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Separation of myoglobin molecular mass markers using non-gel sieving capillary electrophoresis

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Abstract

A non-gel sieving method was described for the separation of myoglobin molecular mass markers (M_r range 2512–16 949). In this system glycerol was added in order to slow the diffusion of peptides. The results are consistent with those obtained with cross-linked polyacrylamide. Deviation from linearity in the log M_r versus migration time plot was observed for the smaller peptides because of their intrinsic net charge. Compared with traditional slab gel electrophoresis this method is simpler and less time consuming.

Keywords: Peptides; Myoglobin

1. Introduction

Size-dependent separations are widely used electrophoretic techniques for the analysis of biomolecules. Traditional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) has been used for over 30 years to separate proteins according to difference in molecular mass [1]. With the development of capillary electrophoresis, the traditional SDS-PAGE has been performed in capillary tubes obtaining very high efficiencies [2]. Compared with slab gel electrophoresis, capillary gel electrophoresis has many advantages due to its high separation electric field, resulting in high resolution. However, this method also presents problems, such as lack of control of polymerization, shorter life time of column, and difficulty of automation [3–10].

In order to overcome these problems, an innovative solution has been developed, in which a dynamic sieving system was created by introducing specific linear polymers into the separation buffer [3,5,6,9–11]. Zhu et al [12,13] first presented some early results of sieving separation of CE with polymer solutions, instead of cross-linked gels in 1989. The polymer solution can be replenished at the beginning of each analysis, enabling a single capillary to be used for up to hundreds of runs with good reproducibility. Several commercial sieving buffers for SDS-proteins (M_r 14 000–200 000) are available now.

With the growth of interest in biological membranes, the demands for an analytical method for the determination of peptide molecular masses is accentuated. A reliable procedure for the separation of SDS-peptides in slab gel has been established by Swank and Munkres [14]. In this paper, a non-gel sieving capillary electrophoresis method was described for the separation of myoglobin molecular mass markers (M_r range 2512–16 949). Compared

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with traditional slab gel electrophoresis this method is simpler and less time consuming.

2. Experimental

2.1. Materials

Peptide markers (M_r 2512–16 949) manufactured from horse-heart myoglobin were purchased from BDH (Poole, UK). α-Lactalbumin, insulin and dextran with different molecular masses were purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl) aminomethane (abbr. Tris), sample buffer and SDS run buffer were purchased from Bio-Rad (Hercules, CA, USA). Boric acid was purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate(SDS) was purchased from Fluka (Buchs, Switzerland). All the buffer solutions were prepared by using Millipore water.

2.2. Instrumentation

All the sieving separations were performed on a commercial capillary electrophoresis instrument, BioFocus 3000 System (Bio-Rad). The capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.3. Conditions

A 24 cm×50 μm I.D. (length to detector: 19.5 cm) uncoated capillary was employed. The inner surface of the capillary was rinsed with 0.1 *M* NaOH (5 min), followed by washing with Millipore water (2 min) before use. All analyses were performed under constant voltage, from negative to positive. Detection was carried out by UV absorption at 220 nm. Temperatures of capillary and autosampler were set at 20 °C.

The separating buffer solution consisted of 0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol unless stated otherwise. The concentrations of dextrans with different molecular masses are described where appropriate in the text. Bio-Rad SDS run buffer was used as washing buffer.

2.4. Protein sample preparation

SDS-protein samples were prepared in 0.1 *M* Tris HCl, 1% SDS, pH 9.2 (Bio-Rad). The final concentration of the peptides was 1 mg/ml. The samples

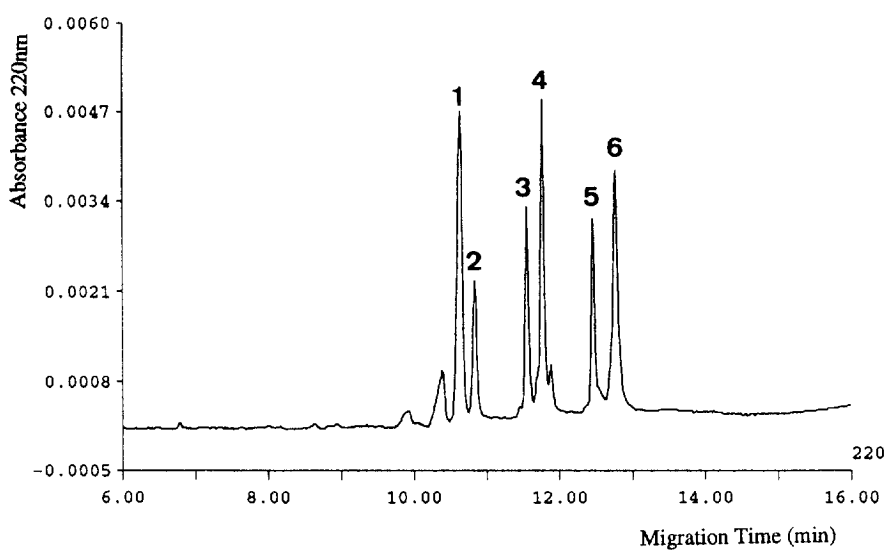


Fig. 1. Electropherogram of peptide markers in non-gel sieving capillary electrophoresis. Instrument, BioFocus 3000 capillary electrophoresis system; capillary, 24 cm×50 μm I.D. uncoated capillary; sample, myoglobin molecular weight mass (M_r 2152–16 949 BDH); buffer, 0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol, 12% dextran (M_r 2 000 000), pH 8.3; voltage, 12 kV, negative to positive; temperature, 20 °C (capillary and autosampler); detection, 220 nm. Peaks numbers as Table 1.

Table 1
Reproducibility of migration times using non-gel sieving buffer

Peak No.	Peptide	M_r	Migration time (min) (mean±S.D., n=9)
1	Globin III (132–153)	2512	10.54±0.030
2	Globin II (1–55)	6214	10.77±0.033
3	Globin I (56–131)	8159	11.53±0.036
4	Globin I+III (56–153)	10 701	11.77±0.036
5	Globin I+II (1–131)	14 404	12.49±0.038
6	Globin (1–153)	16 949	12.82±0.040

were placed in a boiling water bath for 5 min. These prepared solutions were stored at −20 °C and could be reused several times after defrosting.

3. Results and discussion

Fig. 1 shows the electropherogram of standard peptides (M_r 2512–16 949) separated in the 12% dextran (M_r 2 000 000) buffer solution (0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol, pH 8.3). The applied electric field was 500 V/cm. Complete separation of all the peptide markers was achieved within 15 min. Good reproducibility was observed provided that the capillary was washed with Bio-Rad SDS run buffer before each run to suppress electro-osmotic flow.

Table 1 shows the statistical data for the run-to-run reproducibility of migration times. Fig. 2 shows the calibration curve of log M_r vs. migration time. In the M_r range 2500–6000, obvious deviation was noticed for the horse heart muscle myoglobin fragments from 1–55, where unique structural features have an important effect on electrophoretic mobilities [15]. This result agrees with that obtained from traditional slab gel electrophoresis (Fig. 3).

3.1. Effect of concentration of dextran (M_r 2 000 000) on the separation of SDS-peptides

The migration of SDS peptides in non-gel sieving buffers with different concentrations of dextran (M_r 2 000 000) was studied in this work. The results shown in Figs. 4 and 5 shows the relationship between migration times of peptides and the concentrations of dextran in the separation buffers.

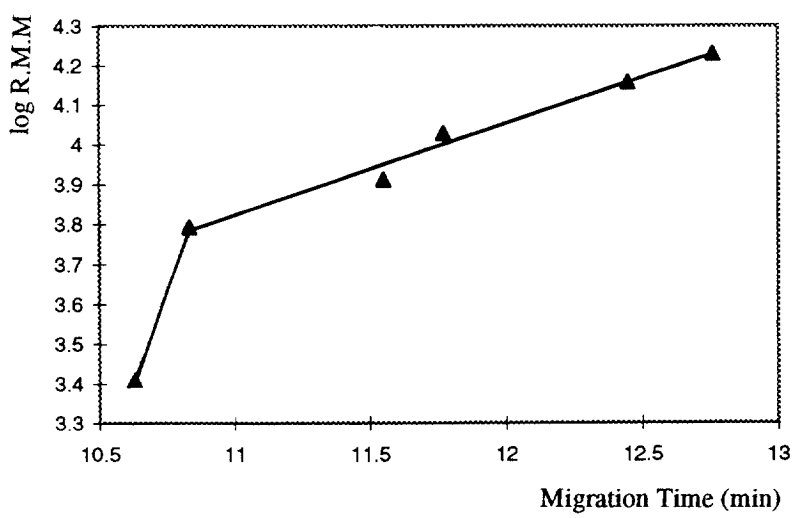
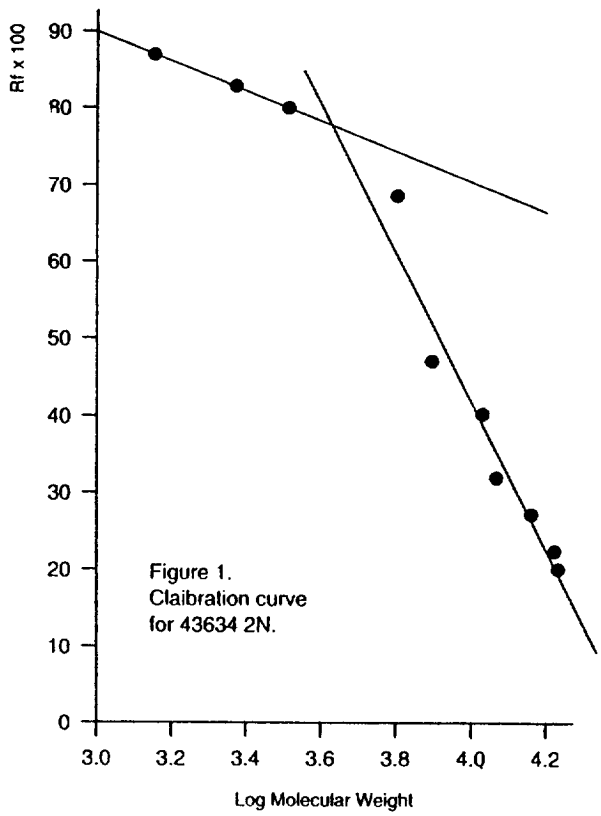


Fig. 2. Calibration curve of peptide markers obtained in non-gel capillary electrophoresis



1,450 - 17,572	
Lentil Lectin[4]	17,572
Globin	16,949
Globin I + II	14,404
Cytochrome C equine[5]	12,300
Globin I + III	10,701
Globin I	8,159
Globin II	6,214
Glucagon, bovine[6]	3,485
Globin III	2,512
Bacitracin	1,450

Fig. 3. Calibration curve of peptide markers obtained in traditional slab gel electrophoresis

In free solution without dextran, all the six standard peptides migrated out within 5 min as overlapping peaks due to the identical charge densities of SDS-proteins (Fig. 4A). When the concentration of dextran in the buffer increases, the resolution of peaks improves, indicating that the concentration of sieving polymer influences separation power significantly. In this case, it was found that the higher the concentration of dextran, the higher was the sieving power for peptides although an increase of migration time was also observed. The best separation was obtained when dextran concentration was at 12%.

3.2. Effect of molecular mass of dextran on the separation of SDS-peptides

The results of the separation of the SDS-peptides using dextrans with different molecular mass are shown in Fig. 6. In separation buffers with 12% dextran of M_r 9300, only two peptides (M_r 2512 and 6214) were well separated. The remaining four peaks were overlapping. In buffer solutions with 12% dextran of M_r 70 000, four small peptides were baseline separated while two bigger ones comigrated. This indicated that the higher the molecular mass of dextran the higher was the resolving power for peptides. These results agree with those obtained by Mohammed et al [6] in the separation of proteins using dextrans with different molecular mass: M_r 2 000 000, 500 000 and 70 000. Ganzler et al [5] also reported that the selectivity of proteins was enhanced with an increase in the molecular mass of the dextran from M_r 9300 to 2 000 000.

We also found that the electroosmotic flow increases while the molecular mass of dextran used in the separation buffer decreases. To prevent excessive changes in electroosmotic flow, a Bio-Rad SDS run

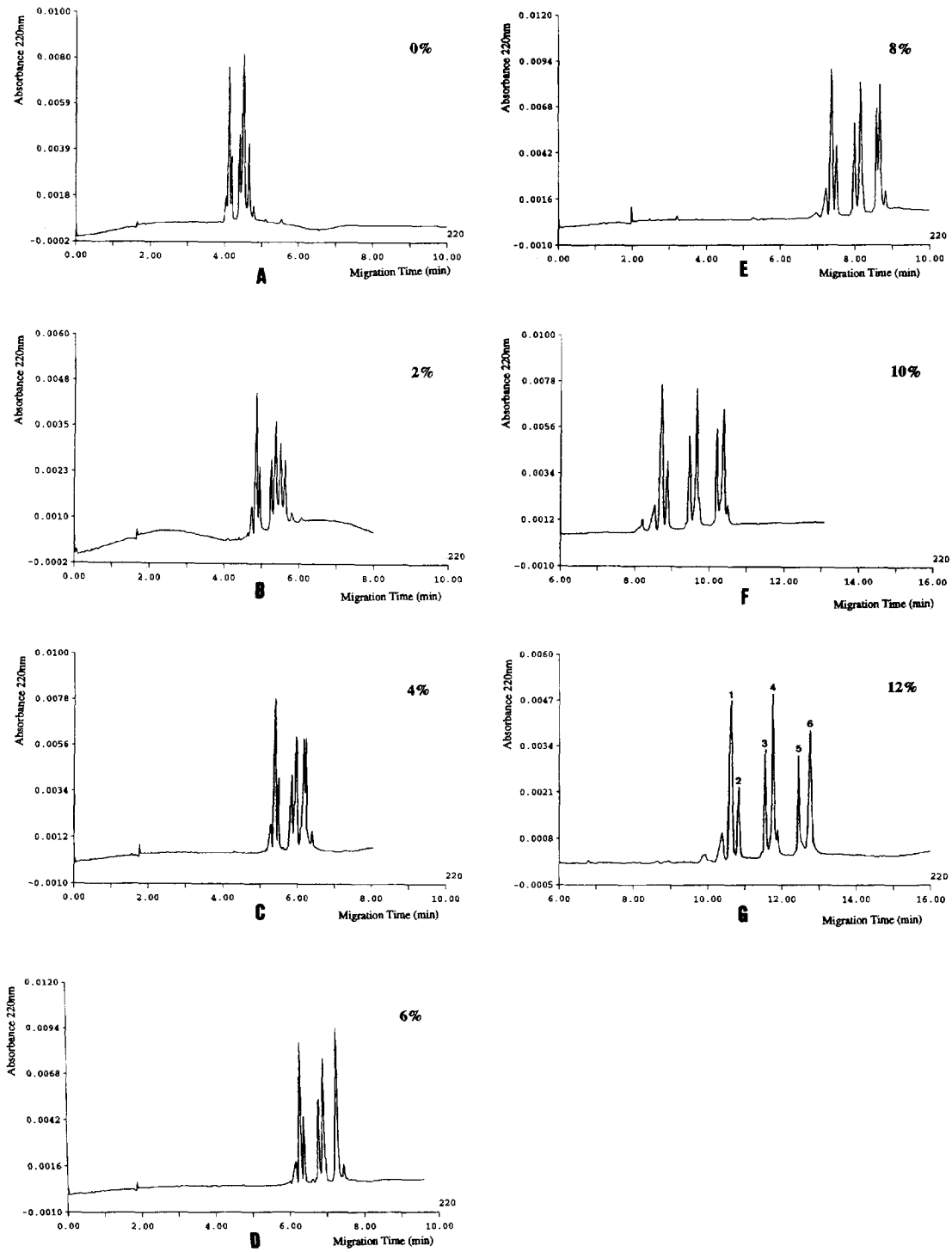


Fig. 4. Effect of different concentration of dextran (M_r 2 000 000) on the separation of peptides in non-gel sieving capillary electrophoresis. buffer, 0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol, with (A) 0%, (B) 2%, (C) 4%, (D) 6%, (E) 8%, (F) 10%, (G) 12% dextran (M_r 2 000 000), pH 8.3. Other conditions are the same as Fig. 1.

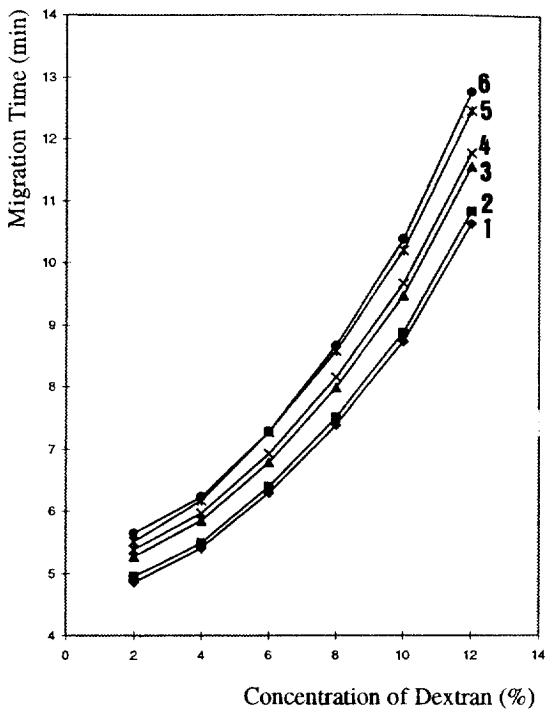


Fig. 5. Plot of migration times of peptide vs. concentrations of dextran in separation buffer

buffer was introduced in which a sort of positive-charged polymer was added to act as a dynamic coating reagent. This ensures that a good reproducibility was obtained, especially when solutions with lower molecular mass dextran were used. A molecular mass dependent reduction of electroosmotic flow could be due to two reasons. One is that electroosmotic flow can be affected by high viscosity polymer solutions. This is because at the same concentration, the higher the molecular mass of the polymer, the higher is its viscosity. A second could be that the adsorption of the dextran with high molecular mass onto the inner wall of the capillary decreases the electroosmotic flow.

3.3. Effect of glycerol on the separation of SDS-peptides

The effect of glycerol on the separation of SDS-peptides was studied by comparing the separation results obtained by using non-gel sieving solution with and without 10% glycerol. The result is shown in Fig. 7. In Fig. 7A, the resolution of SDS-peptides decreases with an accompanying decrease in migration times when a buffer solution without glycerol was used. Two peaks of the standard peptides (M_r 8159 and 10 701) overlapped. Addition of glycerol resulted in slower diffusion and thus retarded migration of SDS-peptides while the high applied potential was applied. As a result, good resolution of peptides which have smaller molecular masses could be obtained. This effect was also observed in the separation of peptides using conventional slab gel electrophoresis although it would take at least 12 h to finish the separation of standard peptides. We also found that the separation current increases when 10% glycerol was added in the buffer solution.

3.4. Effect of ionic strength on the separation of SDS-peptides

Fig. 8 shows the separation of peptide markers obtained with different ionic strength sieving buffers. According to these results, the ion-strength of the buffer of 0.4 *M* Tris–borate was recommended. In this case, higher ion strength gave better resolution and longer migration times. The effect of Joule heating was not significant due to the relatively low currents obtained for the non-gel sieving buffers.

3.5. Separation of other proteins with non-gel sieving buffer

The molecular masses of insulin and α-Lactalbumin were tested using dextran non-gel sieving buffers. The results are shown in Figs. 9 and 10. According to the calibration curve for peptides markers, the molecular masses of the insulin and α-lactalbumin samples are 6500 and 13 500, respectively.

Acknowledgments

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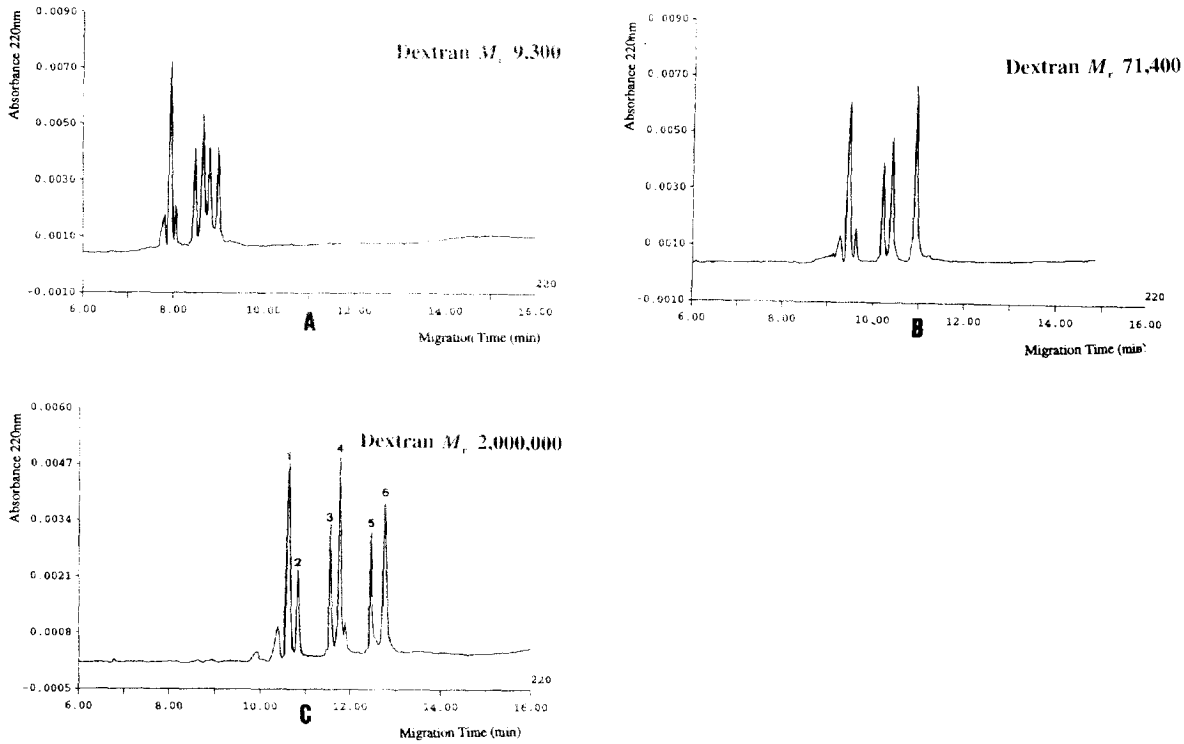

Fig. 6. Effect of dextran with different molecular masses on the separation of peptides in non-gel sieving capillary electrophoresis. Buffer, 0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol, 12% (A) dextran (M_r 9300), (B) dextran (M_r 71 400), (C) dextran (M_r 2 000 000), pH 8.3. Other conditions are the same as Fig. 1.

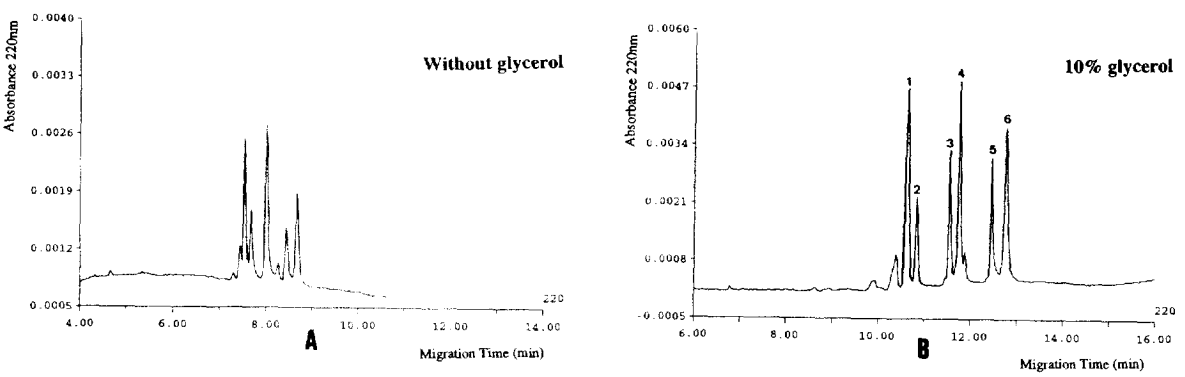

Fig. 7. Effect of glycerol on the separation of peptides in non-gel sieving capillary electrophoresis. Buffer, 0.4 *M* Tris–borate, 0.1% SDS, 12% dextran (M_r 2 000 000), (A) without, (B) with 10% glycerol, pH 8.3. Other conditions are the same as Fig. 1.

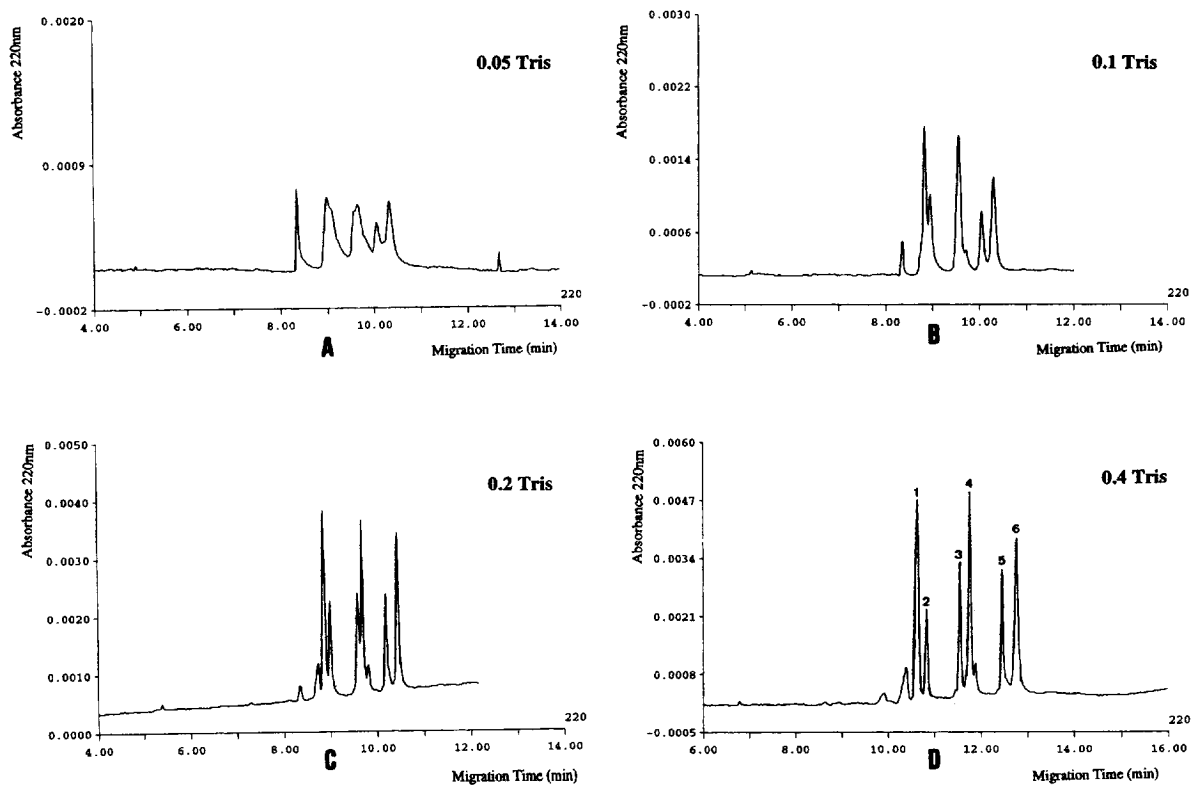

Fig. 8. Effect of ionic strength on the separation of peptides in non-gel sieving capillary electrophoresis. Buffer, (A) 0.05 *M*, (B) 0.1 *M*, (C) 0.2 *M*, (D) 0.4 *M* Tris–borate, 0.1% SDS, 10% glycerol, 12% dextran (M_r 2 000 000), pH 8.3. Other conditions are the same as Fig. 1.

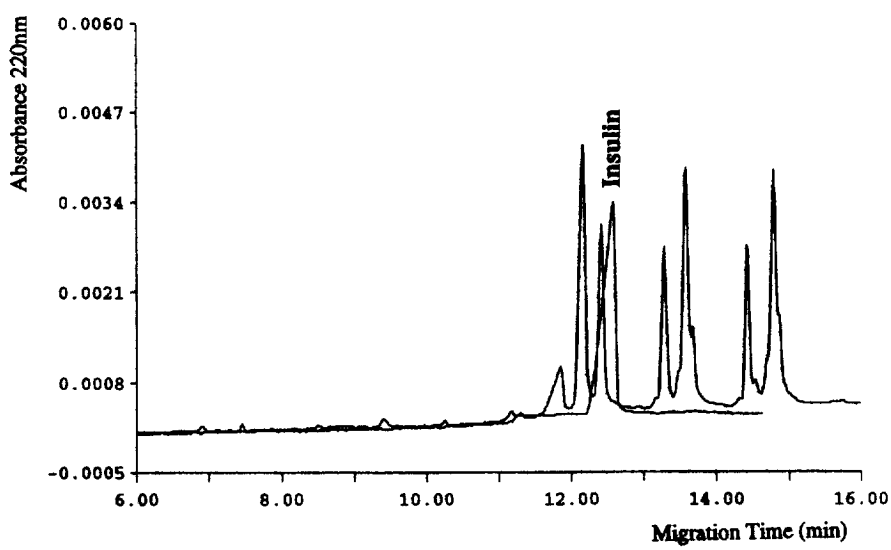

Fig. 9. Electropherogram of insulin in non-gel sieving capillary electrophoresis. The separation conditions are the same as Fig. 1.

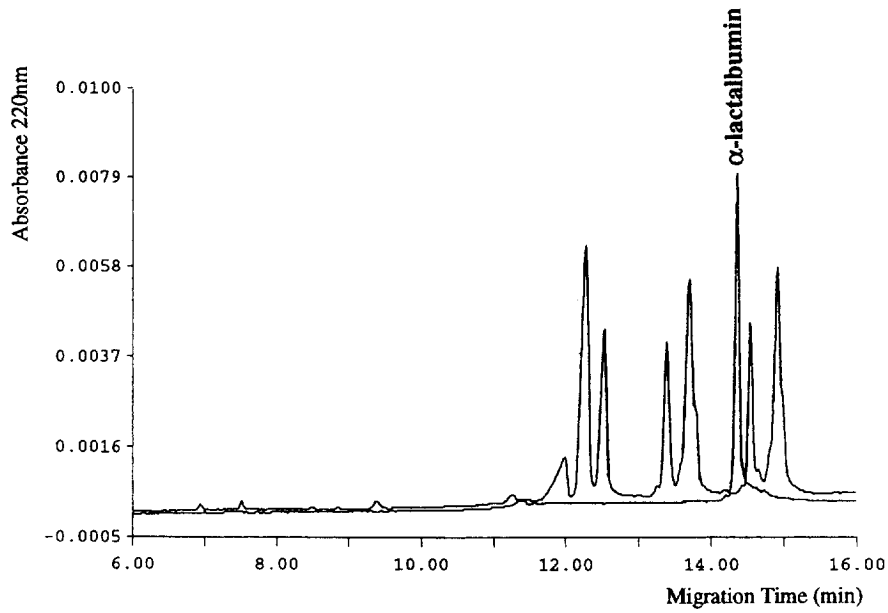

Fig. 10. Electrophoresis of α-lactalbumin in non-gel sieving capillary electrophoresis. The separation conditions are the same as Fig. 1.

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