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Improved capillary zone electrophoretic separation of basic proteins in uncoated fused-silica capillary by using ethylene diamine as a buffer additive

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ABSTRACT

Ethylene diamine was used as a buffer additive to improve capillary zone electrophoretic separation of basic proteins in an uncoated fused-silica capillary and theoretical plate numbers of the order of a hundred thousand were obtained for all five standard proteins at pH 6.5, 8.0 and 9.5. The critical factors that affected the separation efficiency were the ethylene diamine concentration and the applied voltage at each pH value. Adsorption of ethylene diamine ions to the capillary inner wall significantly reduced the charge density of the wall, and therefore reduced the adsorption of basic proteins. Increasing the concentration of ethylene diamine increased this reduction, and caused an increase in the theoretical plate numbers of all proteins. Increasing the applied voltage caused an increase in the theoretical plate numbers of all proteins in the low-voltage region and a decrease in high-voltage region, which maybe attributed to excessive Joule heat.

INTRODUCTION

Since Jorgenson and Lukacs [1,2] established the theoretical basis of capillary zone electrophoresis (CZE) in the early 1980s, analysts have made great efforts to make it a routine method of separating macromolecules such as polypeptides, proteins and DNA bases. In theory, because these macromolecules have very low diffusion coefficients, separation efficiency can reach theoretical plate numbers of the order of millions [1,3,4]. However, adsorption of the macromolecules onto the capillary inner wall causes serious problems, including tailing peaks, resulting in low efficiency and low resolution. Sometimes it can even result in the absence of signal response after an injection [5]. Adsorption also continuously changes the property of the capillary inner wall, resulting in continuous change in the electroosmotic flow and therefore non-reproducible elution time [6].

The reason for adsorption is the opposite charge of the capillary inner wall and basic proteins. Fused silica always carries a negative charge in aqueous solution ($pI < 2$), while basic proteins carry a significant net positive charge when operating in the typical pH range used for CZE, resulting in strong coulombic attraction. Three main techniques have been designed to prevent the capillary wall–protein attraction: (1) by directly controlling charge density on the capillary inner wall through an additional electric field applied from the outside of the capillary; (2) by changing the composition of the carrier electrolyte; and (3) by modifying the capillary inner wall. Though all these techniques succeed in reducing adsorption to some extent, we still think that changing the composition of the carrier electrolyte is a simpler and more acceptable technique; it can also be combined with other techniques for even greater success.

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In the technique of changing the composition of carrier electrolyte, Lauer and McManigill [3] raised the pH of the carrier electrolyte above the p*I* of basic proteins, so that coulombic repulsion suppressed adsorption. However, pH 11.0 solutions could decompose basic proteins. McCormick [7] demonstrated successful separation of peptides and proteins at low pH values at which the charge on the capillary inner wall approaches zero, but protonization of proteins caused low mass to charge ratios and prohibited high-resolution separation. Green and Jorgenson [8] added a high concentration of alkali metal salts to the carrier electrolyte to suppress the adsorption, but although they were successful Joule heat remained a problem. Bushey and Jorgenson [9] partly solved this problem by using a combination of a high concentration of zwitterionic buffer and alkali metal salts. Recently, Chen *et al.* [10] also demonstrated the value of using a high concentration of alkali metal salts in suppressing adsorption of basic proteins. Waters Chromatography Division of Millipore produced a zwitterionic modifier, AccuPure Z1-Methyl (a registered trademark), and Chen *et al.* [10] used a proprietary zwitterionic additive ZB-4; both improved the separation of basic proteins. Emmer *et al.* [11] improved capillary zone electrophoretic separation of basic proteins by using a fluorosurfactant buffer additive, FC134.

In this report, the use of ethylene diamine as a buffer additive is proposed for the first time, and high separation efficiency of basic proteins was obtained. Although amines such as spermine [12], morpholine [13], 1,3-diaminopropane [14], 1,4-diaminobutane [3,15] and 1,5-diaminopentane [16] have been used as buffer additives, we have found that a series of relatively simple buffers in uncoated fused-silica capillaries at pH values below the isoelectric points of basic proteins can give excellent separation. We also found that electroosmotic mobility varies with the concentration of ethylene diamine and the applied voltage, and that there is a linear relationship between electroosmotic flow and current. Since ethylene diamine is a common reagent in the laboratory, it is a more acceptable additive than some proprietary ones.

EXPERIMENTAL

Apparatus

The capillary electrophoresis apparatus used in this study resembles that reported earlier by Jorgenson and Lukacs [1,2]. It was constructed by the Peking Institute of New Technology Application (Peking, China). It consists of a high-voltage d.c. power supply delivering up to ±30 kV, a UV detector which has several optional wavelengths with fixed and removable devices for on-column detection, a Plexiglass box with a safety interlock and a syringe installation used to flush the capillary. The electrophoregrams were recorded with an HP3390A integrator (Hewlett-Packard, Avondale, PA, USA).

Reagents and materials

Lysozyme from egg white (p*I* 11.0) was purchased from Fluka Buchs, Switzerland). Ribonuclease from bovine pancreas (p*I* 9.45) was purchased from Merck (Darmstadt, Germany). Cytochrome *c* (p*I* 10.5) from horse heart, trypsinogen from bovine pancreas (p*I* 9.3) and α-chymotrypsinogen A from bovine pancreas (p*I* 9.1) were purchased from Sigma (St. Louis, MO, USA). Ethylene diamine anhydrous, sodium sulphate anhydrous, dimethyl sulphoxide and all other reagents were analytical grade and purchased in China. In all experiments, deionized water was used.

Stock protein solutions with individual concentrations of about 2.0 mg/ml were prepared and stored in a freezer. Sometimes 1% dimethyl sulphoxide (DMSO) was added to protein solutions as a neutral marker. Carrier electrolytes were prepared using the corresponding amounts of anhydrous ethylene diamine and anhydrous sodium sulphate, and adjusting the pH with phosphoric acid. Sample injection was accomplished by siphoning for 10 s at 8 cm.

Electrophoresis

A polyimide-coated fused-silica capillary (Yongnian Photoconductive Fibre Factory, Hebei, China) of 50 μm I.D. and 375 μm O.D. was used with a total length 75 cm, in which the detector was placed 55 cm from the capillary

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inlet. Detection was monitored at 214 nm for all proteins and DMSO. Before using, a new capillary was flushed with 1 *M* KOH for 1 h and then equilibrated with carrier electrolyte overnight by using a syringe to force the solution through it. When a change of carrier electrolyte was needed, the same procedure was followed. Between two runs, the capillary was first flushed with one capillary volume of 1 *M* KOH, then with carrier electrolyte for 5 min. All analyses were run at ambient temperature between 16 and 18°C without temperature control.

RESULTS AND DISCUSSION

Effect of pH value and ethylene diamine concentration on the separation of basic proteins

When ethylene diamine was added to the carrier electrolytes, excellent separation of basic proteins could be obtained. Three pH values, 6.5, 8.0 and 9.5, were arbitrarily chosen to examine the effect of ethylene diamine, and at each pH value four concentrations of ethylene diamine between 20 m*M* and 80 m*M* were selected. In all experiments, 35 m*M* sodium sulphate was added to every carrier electrolyte, aiding in increasing the overall ionic strength and reducing attractions between proteins and capillary inner wall. The applied voltage was 12 kV for each run. Separations of five standard basic proteins at pH 8.0 are demonstrated in Fig. 1.

The results showed that raising the concentration of ethylene diamine could effectively reduce attraction between proteins and the capillary inner wall and improve the separation of basic proteins. When the concentration of ethylene diamine was low, noticeable peak tailing and low resolution were observed. But when the concentration of ethylene diamine was high enough, several unresolved peaks were baseline separated. Ribonuclease, trypsinogen and α-chymotrypsinogen A had very similar electrophoretic mobility, but at any pH value three peaks could be obtained with enough ethylene diamine in the carrier electrolytes. Impurity in lysozyme could also be successfully separated from lysozyme at pH 6.5 and pH 8.0 with enough ethylene diamine. Raising the concentration of ethylene diamine was not always beneficial; too high a concentration can result in low electoosmotic flow and long elution times.

Theoretical plate numbers calculated for individual proteins at different pH values and different concentrations of ethylene diamine are summarized in Table I. It is observed that theoretical plate numbers higher than a hundred thousand were obtained for all proteins, of which the highest was 740 000 for α-chymotrypsinogen A at pH 8.0/60 m*M* ethylene diamine. Because retention time increased with increasing concentration of ethylene diamine, the theoretical plate number of the neutral marker DMSO decreased in accordance with the theory of Jorgenson and Lukacs [1,2], expressed as follows:

$$N = \frac{(\mu_e + \mu_{osm})Vl}{2DL} \quad (1)$$

where N is the theoretical plate number, μ_e is the electrophoretic mobility of the analyte, μ_{osm} is the electroosmotic mobility, V is the applied voltage, l is the distance between the capillary inlet and the detector window, D is the diffusion coefficient of the analyte and L is the total length of the capillary. But, in contrast, the theoretical plate numbers of all proteins showed an obvious tendency to increase with increasing concentration of ethylene diamine, although the theoretical plate numbers of some proteins decrease at too high a concentration of ethylene diamine.

Raising the pH from 6.5 to 8.0 to 9.5 speeded up electroosmotic flow and caused short elution times for all proteins, but low resolution. Impurity in lysozyme could not be separated from lysozyme at pH 9.5. Changing the pH also caused changes in the elution sequence, especially for DMSO. At pH 6.5, its peak was far behind all the proteins; at pH 8.0, its peak overlapped with the three near peaks of ribonuclease, trypsinogen and α-chymotrypsinogen A, except that at up to 80 m*M* ethylene diamine it was separated from them; at pH 9.5, its peak was ahead of the three peaks, which indicated that ribonuclease, trypsinogen and α-chymotrypsinogen A were negatively charged.

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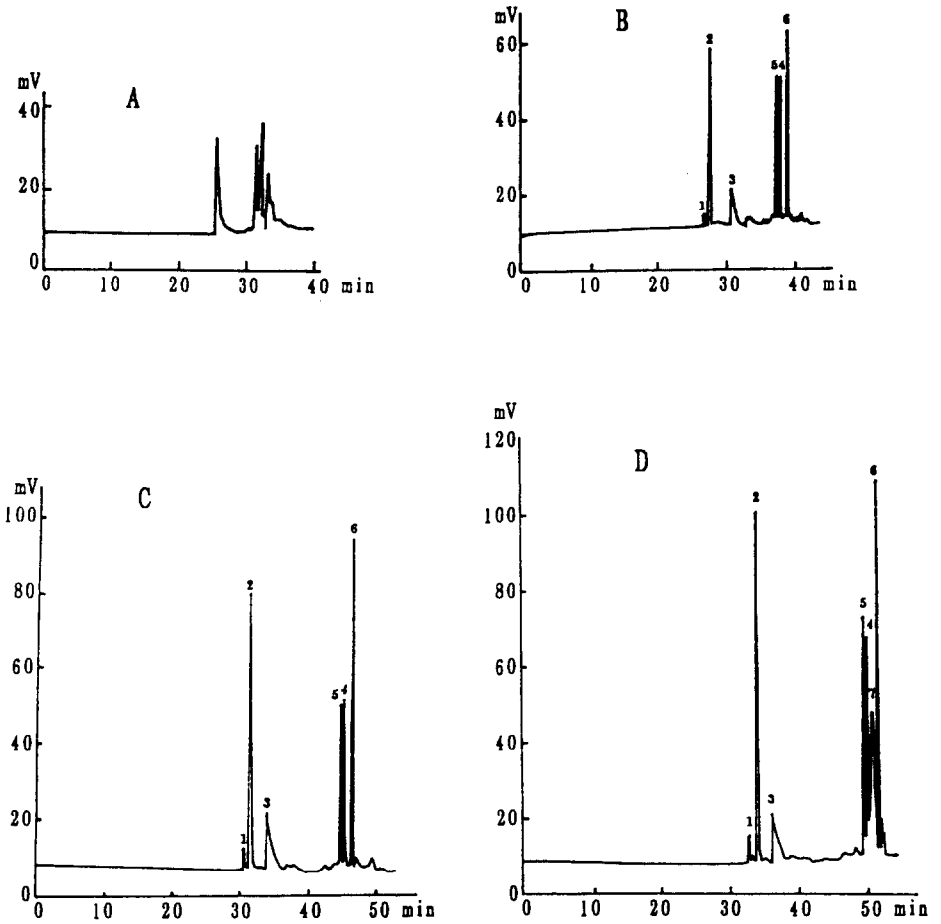

Fig. 1. Effect of ethylene diamine concentration on the separation of basic proteins at pH 8.0. Peaks: 1 = impurity of lysozyme; 2 = lysozyme; 3 = cytochrome *c*; 4 = ribonuclease; 5 = trypsinogen; 6 = α-chymotrypsinogen A; 7 = DMSO. Conditions: (A) 20 m*M* ethylene diamine, 27 μA; (B) 40 m*M* ethylene diamine, 31 μA; (C) 60 m*M* ethylene diamine, 34 μA; (D) 80 m*M* ethylene diamine, 38 μA.

Effect of applied voltage on the separation of basic proteins

Another factor which significantly affected the separation efficiency and separation resolution was the applied voltage. Three buffers, pH 6.5/60 m*M* ethylene diamine, pH 8.0/60 m*M* ethylene diamine and pH 9.5/80 m*M* ethylene diamine, were chosen to examine this effect. The results are demonstrated in Figs. 2 (pH 6.5) and 3 (pH 9.5). As was anticipated, increasing the applied voltage resulted in short elution times and low resolution at pH 6.5 and pH 8.0. In contrast, at pH 9.5 short elution times but high resolution of ribonuclease, trypsinogen and α-chymotrypsinogen A were obtained, which maybe attributed to the negative charge on the three proteins at pH 9.5. Theoretical plate numbers calculated for individual proteins at different pH values and different applied voltages are summarized in Table II. It is observed that the theoretical plate number of DMSO and all proteins showed a tendency to increase with increasing applied voltage in the low-voltage region, which conformed to the theory of Jorgenson and Lukacs [1,2] as expressed in eqn. 1. It is also observed that their efficiency decreased in the high-voltage region.

It is shown above that as a buffer additive

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TABLE I

THEORETICAL PLATE NUMBER FOR BASIC PROTEINS AT DIFFERENT pH VALUES AND DIFFERENT CONCENTRATIONS OF ETHYLENE DIAMINE

Operation pH	Concentration of ethylene diamine (m*M*)	Current (μA)	Theoretical plate numbers · 10^{-4} [a]						
			Lysozyme[b]	Lysozyme	Cytochrome	Ribonuclease	Trypsinogen	Chymotrypsinogen	DMSO
6.5	20	27	[c]	1.1	[c]	[c]	6.8	[c]	5.1
	40	31	12.0	7.0	2.0	26.6	24.7	23.7	4.4
	60	34	17.8	9.1	2.4	30.1	35.5	30.7	3.6
	80	38	35.6	20.4	12.4	29.8	30.1	24.6	3.1
8.0	20	27	[c]	[c]	[c]	[c]	[c]	[c]	[d]
	40	31	17.1	11.0	1.2	30.9	35.3	51.5	[d]
	60	34	22.6	13.2	1.5	20.1	20.4	74.0	[d]
	80	38	31.8	23.5	7.5	25.3	34.2	43.2	[d]
9.5	30	30	[c]	4.8	3.5	5.1	[c]	[c]	8.1
	50	32	[c]	10.7	13.8	6.3	[c]	[c]	8.0
	60	34	[c]	12.1	14.9	12.2	[c]	[c]	7.8
	80	38	[c]	18.8	4.2	25.7	24.6	23.8	7.1

[a] $N = 5.54(RT/WI)^2$, where RT is the retention time and WI is the peak width at half-height.
[b] Impurity in lysozyme.
[c] Unresolved peaks.
[d] Did not measure theoretical plate numbers.

ethylene diamine is suitable over a wide pH range to improve the capillary zone electrophoretic separation of basic proteins. The critical factors that affect the separation efficiency and separation resolution are its concentration, the pH of the carrier electrolyte and the applied voltage. Because the pH values were arbitrarily chosen, it is anticipated that intermediate pH values between 6.5 and 9.5 can also be successfully used. pH values below 6.5 were also tried, but were unsuccessful. At pH 5.0 (adjusting pH with acetic acid), although four peaks were detected for five proteins, the separation efficiencies were low and elution times were very long. At pH 3.5, no signal was observed at the detector for a long time, though the electrode polarity was ever exchanged. The separations depicted in this preliminary work were all obtained on mixtures of proteins standards; no separation of complex matrices such as biological fluids was accomplished. But it is anticipated that, by choosing an appropriate pH value of the carrier electrolyte, concentration of ethylene diamine and applied voltage, an efficient and rapid separation of complex matrices will be obtained.

Reasons for the effect on separation efficiency of proteins

In order to study the effect on separation efficiency of proteins, the electroosmotic mobilities of the capillary at different pH values and different concentrations of ethylene diamine as well as at different pH values and different applied voltages were measured and are illustrated in Figs. 4 and 5.

It is observed from Fig. 4 that the electroosmotic mobilites of the capillary with ethylene diamine buffer at each pH value were much lower than those with metal salts buffer described in ref. 5. Unlike metal ions, positively charged ethylene diamine ions adsorbed tightly to the capillary wall, dramatically reducing the charge density of the wall and causing a dramatic reduction in electroosmotic mobility and adsorption of basic proteins to the capillary inner wall. Increasing the concentration of ethylene diamine enhanced these effects, and hence caused a dynamic decrease in electroosmotic mobility but an increase in the separation efficiency of all basic proteins.

It is not thought that increasing the applied voltage caused the increase in electroosmotic

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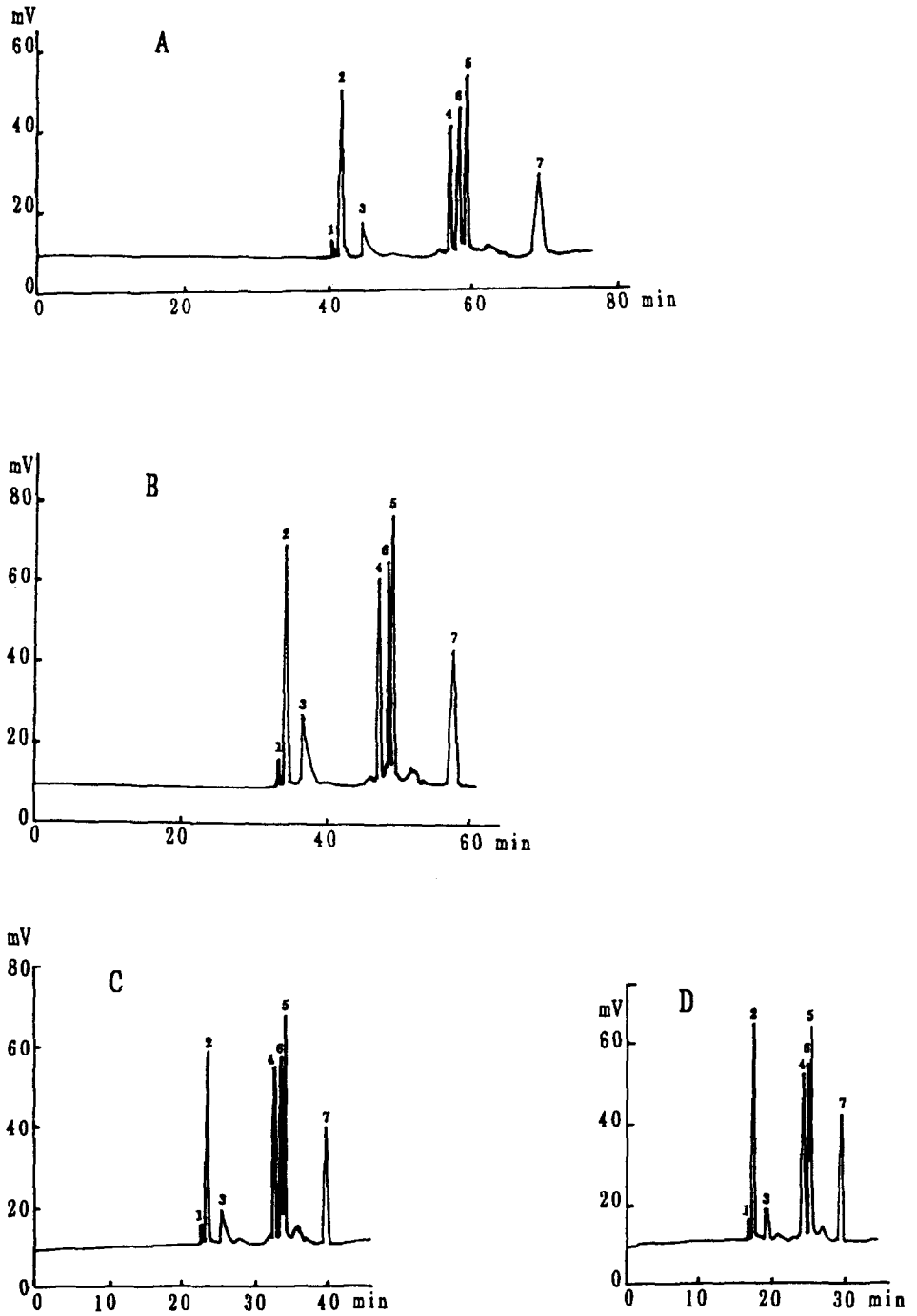

Fig. 2. Effect of applied voltage on the separation of basic proteins at pH 6.5. Peaks: 1 = impurity of lysozyme; 2 = lysozyme; 3 = cytochrome *c*; 4 = ribonuclease; 5 = trypsinogen; 6 = α-chymotrypsinogen A; 7 = DMSO. Conditions: (A) 9 kV, 27 μA; (B) 12 kV, 34 μA; (C) 15 kV, 50 μA; (D) 18 kV, 64 μA.

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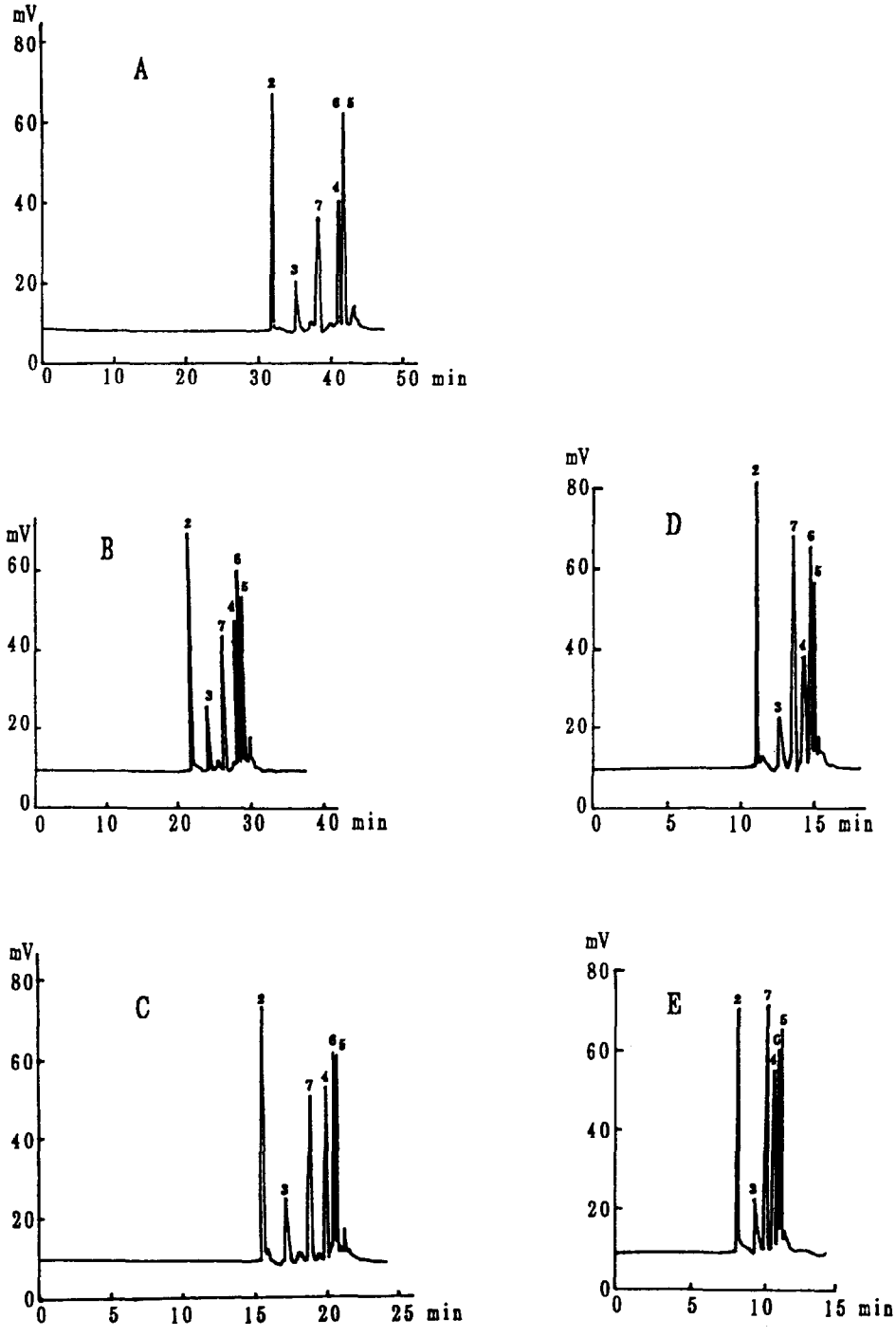

Fig. 3. Effect of applied voltage on the separation of basic proteins at pH 9.5. Peaks: 2 = lysozyme; 3 = cytochrome *c*; 4 = ribonuclease; 5 = trypsinogen; 6 = α-chymotrypsinogen A; 7 = DMSO. Conditions: (A) 9 kV, 25 μA; (B) 12 kV, 37 μA; (C) 15 kV, 52.5 μA; (D) 18 kV, 71.5 μA; (E) 21 kV, 94 μA.

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TABLE II

THEORETICAL PLATE NUMBER FOR BASIC PROTEINS AT DIFFERENT pH VALUES AND DIFFERENT APPLIED VOLTAGES

Operation pH	Applied voltage (kV)	Current (μA)	Theoretical plate numbers · 10^{-4} [a]						
			Lysozyme[b]	Lysozyme	Cytochrome	Ribonuclease	Trypsinogen	Chymotrypsinogen	DMSO
6.5	9	27	14.4	11.6	1.6	26.5	24.6	23.2	2.8
	12	34	17.8	9.1	2.4	30.1	35.4	30.7	3.6
	15	50	12.0	10.1	5.4	25.9	21.5	20.3	4.9
	18	64	15.6	8.2	1.7	11.3	[c]	[c]	4.9
	21	87–91	[c]	7.3	1.2	12.4	[c]	[c]	[c]
8.0	9	23	15.0	8.3	0.7	5.8	4.4	61.7	[d]
	10.5	29	28.4	14.0	0.9	29.5	32.0	70.6	[d]
	12	34	22.6	13.2	1.5	20.1	20.4	74.0	[d]
	13.5	40	9.2	8.1	1.1	[c]	[c]	65.2	[d]
9.5	7.5	20	[c]	18.7	3.2	19.6	[c]	[c]	4.5
	9	25	[c]	18.1	3.3	27.8	[c]	[c]	5.1
	10.5	31	[c]	19.1	3.7	27.0	[c]	[c]	6.4
	12	37	[c]	18.8	4.2	24.8	24.6	23.8	7.1
	15	52–53	[c]	15.1	3.1	25.7	23.7	19.3	7.7
	18	70–73	[c]	12.7	2.3	18.5	16.1	19.5	6.5
	21	92–96	[c]	6.8	1.3	14.5	17.0	13.5	6.6

[a] $N = 5.54(RT/WI)^2$, where RT is the retention time and WI is the peak width at half-height.
[b] Impurity in lysozyme.
[c] Unresolved peaks.
[d] Did not measure theoretical plate numbers.

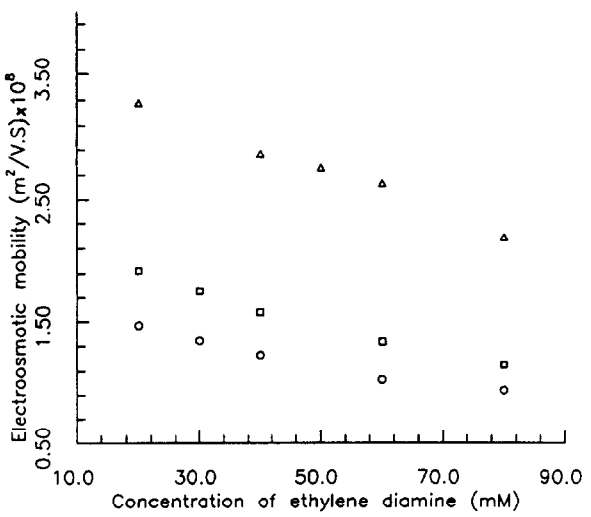

Fig. 4. Effect of the concentration of ethylene diamine on the electroosmotic mobility. The applied voltages were all 12 kV. ○ = pH 6.5; □ = pH 8.5, △ = pH 9.5.

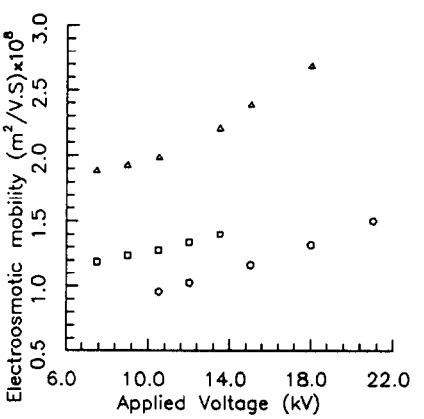

Fig. 5. Effect of the applied voltage on the electroosmotic mobility. ○ = pH 6.5/60 m*M* ethylene diamine; □ = pH 8.5/60 m*M* ethylene diamine; △ = pH 9.5/80 m*M* ethylene diamine.

mobility which is observed in Fig. 5. It was considered that Joule heat caused by high applied voltages across the capillary perhaps contributed to this increase, but it was also considered that Joule heat caused by low voltages such as 7.5, 9 kV, etc. may be efficiently dissipated by

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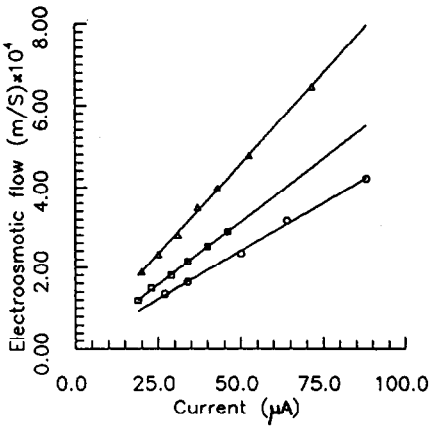

Fig. 6. Linear relationship between the electroosmotic flow and the current through the capillary. ○ = pH 6.5/60 m*M* ethylene diamine; □ = pH 8.5/60 m*M* ethylene diamine; △ = pH 9.5/80 m*M* ethylene diamine.

the capillary wall. According to ref. 17, although Joule heat can cause a non-linear relationship between electroosmotic flow and applied voltage, a linear relationship between electroosmotic flow and the current through the capillary did exist. In this study, a similar linear relationship also existed, as demonstrated in Fig. 6, so it is considered that Joule heat caused by applied voltages contributed to the increased electroosmotic mobility, and also caused the decreased efficiency of all basic proteins, even though the applied voltages were not very high.

ACKNOWLEDGEMENT

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