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Separation and detection of closely related peptides by micellar electrokinetic chromatography coupled with electrospray ionization mass spectrometry using the partial filling technique

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Abstract

Closely related peptides such as neurotensin and angiotensin analogues were separated by capillary zone electrophoresis using a nonionic surfactant, sucrose monododecanoate, as a micelle forming reagent. These peptides were detected by an on-line coupled mass spectrometer using an electrospray ionization interface. However, the presence of the micelles in the separation solution drastically reduced the sensitivity of the mass spectrometer. Therefore, a partial filling technique was employed to prevent the micelles from entering the mass spectrometric interface. A part of the capillary from the injection end was filled with the micellar solution in this technique. Analytes passed through the micellar zone during the electrophoresis and when the separated analytes reached the detection end of the capillary, the micellar zone was still behind the analyte zones, because the nonionic surfactant moved very slowly in acidic conditions. Thus the technique was very useful for mass spectrometric detection for CE when the micellar solution was employed for separation. The optimization of separation and detection conditions was investigated.

Keywords: Partial filling method; Peptides; Neurotensin; Angiotensin; Sucrose monododecanoate

1. Introduction

Mass spectrometry (MS) is a powerful detection method of the separation techniques, because MS has high sensitivity and generates molecular information on the analyte. On-line coupling of capillary electrophoresis (CE) with MS has been reported by several groups [1–5]. Recently, several review articles were also published on the CE–MS system [6–11]. However, very few papers have described MS detection for micellar electrokinetic chromatography (MEKC) [12–14], although MEKC expands the application range of CE to the neutral analytes. This is because the micelles introduced into fast-atom bombardment (FAB) ionization [15] or electrospray ionization (ESI) [16] systems caused several problems: the decrease of sensitivity [16], generation of the surfactant related spectra [1] and probable contamination of the interface by the adsorption of the non-volatile surfactant. To solve these problems several techniques have been proposed. Lamoree et al. [12] employed a heart-cut technique, where neutral analytes were separated by MEKC in the first capillary filled with a micellar solution and the separated zones were transferred to the second capillary through a liquid junction. The second capillary was filled with a buffer solution containing only volatile components and used to transfer the analyte zone to

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the ESI interface. In this technique, almost no surfactant was introduced into the ESI interface and a conventional CE–ESI-MS system showed sensitive detection. We employed a high-molecular-mass surfactant for a conventional CE–ESI-MS system [13]. Butyl acrylate-butyl methacrylate–methacrylic acid copolymer [17] used as a pseudo-stationary phase did not significantly deteriorate ionization efficiency nor interfered with mass spectrum of the analyte. Takada et al. [14] developed an atmospheric pressure chemical ionization (APCI)-MS system for CE–MS. In this system, the adverse effects of an ionic surfactant and non-volatile buffer ions were suppressed and no strong decrease in ionization efficiency was observed up to 50 m*M* sodium dodecyl sulfate.

In this paper, we describe a new technique of MEKC–ESI-MS using a partial filling method. The technique was applied to the separation and detection of closely related peptides. The separation of closely related peptides by using nonionic surfactants in an acidic medium was reported previously [18,19]. Although the technique cannot separate neutral analytes, the separation principle is also based on the differential partitioning to the micelles and the technique meets the same problems in coupling with the MS detection as MEKC. In the partial filling technique, a micellar solution is introduced into a part of the capillary from the injection end, the analytes can be separated during passing through the micellar zone, and then the separated analyte zones are free from micelles when they reach the detection end of the capillary. Therefore, the detection problems caused by the surfactant are successfully avoided.

2. Experimental

2.1. Reagents

Bradykinin, neurotensin derivatives and angiotensin II derivatives (Fig. 1) were purchased from Sigma (St. Louis, MO, USA) and dissolved in 10 m*M* ammonium formate (pH 3.0) to make a 0.5–1 mg ml^{-1} solution. Sucrose monododecanoate (Fig. 1) was purchased from Dojindo Laboratories (Kumamoto, Japan). Separation buffer solutions were 10–30 m*M* ammonium formate (pH 3.0) containing 0 or 100 m*M* sucrose monododecanoate. All reagents were analytical-reagent grade and used as received.

2.2. Apparatus

An outline of the CE–ESI-MS system is shown in Fig. 2. CE was performed with a Jasco Model CE-990 (Hachioji, Tokyo, Japan) which was con-

Peptides

Bradykinin:	**Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg**
[D-Tyr^{11}]-Neurotensin:	**<Glu - Leu - Tyr - Glu - Asn - Lys - Pro - Arg - Arg - Pro - D-Tyr - Ile - Leu**
[D-Phe^{11}]-Neurotensin:	**<Glu - Leu - Tyr - Glu - Asn - Lys - Pro - Arg - Arg - Pro - D-Phe - Ile - Leu**
[D-Trp^{11}]-Neurotensin:	**<Glu - Leu - Tyr - Glu - Asn - Lys - Pro - Arg - Arg - Pro - D-Trp - Ile - Leu**
[Val^{5}]-Angiotensin II:	**Asp - Arg - Val - Tyr - Val - His - Pro - Phe**
[Ile^{5}]-Angiotensin II:	**Asp - Arg - Val - Tyr - Ile - His - Pro - Phe**

Sucrose monododecanoate (β-Fructofuranosyl-α-D-glucopyranoside monododecanoate)

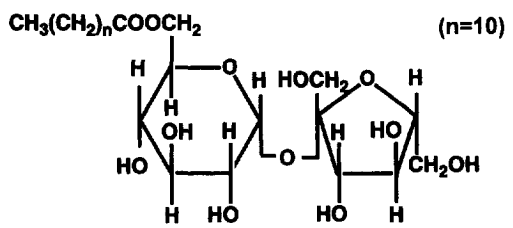

Fig. 1. Structures of peptides and a nonionic surfactant.

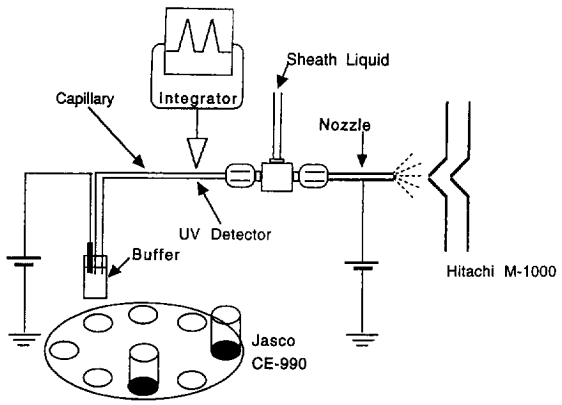

Fig. 2. Schematic representation of the CE–ESI-MS system.

nected to a quadrupole mass spectrometer Hitachi M-1000 (Tokyo, Japan) through the same ESI interface as described by Takada et al. [20]. The nozzle of the ESI interface was made of a 1.6 mm (1/16 inch) (1 inch=2.54 cm) T-union and G28 stainless steel tubing (190 μm I.D., 350 μm O.D.) [13]. A capillary, 100–105 cm×50 μm I.D., 150 μm O.D. (GL Science, Tokyo, Japan) was used for separation. The UV detector of the Model CE-990 was set at 65–70 cm from the injection end of the capillary and run at 215 nm. The mass spectrometer was operated in positive-ion mode only. The applied voltage to the injection end of the capillary was 17 kV or 14 kV for electrophoresis (3 kV for electrospray) and the samples were separated at room temperature. The electrospray ionization potential of the mass spectrometer was 3 kV, the ion guide potential was 700–900 V, the drift voltage was 45–70 V and static lens potential was 100–140 V. The m/z value was not always accurate because the calibration was not performed frequently and the room temperature was not constant. For MS detection, the cyclic-scan mode (from m/z 1 to 1000 at 4 s per scan) was used and relevant single-ion electropherograms and mass spectra were displayed after the run. The sheath flow solution was a mixture of water, organic solvent (methanol, ethanol or acetonitrile) and formic acid and was delivered by a micro-syringe pump (Harvard Model 11, Natick, MA, USA) at the rate of 1–5 μl min^{-1}. For the optimization of the separation conditions with volatile buffer systems, a Beckman Model P/ACE 2100 (Fullerton, CA, USA) was employed with a fused-silica capillary of 50 μm I.D. and 375 μm O.D. and operated at 215 nm.

3. Results and discussion

3.1. Sheath solution

The sheath solution was essential to make the electrospray stable in our CE–ESI-MS system. A mixture of water–organic solvent–formic acid (50:50:1) was employed as a sheath liquid, and the effect of the organic solvent on sensitivity was studied. Methanol, ethanol and acetonitrile did not show any significant differences in sensitivity. The composition of the sheath liquid did not essentially affect sensitivity. Therefore, we used a mixture of water–methanol–formic acid (50:50:1) as the sheath solution hereafter. The dependence of the signal intensity on the flow-rate of the sheath solution is shown in Fig. 3 for the single-ion of bradykinin under CZE condition using a 10 mM formate buffer (pH 3.0). Between 1–5 μl min^{-1}, the signal of the mass spectrometer increased with the decrease of the sheath flow. We set the flow-rate of the sheath solution to 1 μl min^{-1} to compromise the sensitivity of mass spectrometry and the stability of electrospray.

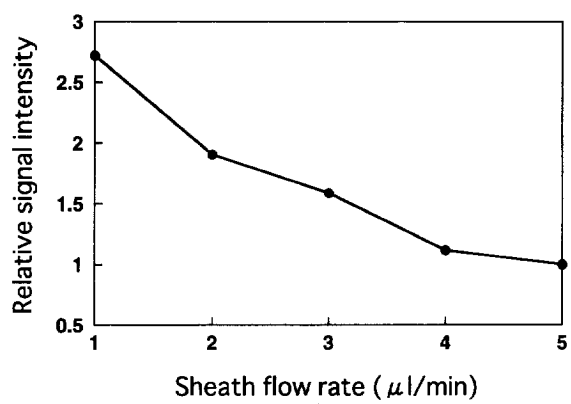

Fig. 3. Effect of the sheath flow-rate on the mass-spectrometric sensitivity. The single-ion intensity of bradykinin separated by CZE was plotted against the sheath flow-rate. Conditions: capillary, 100 cm×50 μm I.D., 150 μm O.D.; running solutions, 10 mM ammonium formate–formic acid buffer (pH 3.0); applied voltage for CZE, 17 kV (−3 kV). See Section 2.2 for ESI-MS conditions.

3.2. Separation solution

In previous papers [18,19], we used acidic sodium phosphate buffers to separate closely related peptides. However, the sodium phosphate buffer generated a strong signal of sodium ion in ESI-MS and signals from peptides were hardly detected. Therefore, we chose ammonium formate and formic acid as buffer components because of their high volatility and low ionic strength compared with the phosphate buffer in order to maximize detection sensitivity. No distinct difference in the back ground noise in ESI-MS was observed between 10 m*M* and 30 m*M* formate buffer. The only difference between these two concentrations was the peak width of the neurotensins. As shown in Fig. 4, peaks were much sharper with 30 m*M* formate than with 10 m*M* formate although these electropherograms were detected by UV absorption at 215 nm. This was probably because the electrophoretic dispersion due to the difference in electrophoretic mobilities between the peptide ion and the buffer component ion (formate) and also slight interaction of the peptides with the capillary wall. These effects are usually less significant at a higher buffer concentration. Thus the concentration of formate was set at 30 m*M* in the rest of the experiments. The effect of surfactant is shown in Fig. 5 for the total-ion electropherogram of bradykinin. The peak intensity was much smaller when 100 m*M* sucrose monododecanoate was added to the separation solution. The results suggest that the presence of the nonionic surfactant also causes low sensitivity in ESI-MS as the presence of the ionic surfactant. Therefore, we decided to apply the partial filling technique to ESI-MS detection to avoid the decrease of sensitivity.

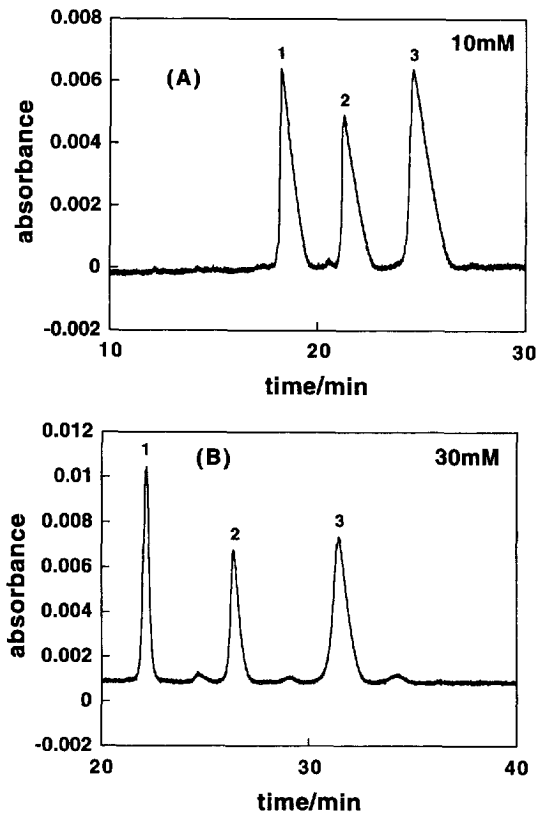

Fig. 4. Effect of the buffer concentration on resolution. Conditions: instrument, Beckman P/ACE2100; capillary length, 67 cm (effective length 60 cm)×50 μm I.D., 375 μm O.D.; running solutions, (a) 10 m*M* ammonium formate–formic acid buffer (pH 3.0) containing 100 m*M* sucrose monododecanoate; (b) 30 m*M* formate–formic acid buffer (pH 3.0) containing 100 m*M* sucrose monododecanoate; peaks: (1) [D-Tyr^{11}]-neurotensin, (2) [D-Phe^{11}]-neurotensin, (3) [D-Trp^{11}]-neurotensin.

3.3. Partial filling method

The partial filling technique was first described by Valtcheva et al. [21] to solve a problem of the wavelength limit in UV detection when a protein was used as an additive to the running solution for enantiomer separation. We successfully employed the technique in affinity electrokinetic chromatography using proteins such as avidin, ovomucoid, bovine serum albumin, etc., as chiral selectors [22]. The basic idea of the partial filling method is shown in Fig. 6. First, we fill the capillary with a running buffer which does not contain micelles, and then a micellar solution is introduced from the injection end into the capillary to a point shorter than the detection window. Finally, we inject a sample solution from the same end as the micellar solution is introduced and the separation voltage is applied after the injection end is returned to the running buffer without the micelle. Under the conditions of this study (pH 3.0), since the electroosmotic flow was very weak and the micelles were uncharged because a nonionic surfactant was used, the micellar zone moved very slowly toward the cathodic end of the

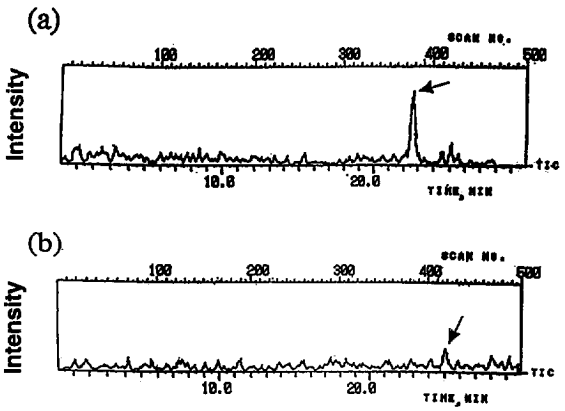

Fig. 5. Total-ion electropherogram of bradykinin. Peaks based on bradykinin are indicated by arrows. Conditions: capillary, 100 cm×50 μm I.D., 150 μm O.D.; running solutions, (a) 10 m*M* ammonium formate–formic acid buffer (pH 3.0); (b) 10 m*M* ammonium formate–formic acid buffer (pH 3.0) containing 100 m*M* sucrose monododecanoate. See Section 2.2 for ESI-MS conditions.

capillary by electroosmosis. By contrast, positively charged peptides moved much faster than the micelle and passed through the micellar zone to reach the detection end faster than the micelle. During passing through the micellar zone, peptides were partitioned to the micellar phase proportionally to their hydrophobicities. Thus, we could achieve the separation of similarly charged peptides and detect by mass spectrometry without the contamination by the surfactant.

The experimental technique and reproducibility of the partial filling method was described previously [22]. The optimum length of the micellar zone depends on the concentration of the micelle and the distribution coefficients. Optimization of the length of the micellar zone will be discussed in detail elsewhere. Therefore, only a brief discussion is given here. If the concentration of the micelle is constant, a longer zone length will give a higher resolution. The maximum length must be theoretically close to the effective length, which is equal to the total length for the MS detection, because the electroosmotic flow is almost completely suppressed and the micelle does not migrate by electrophoresis in this case. However, we did not try to find the maximum length that did not cause the detection problem. Actually, much shorter lengths than the effective length were enough to perform resolution as shown below.

3.4. Mass spectrometric detection of peptides

As shown in Fig. 1, neurotensin derivatives have one acidic and three basic amino acids, that is, one glutamic acid, one lysine and two arginines in the structure, so they are supposed to exist from monovalent to trivalent cations in pH 3.0 solutions. The ESI mass spectrum of [D-Trp^{11}]-neurotensin is shown in Fig. 7. The signal based on the trivalent cation (*m*/*z* 567 in Fig. 7 but should be 566 by calculation) is much larger than the peak based on the divalent cation (*m*/*z* 851 in Fig. 7 but should be 848 by calculation). Thus trivalent cations of neurotensin derivatives were monitored in the rest of the

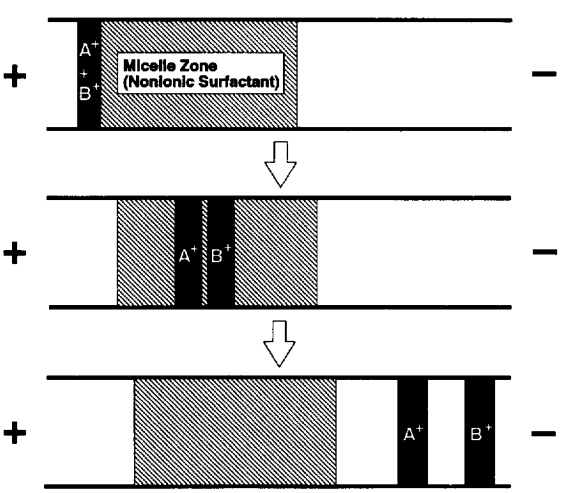

Fig. 6. Schematic diagram of the partial filling method. Closely related peptides are separated only during passing through the micellar zone and after the micellar zone they migrate at the same velocity by electrophoresis.

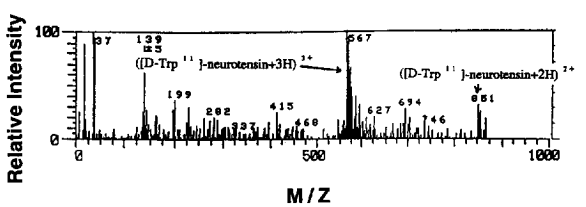

Fig. 7. ESI mass spectra of [D-Trp^{11}]-neurotensin measured by CE–ESI-MS system. CE conditions: 30 m*M* ammonium formate–formic acid buffer (pH 3.0). Other conditions are the same as in Fig. 5a. See Section 2.2 for ESI-MS conditions.

experiments of CE–ESI-MS. The differences between calculated and measured m/z values are probably due to the significant change in room temperature because of poor air-conditioning. In case of angiotensin II derivatives, main peaks were those of divalent cations, which were used to monitor in CE–ESI-MS.

3.5. Separation and detection of neurotensin derivatives

Separation of three neurotensin derivatives that have similar charges was optimized with the partial filling technique. The results are shown in Fig. 8. Fig. 8 (a, upper trace) shows the result when the micellar zone was formed by injecting 50 mb of pressure for 5 min to a 100 cm capillary (50 μm I.D.) and Fig. 8 (b, lower trace) shows the results with the pressure of 50 mb for 15 min. The lengths of micellar zones introduced in the capillary were estimated to be about 6 cm and 18 cm in Fig. 8a and 8b, respectively. The resolution of three neurotensin derivatives was much better when the micellar zone was longer. Three neurotensins were not at all separated without the micelle. These results were expected because the longer micellar zone lead to the better separation since the separation was based on the partitioning to the micellar phase. The flat-top peaks observed at 35–44 min in Fig. 8a and at 27–45 min in Fig. 8b were the micellar zones. In case of neurotensin derivatives, peptides were separated from each other and also from the micellar zone at the position of the UV absorbance detector which was placed between the injection side and mass spectrometric interface in the other side. Single-ion electropherograms monitored with the trivalent cations by ESI-MS at the same time are shown in Fig. 9. For confirmation, a mass spectrum of each peak was taken (Fig. 10).

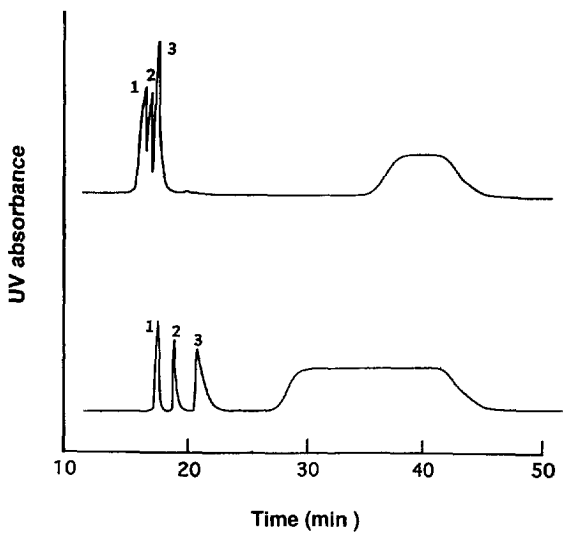

Fig. 8. UV-electropherograms using the partial filling method. Conditions: instrument, Jasco CE 990; capillary, 100 cm×50 μm I.D., 150 μm O.D.; running solutions, 30 m*M* ammonium formate–formic acid buffer (pH 3.0); micellar zone, 30 m*M* ammonium formate–formic acid buffer (pH 3.0) containing 100 m*M* sucrose monododecanoate (upper trace) filled at 50 mbar for 5 min (micellar zone length, ca. 6 cm) and (lower trace) filled at 50 mbar for 15 min (micellar zone length, ca. 18 cm); applied voltage, 17 kV; detection, UV absorption at 215 nm; peaks: (1) [D-Tyr^{11}]-neurotensin, (2) [D-Phe^{11}]-neurotensin, (3) [D-Trp^{11}]-neurotensin.

3.6. Separation and detection of angiotensin II derivatives

Fig. 11 shows the separation of [Ile^5]-angiotensin II (human) and [Val^5]-angiotensin II by CZE monitored by UV absorption and ESI-MS in the buffer solution without micelles. UV-detected peaks of two compounds overlapped each other as shown in Fig. 11a, and mass electropherograms of these two compounds had the same migration times around 29–32

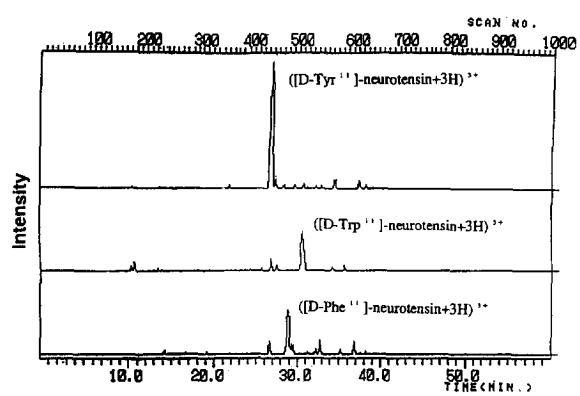

Fig. 9. Single-ion electropherogram of three neurotensin derivatives. CE conditions are the same as in Fig. 8b. See Section 2.2 for ESI–MS conditions.

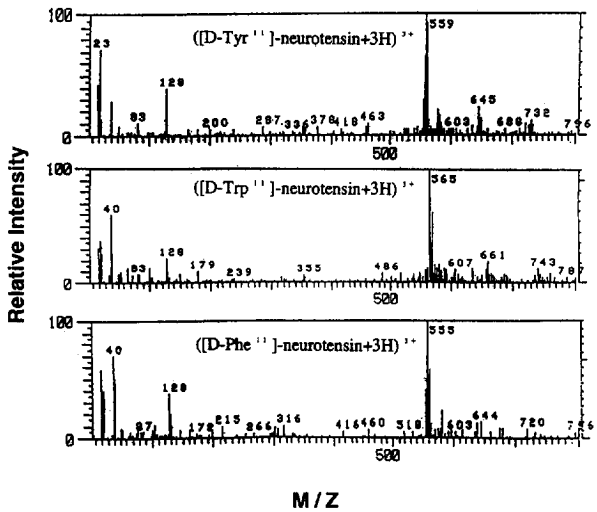

Fig. 10. On-line ESI mass spectra of three neurotensin derivatives separated in Fig. 9. Calculated m/z value of each neurotensin derivatives is 558 for ([D-Tyr^{11}]-neurotensin+3H)/3, 566 for ([D-Trp^{11}]-neurotensin+3H)/3 and 553 for ([D-Phe^{11}]-neurotensin+3H)/3.

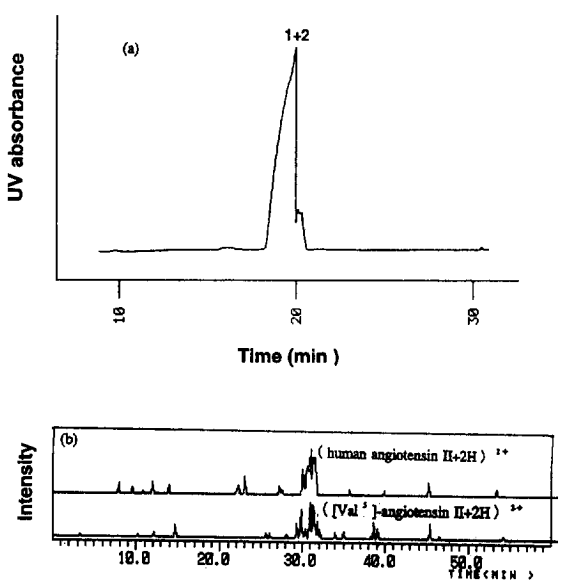

Fig. 11. UV-electropherogram (a) and single-ion electropherogram (b) of two angiotensin II derivatives by CZE–ESI–MS. CE conditions: running solutions, 30 mM ammonium formate–formic acid (pH 3.0).; applied voltage, 17 kV (−3 kV); peaks: (1) [Val^5]-angiotensin II, (2) [Ile^5]-angiotensin II. Other conditions are the same as in Fig. 5. See Section 2.2 for ESI-MS conditions.

min because of their similar charges and molecular masses. Fig. 12 shows the separation obtained by the partial filling technique with 200 mM sucrose monododecanoate. UV-electropherogram (Fig. 12a) showed two distinct peaks of [Ile^5]-angiotensin II and [Val^5]-angiotensin II which were superimposed on a broad peak of the micellar zone seen between 17 min and 39 min. Single-ion mass electropherograms of [Val^5]- and [Ile^5]-angiotensin II also showed different migration times as given in Fig. 12b. Although both angiontensins II detected overlapped over the micellar zone in UV-electropherogram, the peptides were detected free from the nonionic surfactants by ESI-MS because the capillary was long enough to outrun the slowly moving nonionic-micellar band. Mass spectra taken at the peaks of Fig. 12b are shown in Fig. 13. As shown in Fig. 12b, the sensitivity of the ESI-MS detection system was lower than the UV detection. This is probably due to a low ion-transfer efficiency from the ESI interface to the mass spectrometer, and not due to a low ionization efficiency of the ESI interface due to the presence of the surfactant, because the ESI-MS sensitivity was also low even in the absence of the

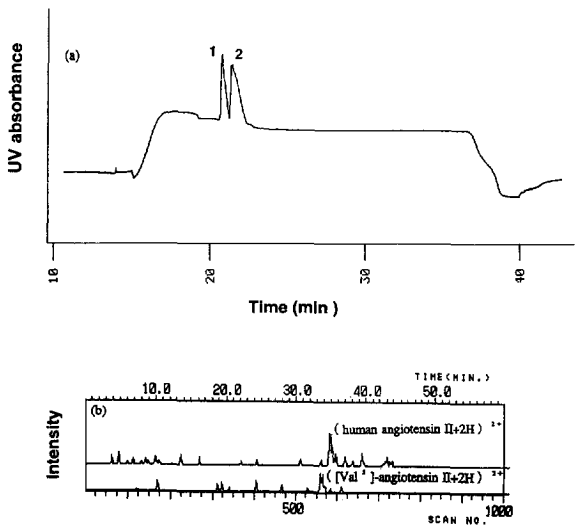

Fig. 12. UV-electropherogram (a) and single-ion electropherogram (b) of two angiotensin II derivatives by partial filling CE–ESI-MS using sucrose monododecanoate. CE conditions: running solutions, 30 m*M* ammonium formate–formic acid (pH 3.0); micellar solutions, 30 m*M* ammonium formate–formic acid (pH 3.0) containing 200 m*M* sucrose monododecanoate; peaks: (1) [Val^5]-angiotensin II, (2) [Ile^5]-angiotensin II. Other conditions are same as in Fig. 11.

surfactant as shown in Fig. 11. Therefore, we are going to improve the transfer efficiency in the next step.

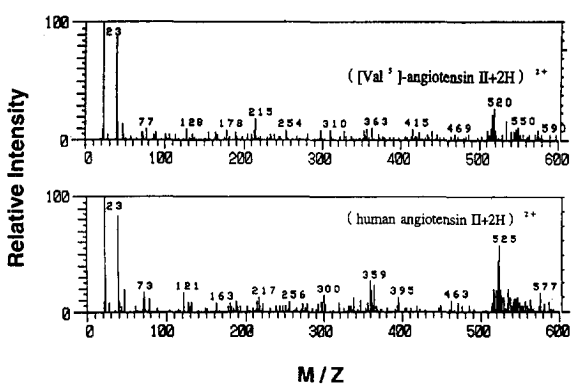

Fig. 13. On-line ESI mass spectra of two angiotensin II derivatives separated in Fig. 12. Calculated *m/z* values are 517 for ([Val^5]-angiotensin II+2H)/2 and 524 for ([Ile^5]-angiotensin II (human)+2H)/2.

4. Conclusion

The partial filling technique was successfully employed in the ESI-MS detection system for the separation of closely related peptides using a nonionic surfactant. An ammonium formate buffer was used not to deteriorate the ionization efficiency of the ESI-MS, and separation conditions with this buffer were optimized. The sensitivity of the ESI-MS system was lower than the UV detection system, but the system allowed the measurement of the mass spectra of the separated peptides. The partial filling technique will be applied to MEKC–ESI-MS with ionic surfactants.

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