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Journal of Chromatography A, 772 (1997) 137–144

JOURNAL OF CHROMATOGRAPHY A

Evaluation of pH gradient formation of carrier ampholytes with synthesized isoelectric point markers in capillary isoelectric focusing

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Abstract

Carrier ampholytes, peptides from protamine digested by α-chymotrypsin and two peptides, Gly–Gly–Gly and Gly–Gly–His, were labeled with dansyl chloride isoelectric point (p*I*) markers for evaluating pH formation in isoelectric focusing. These Dns-labeled ampholytes were partially purified by reversed-phase chromatography and/or preparative isoelectric focusing by rotatory cell and their p*I* values were estimated by comigration with protein-p*I* markers. These labeled markers were used to evaluate the resolution and the range of pH forming ability of carrier ampholytes.

Keywords: pH gradients; Isoelectric point markers; Ampholytes; Peptides; Proteins

1. Introduction

Capillary isoelectric focusing (cIEF) is a potent method for estimation of isoelectric points (p*I*) of proteins [1]. Proteins are separated according to their isoelectric points in a pH gradient generated by amphoteric polyamino-polycarboxylic acids (carrier ampholytes) and the resolution of p*I* on cIEF is mainly dependent on carrier ampholyte which forms the pH gradient in the capillary. The estimation of pH on isoelectric focusing (IEF) is carried out by calibration with protein p*I* markers which were measured by other methods, but the use of IEF is not convenient because the p*I* markers which are composed of proteins cause p*I* changes due to denaturation or conformational changes [3].

Recently a more stable p*I* marker was developed for p*I* estimation by using stable fluorescence-labeled peptides with fluorescence detection [4]. These markers and the modification method of proteins is a powerful, high sensitivity use for detection and estimation of p*I* of proteins. But it is slightly tedious to modify an objective protein with fluorescent dye as the protein for which p*I* is to be determined, and the ratio of modification reaction of the objective protein is not always quantitative. On the other hand, UV absorption by protein is detectable without modification of the protein itself, but has low sensitivity in detection, so it is more convenient to use p*I* markers which have UV absorption. Dansyl chloride (Dns-Cl) derivatives which absorb between 280 nm and 300 nm are able to distinguish those peaks between proteins and Dns derivatives used as p*I* markers by measuring absorption at both wavelengths [5].

IEF is capable of high resolving power, i.e.,

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PII S0021-9673(97)00101-5

proteins with a 0.1 p*I* unit difference can be routinely resolved, while 0.01 p*I* resolution is possible under certain conditions. To approach this resolution level we have to know p*K* values of the components of an ampholyte to be used in IEF. We tried to measure the components of carrier ampholytes as electrolytes by using capillary isotachophoresis (cITP) but we can not elucidate its components especially in the high pH region because of its enforced effect [6–8].

Here, we describe a method for the evaluation of a carrier ampholyte on cIEF by using ampholyte derivatives. To prepare ampholyte derivatives we employed dansylation of amino residue of ampholyte with Dns-Cl, and the modified ampholytes were partially purified on reversed-phase chromatograph and/or on rotatory isoelectric focusing (ROTOFOR) to remove salt from reaction mixture. We applied the Dns-ampholyte to evaluation of pH gradient formation on cIEF with pressure-driven mobilization.

2. Materials and methods

2.1. Materials

Gly–Gly–Gly (GGG) was purchase from Tokyo Kasei (Tokyo, Japan), Gly–Gly–His (GGH) from Protein Institute (Osaka, Japan), protamine from Tokyo Kasei, N,N,N′,N′-tetramethylenediamine (TEMED) from Wako (Tokyo, Japan), α-chymotrypsin from Sigma (St. Louis, MO USA), carrier ampholyte; Servalyte 2-11 from Serva (New York, USA), Bio-Lyte 3-10 from Bio-Rad (Richmond, CA, USA), Pharmalyte 3-10, Pharmalyte 2.5-5, Ampholine 7-9, Ampholine 9-11 from Pharmacia Biotech (Uppsala, Sweden), Ampholine 5-7 from LKB (Bromma, Sweden). eCAP™; CAP cIEF 3-10 Kit purchased from Beckman (Fullerton, CA, USA). This kit includes cIEF GEL, cIEF 3-10 Ampholyte, cIEF protein standards [ribonuclease A (p*I* 9.45), carbonic anhydrase II (p*I* 5.9), β-lactoglobulin A (p*I* 5.1), CCK flanking peptide (p*I* 2.75)] and eCAP neutral capillary. All other reagents were of analytical-grade from Wako, unless otherwise mentioned. A reversed-phase chromatographic column (ODS Sensyu Pak, 250 mm×0.8 mm I.D.) was purchased from Sensyu (Tokyo, Japan).

2.2. HPLC

A reversed-phase column was equilibrated with water at a flow-rate of 1.5 ml/min. A gradient of CH_3CN concentration from 0 to 45% was applied just after application of sample. The yellow pigments were detected by measuring the absorbance at 300 nm.

2.3. Preparative isoelectric focusing

A rotatory isoelectric focusing for preparative scaling (Preparative IEF CELL ROTOFOR; Bio-Rad) was conducted at constant power (12 W and 20°C) with 0.1 *M* phosphoric acid for Anolyte and 0.1 *M* NaOH for Catholyte. The mixture of Dns-Cl treated protamine peptide followed by digestion with α-chymotrypsin was added to 4 μl of TEMED and 30 ml of water, and rotatory isoelectric focusing was applied. The original sample mixture was pH 8.82 and conductivity 1.476 mS/cm. The focusing was operated at constant power, 7 W, and initial voltage was 40 V. After 48 h, the samples were harvested from ROTOFOR chamber when the voltage increased to 80 V. The reaction mixture of Ampholine 9-11 was treated as described above.

2.4. Preparation of dansyl derivatives

Gly–Gly–Gly or Gly–Gly–His was dissolved in 2 ml of 0.1 *M* $NaHCO_3$ at a concentration 22 m*M*. To the peptide solution, 2 ml of 22 m*M* Dns-Cl solved in CH_3CN was added at room temperature and mixture was allowed to react overnight with gently shaking. A 2 ml volume of carrier ampholyte was mixed with 0.5 ml of 0.1 *M* $NaHCO_3$ and 2 ml of 22 m*M* Dns-Cl dissolved in CH_3CN, and the mixture was allowed to react overnight with gentle shaking. Protamine, 1 g, dissolved in water was mixed with 10 ml of 0.5 *M* $NaHCO_3$ and then added to 10 mg of α-chymotrypsin. The mixture was allowed to react overnight at room temperature. To the reaction mixture, 10 ml of 22 m*M* Dns-Cl solved in CH_3CN were added and allowed to react overnight at room temperature. The reaction mixture was centrifuged at 20 000 *g* for 45 min, and the supernatant was filtered with filter paper.

2.5. Capillary isoelectric focusing

In this study we use A P/ACE 5010 (Beckman, Fullerton, CA, USA) capillary electrophoresis instrument controlled by System Gold software (Beckman) with pressure-driven mobilization [9]. Neutral hydrophilic coated capillaries of 50 μm I.D. from eCAP CAP Method Development Kit/Protein (Beckman) were used for cIEF. The total length of the capillary used in this study was 27 cm, with a separation length of 20 cm. The separations were carried out at 20°C with detection 280 nm or 300 nm. The sample solution for cIEF was composed of 2–4 μl ampholyte, 100 μl of cIEF Gel (0.4% methylcellulose) and 2–6 μl of Dns-sample solution and/or protein marker solution. The capillary was first washed with water by applying the rinse mode (20 p.s.i.; 1 p.s.i.= 6894.76 Pa) for 1 min, followed by filling with the sample solution for 1 min and water for 0.5 and then the sample solution reapplied for 2.5 min. This application step can monitor the sample flow in the capillary with absorbance at 280 nm or 300 nm. The anolyte was 91 m*M* phosphoric acid in cIEF gel. The catholyte was 20 m*M* sodium hydroxide. A voltage of 13.5 kV was applied for 2 min focusing step, while maintaining the 13.5 kV voltage, a low pressure rinse (0.5 p.s.i.) was applied to mobilize the focused protein or Dns derivative zones for detection. The mobilization step took 40–50 min. Between runs, the capillary column was rinsed with water for 1 min, 20 m*M* phosphoric acid for 2 min, water for 1 min, 35% ethanol for 1 min and water for 2 min.

3. Results and discussion

3.1. Purification of Dns derivatives and estimation of pI

The Dns derivatives of two peptides were purified by HPLC. One of them, Gly–Gly–His (GGH) derivative, was eluted from ODS column in about 30% CH_3CN and the collected fraction showed a single peak on the HPLC and also a single peak on cIEF. The molecular mass of the Dns-GGH sample was estimated as 504.6 (analyzed by matrix assisted laser desorption-ionization time-of-flight) MS (Model Vision 2000; Finigan MAT). The p*I* of this derivative was estimated as 5.24 according to the calibration curve with the protein markers.

In the case of Gly–Gly–Gly (GGG) the yellow and intense fluorescent fraction was eluted at a lower concentration of CH_3CN and showed a single peak on HPLC. Nevertheless, several peaks around p*I* of CCK flanking peptide (p*I* 2.75) on cIEF were seen. Dns-GGG is expected to migrate to a lower p*I* because the reactive amino residue has only one site on it and if the dansylation occurs at that residue, the derivative will show below and near the catholyte zone in cIEF. Although the later peptide mixture was not purified as a single material and the structure was not elucidated, the absorbance spectrum was the same type as Dns-GGH and no peak was found above p*I* 3. The main peak of this sample was estimated as p*I* 2.86 according to the calibration curve on cIEF so that this mixture was used as a Dns-GGG sample for judgment of the lowest p*I* on cIEF (Fig. 1).

The reaction mixture of protamine was diluted to half with water and was applied to preparative isoelectric focusing. The pH and conductivity profiles are shown on Fig. 2. The focusing was not enough but the fraction number around 14 were shown at relatively low conductivity and high pH. This sample was expected to contain relatively high p*I* ampholytes. The analysis of this sample with carrier ampholyte; eCAP ampholyte 3-10 on cIEF showed at least two main peaks; one around 15 min and another around 35 min on electropherogram in Fig. 3. The former one is near the p*I* of ribonuclease A (p*I* 9.45) and the latter is lower than β-lactoglobulin A (p*I* 5.1). Because the electropherograms at 280 nm and 300 nm of these two peaks almost all showed the same intensity, these peaks might be dansylated as expected. Further purification of this sample was not carried out but used as a Dns-protamine peptide sample.

The ampholytes; Pharmalyte 3-10, Pharmalyte 2.5-5, Ampholine 5-7, Ampholine 7-9, and Ampholine 9-11, were also reacted with Dns-Cl. These Dns-ampholyte samples, except Ampholine 9-11, were adsorbed on an ODS column and washed with water, then eluted at below 10% concentration of CH_3CN from the ODS column on HPLC to eliminate salts. The Ampholine 9-11 reaction mixture was applied to

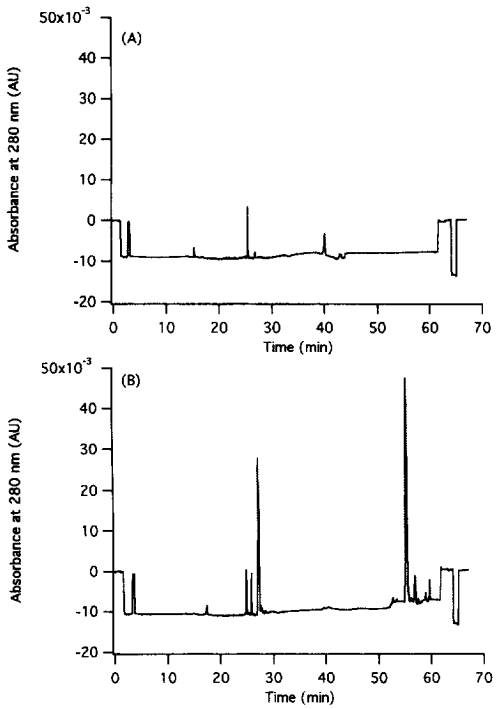

Fig. 1. A cIEF electropherogram of Dns-Gly–Gly–His, Dns-Gly–Gly–Gly and protein markers with simultaneous pressure and voltage mobilization is shown of all processes. These are the pre-rinse step, the sample injection, the focusing and mobilization steps, and the post-rinse steps. One microliter of Dns-Gly–Gly–His and 1 μl of Dns-Gly–Gly–Gly were mixed with 1 μl of 3.5% TEMED, with (A) 2 μl of the mixture of protein markers (ribonuclease A, carbonic anhydrase, β-lactoglobulin A, and CCK Flanking Peptide) and (B) 2 μl of water and 100 μl of cIEF GEL, and was injected for 2.5 min using 20 p.s.i. pressure after pre-rinse of the capillary with water for 2.5 min using the same pressure. The electrophoresis voltage, 13.5 kV, was applied at 5 min followed by 2 min of focusing step and the duration of mobilization step was 54 min. The field strengths of the focusing and mobilization steps were 500 V/cm.

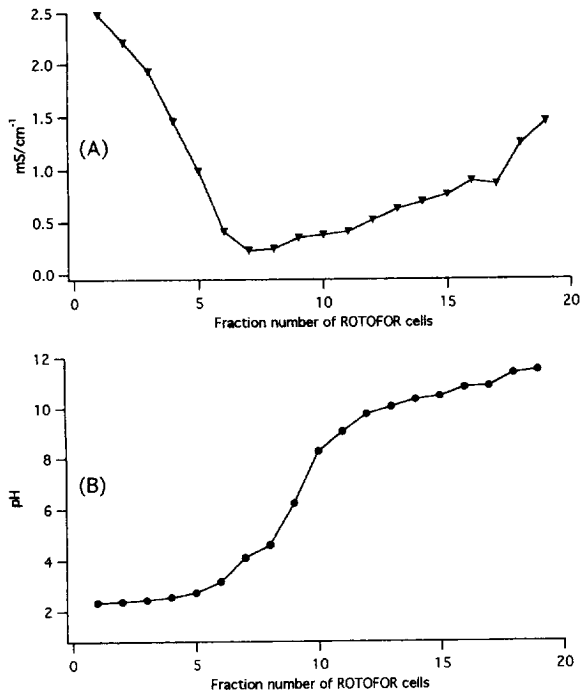

Fig. 2. (A) conductivity and (B) pH profiles of Dns-protamine peptide sample on a rotatory isoelectric focusing, ROTOFOR cell. Dns-protamine peptide followed by digestion with α-chymotrypsin was added with 4 μl of TEMED and 30 ml of water and was applied to a rotatory isoelectric focusing. The original sample mixture was pH 8.82 and conductivity was 1.476 mS/cm. The focusing was operated according to constant current 15 mA and initial voltage was 40 V. After 48 h, the samples were fractionated from ROTOFOR chamber when the voltage increased to 80 V.

preparative isoelectric focusing. Separation was not good enough by monitoring of the pH, but the conductivity of the fractions 3–18 decreased to hundreds μS/cm (data not shown). The cIEF of this sample with eCAP ampholyte 3-10 showed that this sample contained ampholytes above p*I* 6.0 and no peaks were found below p*I* 6.0. (Fig. 4). This result means that the residues of the basic residue were not all coupled with Dns-Cl but there seems to be dissociation of some unreacted residue, *sec.*- or *tert.*-amines, resulting in the formation of a higher p*I* ampholytes mixture. The other dansylated ampholytes were also analyzed on cIEF and mixed with each other in an appropriate ratio of ampholyte mixtures to use as a Dns-ampholyte sample for estimating pH forming. All dansylated samples were freeze-dried and resolved in water then used as the samples on cIEF.

3.2. Capillary IEF of Dns derivatives in carrier ampholyte

Commercially available carrier ampholytes are not the same pH and conductivity as electrolytes shown in Table 1. We have already described the analysis of the electrolytes by cITP by using the dPG method and from these results there were the different kinds and amounts of electrolyte between the ampholytes

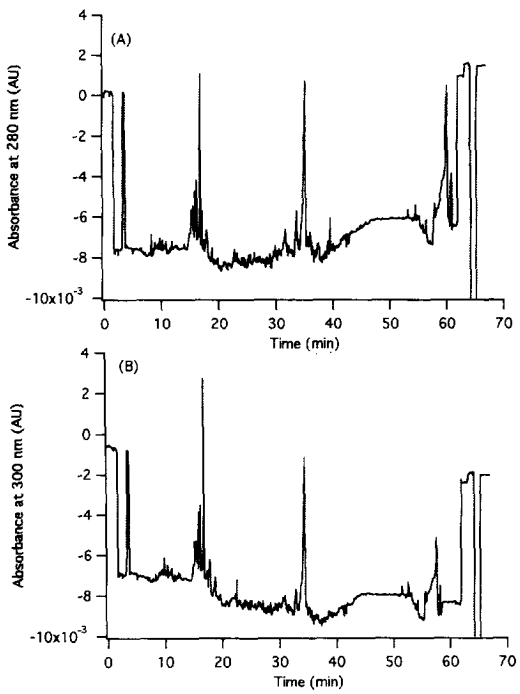

Fig. 3. A cIEF electropherogram (A) at 280 nm and (B) at 300 nm of Dns-protamine peptides with simultaneous pressure and voltage mobilization is shown from the pre-rinse step, the sample injection, the focusing and mobilization steps, and post-rinse steps. Two microliters of the sample was mixed with 2 μl of cIEF Ampholyte 3-10 (Beckman kit), 1 μl of 3.5% TEMED and 100 μl of cIEF Gel and was injected for 2.5 min using 20 p.s.i. pressure after pre-rinse of the capillary with water for 2.5 min using same pressure. The electrophoresis voltage, 13.5 kV, was applied at 5 min followed by 2 min of focusing step and the duration of mobilization step was 54 min. The field strengths of the focusing step and mobilization step were 500 V/cm.

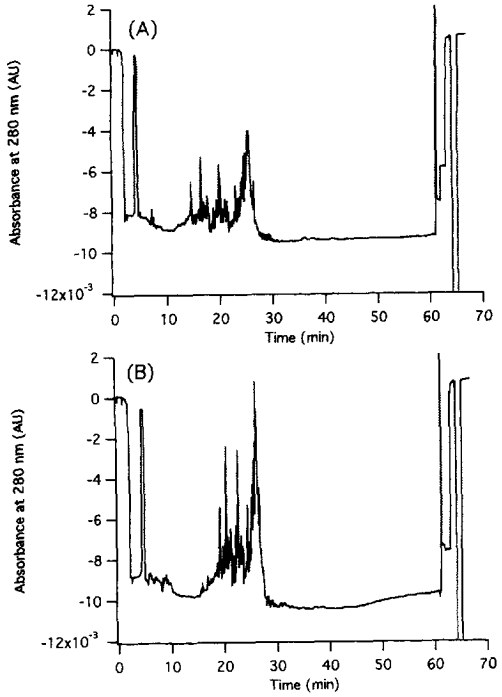

Fig. 4. A cIEF electropherogram of Dns-ampholyte mixture with simultaneous pressure and voltage mobilization is shown of the entire process, these are the pre-rinse step, the sample injection, the focusing and mobilization steps, and post-rinse steps. Two microliters of the sample were mixed with 2 μl of cIEF Ampholyte 3-10 (Beckman kit), (A) 1 μl of 3.5% TEMED or (B) 1 μl of water and 100 μl of cIEF Gel, and was injected for 2.5 min using 20 p.s.i. pressure after pre-rinse of capillary with water for 2.5 min using same pressure. The electrophoresis voltage, 13.5 kV, was applied at 5 min followed by 2 min of focusing step and the duration of the mobilization step was 54 min. The field strengths of the focusing step and mobilization step were 500 V/cm.

[6–8]. We tried to compare the pH forming range and capability of high resolution between the ampholytes on cIEF by using the Dns derivatives and TEMED which extend the pH range of IEF [10,2,11].

One microliter of Dns-protamine peptide sample was mixed with 1 μl of 3.5% TEMED, 2 μl of 3.5% TEMED and 2 μl of (A) eCAP cIEF 3-10, (B) Pharmalyte 3-10 (C) Servalyte 2-11, or (D) Bio-Lyte 3-10 and 100 μl of cIEF GEL and analyzed on cIEF (Fig. 5). The basic Dns-derivative around 20 min and the weak acidic Dns derivative around 30 min were focused respectively in the electropherogram by using each ampholyte. The times from peak to peak between the basic and weak acidic derivative were 14 min for (A), 12 min for (B), 18 min (c), and 21 min for (D), respectively. These net migration times

Table 1
Conductivity and pH of some of commercially available carrier ampholytes

Ampholyte	Manufacturer	pH range	ms/cm	pH
Pharmalyte	Pharmacia	3–10	1.002	6.68
Servalyte	Serva	2–11	0.240	7.23
Bio-Lyte	Bio-Rad	3–10	0.193	7.13
cIEF Kit[a]	Beckman	3–10	2.480	8.76

[a] cIEF Kit of Beckman contains TEMED. All ampholyte samples were measured in one tenth dilution with water at room temperature.

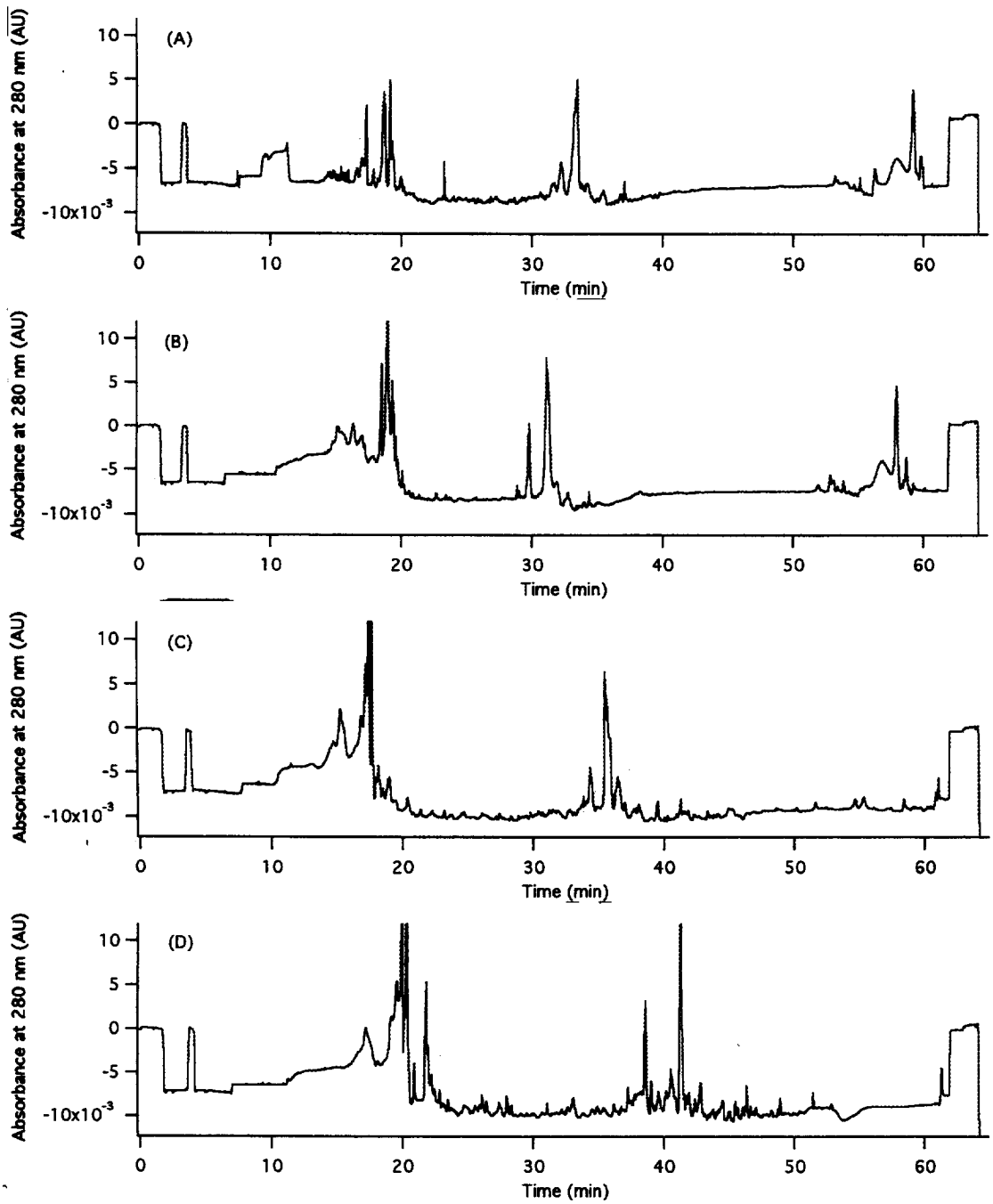

Fig. 5. A cIEF electropherogram of Dns-protamine peptide sample with simultaneous pressure and voltage mobilization. One microliter of DNS-protamine peptide sample was mixed with 2 μl of 3.5% TEMED and 2 μl of (A) cIEF 3-10 Ampholyte, (B) Pharmalyte, (C) Servalyte, or (D) Bio-Lyte and 100 μl of cIEF Gel, and was injected for 2.5 min using 20 p.s.i. pressure after pre-rinse of capillary with water for 2.5 min using the same pressure. The electrophoresis voltage, 13.5 kV, was applied at 5 min followed by 2 min of focusing step and the duration of the mobilization step was 54 min. The field strengths of the focusing step and mobilization step were 500 V/cm.

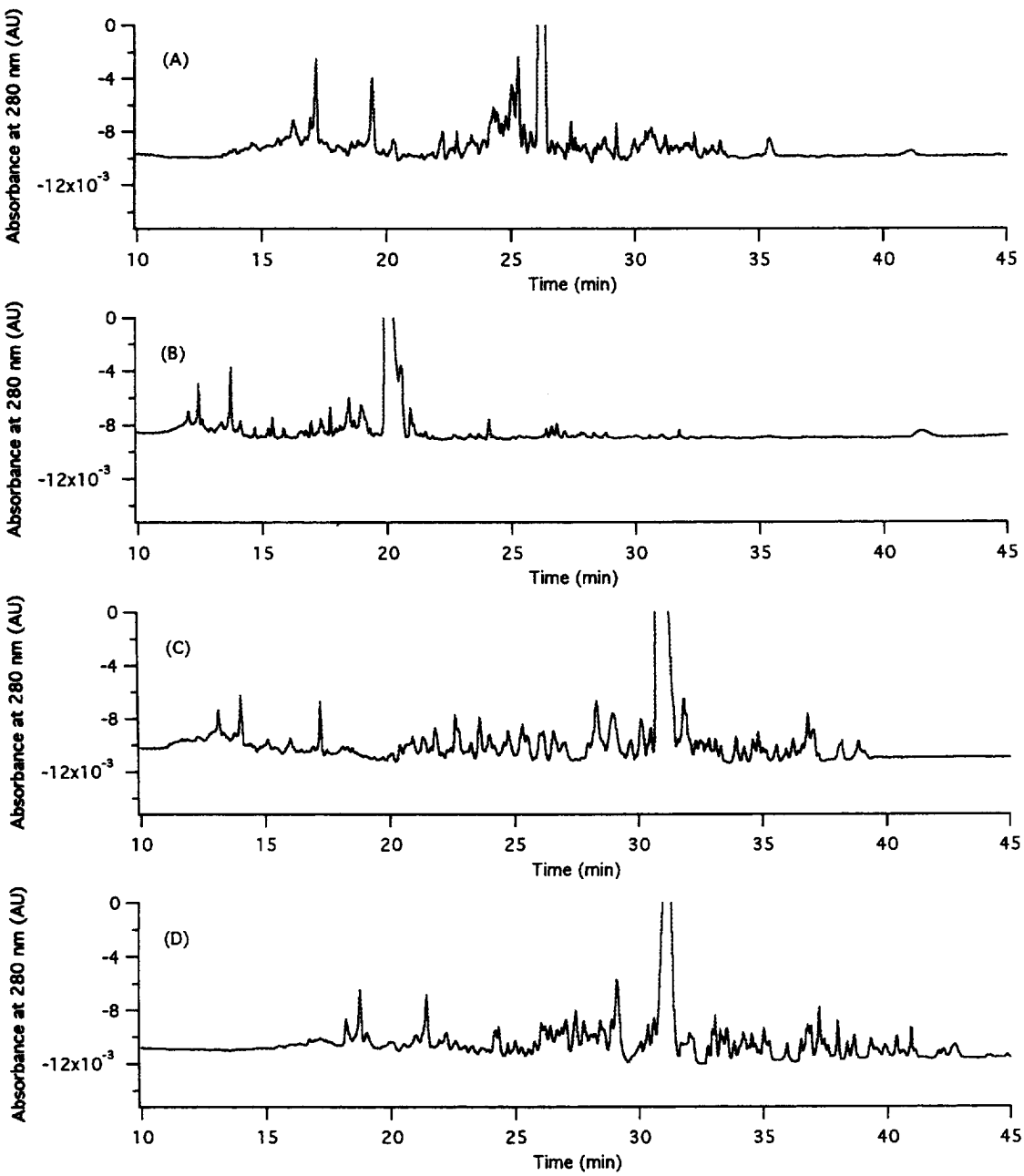

Fig. 6. A cIEF electropherogram of Dns-protamine peptide sample and Dns-ampholyte sample with simultaneous pressure and voltage mobilization. Four microliters of Dns-protamine peptide sample were mixed with 2 μl of 3.5% TEMED and (A) cIEF 3-10 Ampholyte, (B) Pharmalyte, (C) Servalyte or (D) Bio-Lyte and 100 μl of cIEF Gel, and was injected for 2.5 min using 20 p.s.i. pressure after pre-rinse of capillary with water for 2.5 min using the same pressure. Following this injection the Dns-Gly–Gly–His sample was injected for 0.17 min using the same pressure. The electrophoresis voltage, 13.5 kV, was applied at 5 min followed by 2 min of focusing step and the duration of mobilization step was 54 min. The field strengths of the focusing step and mobilization step were 500 V/cm.

correspond to pH forming ability between those two peaks. It can be considered that the pH forming ability is not dependent on electrolyte components, or ampholyte components, which were listed in Table 1. Concerning satellite peaks near the weak acidic peak, the distance from the main peak and peak half width were different profiles from each other. Another analysis of the ampholytes by using the Dns-ampholyte sample and Dns-Gly–Gly–His revealed more and larger differences among the ampholytes (Fig. 6). The excess amount of Dns-Gly–Gly–His divided into two parts in the acidic and basic regions with the wide half width peaks in the center of the electropherograms. The satellite peaks around the Dns-Gly–Gly–His were those of the Dns-ampholyte and the numbers of those peaks might be reflected in the resolution depending on the ampholytes on cIEF.

4. Concluding remarks

The Dns derivatives of peptides, protamine α-chymotrypsin digested peptide, and carrier ampholytes can elucidate a part of the ability of pH forming on cIEF. It is known that IEF is capable of high resolving power, i.e., proteins with 0.1 p*I* unit difference can be routinely resolved, whereas a 0.01 p*I* resolution is possible under certain conditions. How can a reliable estimation of p*I* be made? To know more details of ampholyte we need wide-range p*I* markers to ensure the measurement of p*I* on cIEF. The modifier of amino acid residue decreases the p*I* of the amphoteric compound itself so that it is not easy to produce the high p*I* marker. We tried to make a high p*I* marker by using protamine hydrolyzate and partially succeeded with Dns-protamine peptide, but unfortunately the purification was not completed. Nevertheless the peptides from protamine will become suitable p*I* markers for the high p*I* range.

In this study we demonstrate the limitation of detection of a small number of compounds because commercially available ampholytes are not clear in the UV region. Shimura has also pointed out in his paper [4] this problem of low *S/N* in UV absorbance. The development of a p*I* marker will promote the manufacture of good ampholytes for cIEF at high resolution and good *S/N* of UV absorption.

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