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Short communication

Purity control of different bee venom melittin preparations by capillary zone electrophoresis

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Abstract

A rapid capillary zone electrophoresis method was developed for the determination of melittin during its purification from bee venom by column chromatography. Low-pH buffer was selected to decrease the adsorption of the analytes on capillary wall. The average relative standard deviations of the migration time of melittin and of the peak area were 1.2 and 2.3%, respectively.

1. Introduction

Bee venom is a complex mixture containing many peptides, enzymes, biogenic amines, etc. [1]. The main component is melittin, a peptide consisting of 26 amino acid residues: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2 [2]. The other major components are phospholipase A_2, hyaluronidase, MCD (mast cell degranulation) peptide and apamine, which are basic enzymes and peptides [1,3–5]. Melittin interacts strongly and reversibly with amphiphiles, alters membrane permeability and interferes with several membrane-associated enzymatic systems. Owing to its biochemical properties, melittin has been applied in cell membrane studies and clinical treatments of rheumatism, hypertension, etc. [1,6]. The coexisting phospholipase A_2 and biogenic amines always exert some adverse effects on the clinical application of melittin [1]. Purification is essential for the biomedical application of melittin because even a small amount of phospholipase A_2 activity in melittin preparation leads to extensive phospholipid degradation [7].

Purification of melittin always needs multiple steps. First, the bee venom is fractionated by size-exclusion chromatography, the most frequently used columns being Sephadex G-50F and Sephadex-25M. Second, the phospholipase A_2 still present in the melittin fraction is destroyed by sequential sulfitolysis and cyanogen bromide cleavage. The final steps uses cation-exchange chromatography on a carboxymethylcellulose-52 column [7].

The analytical methods used for monitoring melittin purification are size-exclusion chromatography with a Sephadex G-50F column [7,8] or an I-25 protein column [8], reversed-phase

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HPLC on a C_{18} or C_4 column and the use of an amino acid analysers [5,7].

Another possible method for the determination of proteins and peptides is capillary zone electrophoresis (CZE), which is based on a different mechanism to HPLC. The main problem in the application of CZE to biomolecules such as proteins and peptides is their inherent tendency for adsorption on capillary wall owing to the electrostatic attractive forces between the silanol groups on the wall surface and the positively charged protein molecules [9,10]. To decrease or eliminate the adsorption, various measures have been taken, such as modifying the capillary inner wall by binding it with hydrophilic, non-ionic molecules such as γ-glycidoxylpropyltrimethoxysilane, polyethylene glycol [9] or polyacrylamide [11], coating dynamically with an ionizable additive [12] or adjusting the pH of the buffer to some extreme value either greater than or smaller than the isoelectric points (p*I*s) of the proteins under investigation [13–17]. At $pH < 3$, the dissociation of silanol groups is substantially suppressed. With $pH > 9$, which is above the p*I*s of nearly all proteins and peptides, the solutes become negatively charged, leading to decreased adsorption owing to Coulombic repulsion between the solutes and the capillary inner wall.

In this work, to decrease or eliminate the adsorptive interferences due to the presence of protein components in the complex bee venom sample, we tried to apply CZE with an uncoated capillary at low pH and monitor the degree of enrichment of the melittin fraction during its purification process.

2. Experimental

2.1. Apparatus

The CZE system was assembled with a laboratory-made power supply that can provide a voltage range from 0 to 30 kV. The platinum wires connected to the anode and the cathode of the power supply were immersed into two 5-ml buffer vials.

A 60 cm × 375 μm O.D. × 75 μm I.D. fused-silica capillary (Yongnian Optical Factory, Hebei, China), 40 cm to the detector window from the anode, was used as a separation capillary. Detection was performed by the on-column measurement of UV absorption at 214 nm with a CV^4 UV detector (ISCO, Lincoln, NE, USA). The electropherograms were recorded with an SE 120 recorder (ABB Goerz Instruments, Vienna, Austria) and an HP3394 integrator. A pHs-3C pH meter with an E-201-C combination electrode (Rex Instruments Factory, Shanghai, China) was used for pH measurement.

2.2. Materials

Melittin and phospholipase A_2 standard sample (purity 70% by HPLC) was purchased from Sigma (St. Louis, MO, USA). A bee venom fractionated sample that was purified according to the literature [7] was provided by the Bee Institute of the Chinese Academy of Agricultural Sciences (Beijing, China). Phosphoric acid and sodium dihydrogenphosphate were of reagent grade, and phenol was of analytical-reagent grade. The water used to prepare buffer and sample solution was triply distilled.

2.3. CZE procedure

A 0.03 mol/l phosphate buffer of pH 2.40 was prepared and degassed ultrasonically before use. The sample solutions prepared with triply distilled water were kept at −20°C prior to use and were filtered with a 0.45-μm membrane before injection. Before each run the capillary was rinsed with 0.1 mol l^{-1} sodium hydroxide solution for 8 min for about 20 capillary column volumes, followed by rinsing with water and buffer for 2 min.

CZE operations were run at a voltage of 20 kV and injections were performed at 8 kV for 12 s. For the measurement of electroosmotic flow-rate, phenol was used as a neutral marker and

gravity injection was performed for 10 s with the sample end 5 cm higher than the collection end.

3. Results and discussion

3.1. Selection of buffer pH

As the melittin sample contains not only many basic peptides but also significant amounts of basic proteins as impurities [1,8], the adsorption problem is serious and should be avoided. Although protein separations by CZE are more frequently performed in neutral or alkaline buffers than in acidic buffers [13], a low running buffer pH of 2.40 was preferred for the following reasons.

(1) Lowering the surface negative charge density of the capillary inner wall can decrease protein adsorption. As shown by McCormick [13] in a study of the dependence of electroosmotic flow (EOF) on the buffer pH, the p*I* of the silica wall was lower than 1.5 and the EOF increased only slightly with increase in pH from 1.5 to 3, which means that at pH 2.40 employed here the silanol groups on the capillary wall dissociated only slightly and the negative charge density on the wall surface was insignificant. As the basic peptides and proteins in the sample are positively charged in any reasonable pH range, at pH 2.40 the Columbic attractive force between these positively charged proteins and peptides molecules and the slightly negatively charged wall surface becomes greatly suppressed. This suggestion was confirmed by the appearance of more peaks in the electropherogram at pH 2.40 (Fig. 1) in contrast with that at pH 4.54, where many peaks disappeared owing to serious adsorption losses (Fig. 2).

(2) At low pH good reproducibility of the migration time can be obtained. The migration time of a solute is determined by the effective column length, the electric gradient applied, the electroosmotic flow and electrophoretic mobility of the solute under investigation. Among these, the electroosmotic flow of a solute is sensitive to variations in the ionic strength and pH of the buffer solution and the capillary temperature. At lower buffer pH the electroosmotic mobility of a solute gradually diminishes, hence the migration time of a solute becomes less sensitive to these experimental parameters and the reproducibility of the migration time of a solute improves. This is confirmed by the data given in Table 1.

(3) At pH 2.40, which is near the pK_{a_1} of H_3PO_4 (2.1), the buffer has a high buffer capacity against the "electrolysis effect" during the CZE process, making the migration times of the solutes more reproducible [18].

For determining EOF, phenol was used as a neutral marker and its migration time at pH 2.40 and 15 kV was determined to be 123.5 min. The EOF was found to be $2.17 \cdot 10^{-5}$ cm^2 s^{-1} V^{-1}.

3.2. Reproducibility of migration time and peak area

It should be emphasized that at pH 2.40, the EOF, although greatly reduced, was not completely eliminated. Although separation at this pH could be obtained with high efficiency and resolution, the influence of adsorption still existed. It was shown that the components of bee venom, some of which are basic proteins or peptides, were still partially adsorbed on the silica wall at pH 2.40. Experiments revealed that if the capillary was not purged with an alkaline solution between runs, the migration times, peak areas and even the peak number could not be reproduced. The adsorption of melittin at pH 2.40 was still pronounced as melittin has 26 residues, of which six are positively charged, the α-amino of the terminal glycine, three ε-amino groups of lysines at positions 7, 21 and 23 and two guanidino groups of arginine at positions 22 and 24, four of them (two Lys, two Arg) near the carboxylic terminal of the molecule, which is amidated.

Three samples were analysed to show the reproducibility: dried whole bee venom prior to purification, the main melittin-containing fraction after column chromatographic separation and desalting and the melittin standard (Sigma,

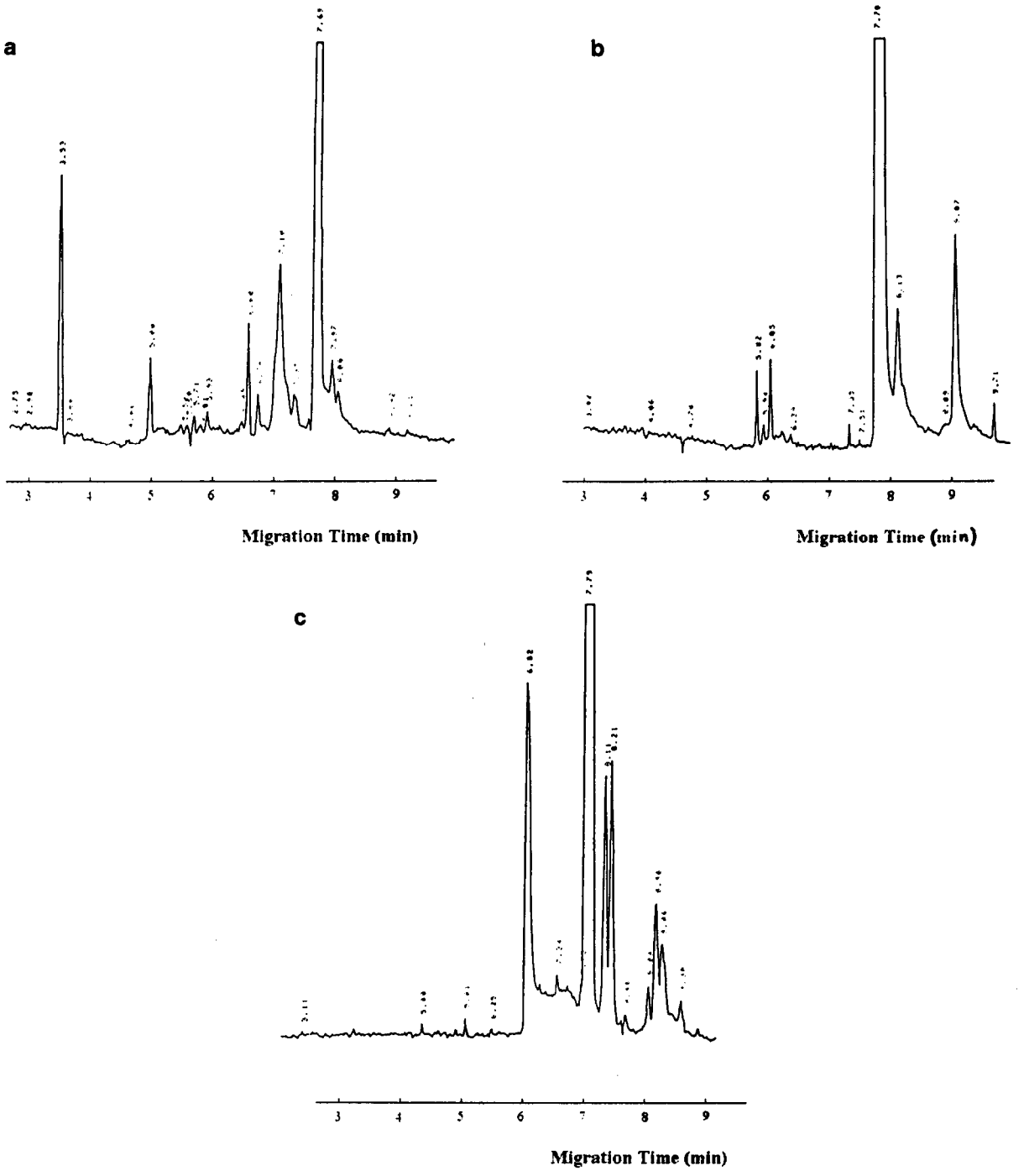

Fig. 1. (a) Electropherogram of the whole bee venom. Conditions: 30 mmol l^{-1} phosphate buffer (pH 2.40); running voltage, 20 kV; injection voltage, 4 kV for 12 s; detection at 214 nm; capillary dimensions, 60 cm (40 cm to detector) × 75 μm I.D. × 375 μm O.D. (b) Electropherogram of the melittin fraction. Conditions as in (a). (c) Electropherogram of the melittin standard. Conditions as in (a).

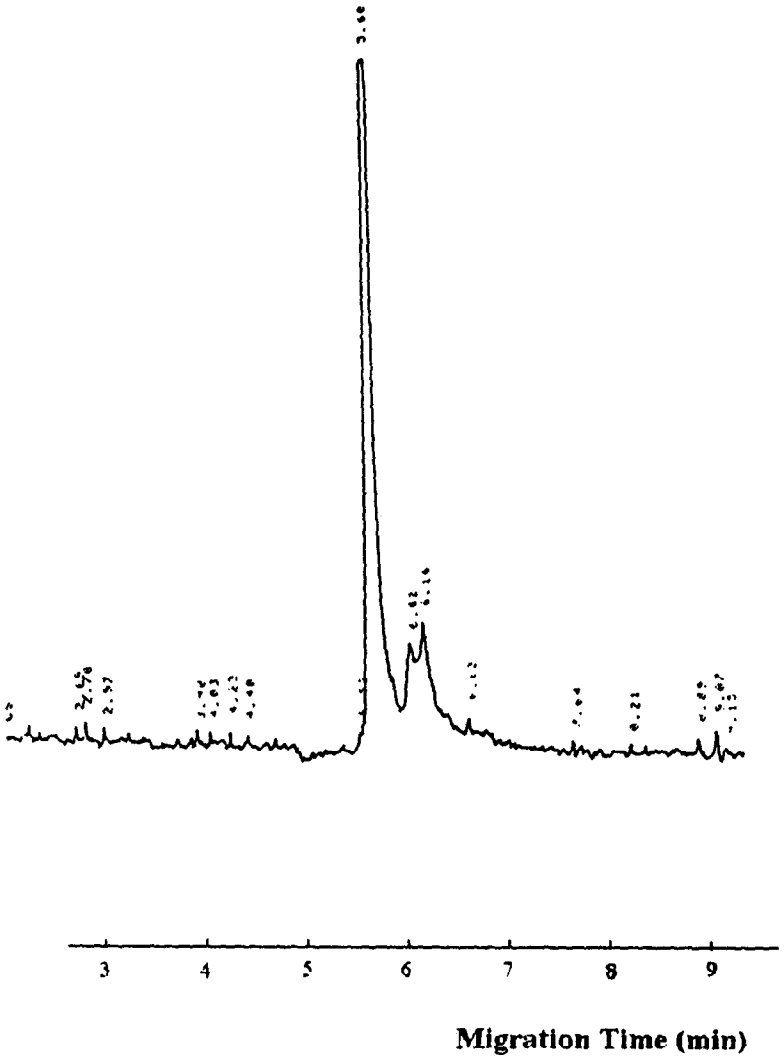

Fig. 2. Electropherogram of the bee venom at pH 4.54. Conditions: 30 mmol l^{-1} acetate buffer; running voltage 20 kV; injection voltage, 4 kV for 12 s; detection at 214 nm; capillary dimensions, 60 cm (40 cm to detector) × 75 μm I.D. × 375 μm O.D.

70%, by HPLC). The average relative standard deviations (R.S.D.s) of the migration time and peak area were 1.2 and 2.3%, respectively (see Table 1).

3.3. Application of the method

The CZE separation of bee venom was found to be fast with all the solutes passing through the detector window within 10 min even though the electroosmotic flow was greatly decreased. The plate number for melittin was about 145 000 m^{-1}. The results of melittin analysis were expressed in terms of area percentage (Table 1).

Comparison between the electropherograms of the melittin fraction and the PLA_2 sample solution showed that the melittin fraction was free from PLA_2 after purification (Fig. 3). This means that the purification process was effective.

A commercially available standard melittin sample with a specified purity of 70%, analysed

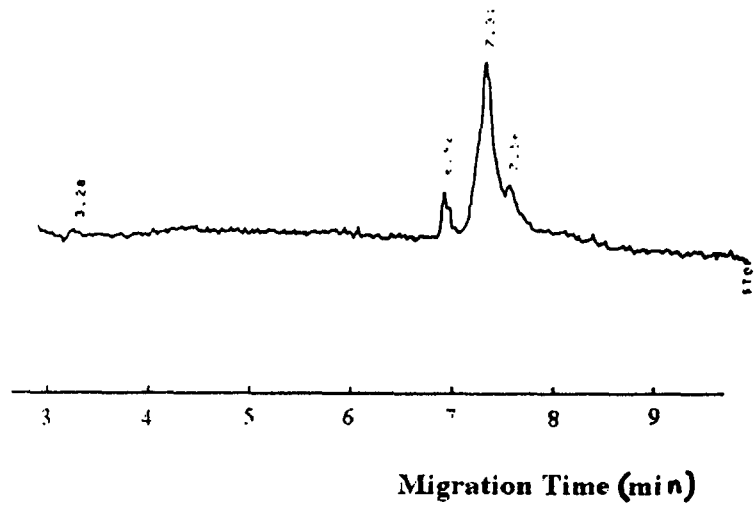

Fig. 3. Electropherogram of phospholipase A_2. Conditions as in Fig. 1.

by HPLC, was found to contain only 64.1% of melittin when determined by the CZE method proposed here. The melittin area percentage of the purified fraction was 82.4% whereas that of the dried whole bee venom was 56.1%.

The proposed CZE method shows high efficiency, good reproducibility and high speed, and is convenient for the determination of melittin in bee venom and its purification product. Compared with the existing chromatographic methods [5,7,8], the CZE method provides better resolution.

Table 1
Reproducibility of the CZE of melittin in bee venom ($n = 3$)

Parameter[a]	Sample[b]		
	I	II	III
t_{r_a} (min)	7.55	7.83	7.68
σ_t (min)	0.11	0.07	0.09
R.S.D.$_t$ (%)	1.4	0.9	1.2
A_a (%)	56.1	82.4	64.1
σ_A (%)	0.7	1.1	2.8
R.S.D.$_A$ (%)	1.2	1.3	4.3

[a] t_{r_a} = Average migration time; A_a = average peak area percentage; σ = standard deviation.
[b] I = Dried whole been venom; II = main fraction of melittin after purification; III = melittin standard.

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