peptides as those obtained for proteins.

3.2. Extension of separation range using ampholyte 9–11

Instead of using TEMED, 1% ampholyte 9–11 was mixed with 1% pharmalyte 3–10 in an effort to enhance the resolving power at or near the basic pH regions. By comparing the results shown in Figs. 2A and 4A, the use of ampholyte 9–11 not only prevented cytochrome *c* (p*I* 10.3, labeled as P10 in Figs. 2A and 4A) from focusing in sections of the capillary passing the detection point, but also significantly enhanced the separation resolution of basic proteins. No attempt was made to correlate the migration time with the p*I* of proteins from the high-p*I* calibration kit due to the anticipated nonlinear pH profile inside the capillary.

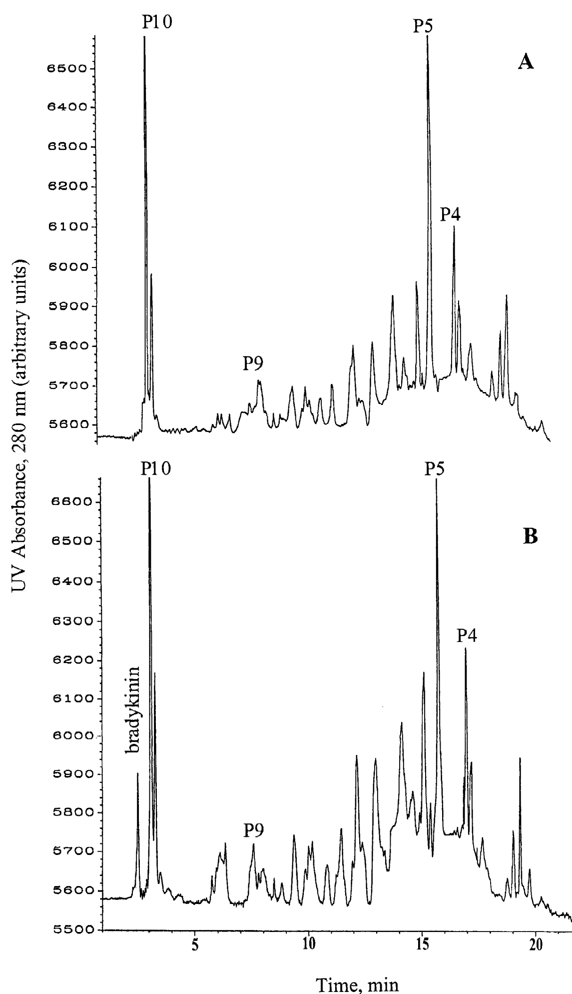

Fig. 4. Extension of cIEF separation range using ampholyte 9–11. Capillary, hydroxypropylcellulose coating, 37 cm×50 μm I.D.×192 μm O.D.; sample mixtures, 1% pharmalyte 3–10, 1% ampholyte 9–11, and (A) 25 μg/ml for each protein in high-p*I* calibration kit (see Table 1) or (B) mixture of high-p*I* protein calibration kit with 0.5 mg/ml bradykinin; anolyte, 20 m*M* phosphoric acid; catholyte, 40 m*M* sodium hydroxide; electric field strength, 500 V/cm; hydrodynamic mobilization; detection, UV absorbance at 280 nm, 7 cm from cathodic end.

To our surprise, the addition of ampholyte 9–11 further facilitated baseline resolution between bradykinin (p*I* 12) and cytochrome *c* (p*I* 10.3) as shown in Fig. 4B. In order to detect the presence of bradykinin, the peptide had to focus in the capillary section ahead of the detection point. Based on a migration time of ~2.6 min (see Fig. 4B) and a

mobilization velocity of ~1 cm/min, the focused bradykinin band was located at ~2.6 cm upstream of the UV detection window.

The peak intensity of bradykinin decreased on reducing the concentration of ampholyte 9–11 from 1% to 0.5% while maintaining constant concentration of pharmalyte 3–10 (data not shown). Complete disappearance of bradykinin peak was observed at ampholyte concentrations below 0.5%. It is not clear about the exact pH profile inside the capillary and how the combination of pharmalyte 3–10 and ampholyte 9–11 precludes the tendency of bradykinin from migrating into the catholyte by either diffusion or gradient shift.

4. Conclusion

TEMED, which is a highly basic organic compound, is typically employed in cIEF to prevent the peptides/proteins from focusing in sections of the capillary passing the detection. In this study, the use of TEMED further contributes to the extension of separation range in cIEF. High-resolution cIEF separation over a wide p*I* range of 3.7–12 is demonstrated using a sample mixture containing bradykinin, the high-p*I* protein calibration kit, and cytochrome *c* digest. Excellent linear correlation between the p*I* of peptide or protein and the migration time is obtained and can be utilized for the determination of the p*I* of other proteins/peptides.

The combination of ampholyte 9–11 with pharmalyte 3–10 clearly enhances the resolving power of cIEF at or near the basic pH regions. Additionally, the presence of ampholyte 9–11 provides unexpected separation resolution among basic biomolecules such as cytochrome *c* (p*I* 10.3) and bradykinin (p*I* 12). Further studies are needed for investigating the exact non-linear pH profile inside the capillary for precluding bradykinin from migrating into the catholyte by either diffusion or gradient shift.

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