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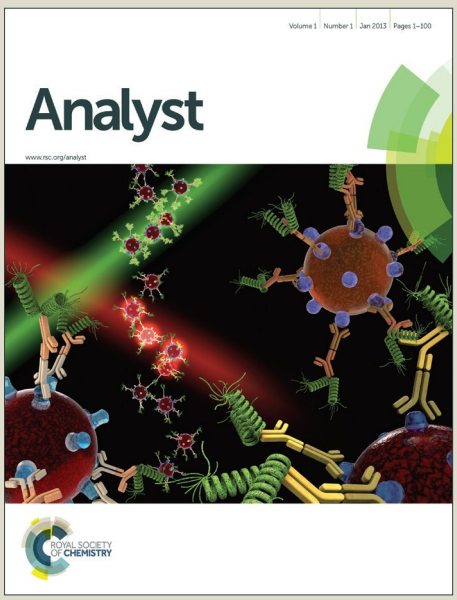

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ARTICLE

A method developed to fractionate intact proteins based on capillary electrophoresis

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Reduction in sample complexity enables more thorough intact protein analysis using MS-based proteomics. A capillary electrophoresis method, namely the velocity gap mode of capillary electrophoresis (VGCE), is proposed to separate protein mixtures in high resolution. Although the separation mechanism of VGCE is also based on the difference of the mass-to-charge ratio of the proteins, it fractionates the sample zone into small pieces of subunits. By means of this way, the resolution can be dramatically improved due to less longitudinal dispersion of the sample. The effect of the new approach is evaluated by separation of three groups of reference protein mixtures, i.e., a mixture of lysozyme, BSA; a mixture of lysozyme, β-lactoglobulin, ribonuclease A; and a mixture of cytochrome C, lysozyme, BSA, β-lactoglobulin, ribonuclease A, conulbumin, carbonic anhydrase, hemoglobin. Results indicate that the new approach shows great potential to couple with MS for top-down analysis of complex mixtures.

1. Introduction

Study of proteomics based on mass spectrometry traditionally includes two strategies: bottom-up approach and top-down approach. Bottom up approach is a well-developed method for the routine identification of proteins in complex mixtures. It entails enzymatic digestion of proteins prior to their introduction to a mass spectrometer, thus it cannot achieve full characterization of a whole protein.[1,2] Top-down approach is complementary to bottom-up approach as it introduces intact proteins into a mass spectrometer, so 100% sequence coverage and post-translation modifications (PTMs) can be guaranteed.[3,4] But the top-down approach cannot be performed at proteomics scale owing to the lack of effective intact protein fractionation strategies.[5,6]

Multidimensional separation techniques have been developed or implemented for intact protein fractionation.[7] Two-dimensional (2D) electrophoresis is considered essential for proteomics research and it is still a method of choice in most biological laboratories.[8] However, it suffers from some limitations, such as poor sensitivity, labour intensive operation, low recovery rate, which gave rise to exploration of liquid phase-based alternatives.[9] Chromatography methods are undoubtedly among the most promising alternatives, given that various techniques can be employed, i.e., reverse phase liquid chromatography.[10–14] John C. Tran et al developed a 4D separation technique which combined solution isoelectric focusing (sIEF), gel-eluted liquid fraction entrapment electrophoresis (GELFrEE), nanocapillary liquid chromatography and MS/MS to analyze human cells. High resolution of the 4D technique led to the confident identification of 1043 gene products from human cells.[15]

However, proteins in low abundance can be easily overshadowed by abundant proteins in the sample, leading to difficulty in qualitative and quantitative analysis.[16,17,18] To address this problem, the strategy was usually to enhance the resolutions of the separations. We proposed VG effect to enhance the resolutions by enlarging the gap distance between two moving analytes.[19,20] But a prerequisite was required, i.e., a gap must have been generated between two analytes before using VG effect. This can lead to no resolution achieved in the mixed section if two peaks were partly overlapped, even though they owned different charge-to-mass ratios. Fortunately, we found that a small amount of analytes contributed to a higher resolution in the separation due to less longitudinal dispersion.[21] In other words, longitudinal dispersion was proportional to the amount of the sample. Less amount of sample caused a better separation. In this paper, we proposed a method, i.e., the velocity gap mode of capillary electrophoresis (VGCE), to fractionate mixed proteins. By means of VGCE, it was possible to separate proteins in low abundance from abundant proteins. Moreover, partly peak overlap of two abundant proteins could be avoided. The feasibility of VGCE was confirmed by the separation of reference proteins.

2. Experimental

2.1 Chemicals and solutions

Formic acid, ammonium acetate and dimethylsulfoxide (DMSO) were purchased from Tianjin Jiangtian Chemical Regent Co., Ltd (Tianjin, China).

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Acetonitrile was purchased from kangkede company (Tianjin, China). Lysozyme, conulbumin (chicken egg) and β-lactoglobuline (bovine) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cytochrome C (horse heart) was obtained from Wolsen (Xi'an, China). Ribonuclease A (bovine pancreas), carbonic anhydrase (bovine erythrocytes) and haemoglobin were obtained from Sigma (Steinheim, Germany). BSA was purchased from Huamaike Company (Beijing, China). Fused silica capillary (50 μm/375μm, ID/OD) was from Yongnian Optic Fiber Plant (Handan, Hebei, China). All reference proteins were dissolved in deionized water with stock concentration of 4 mg mL^{-1}, and were stored in fridge at -20□. Each of them was diluted with deionized water to the final concentration of 0.5 mg mL^{-1} before use.

2.2 Apparatus of VGCE

As shown in Fig. 1, all experiments are carried out on a custom-made CE system.[21] Briefly, the separation channel is composed of two pieces of capillaries that linked together through a joint (VG interface). A custom-made high-voltage DC power supply with three electrodes is used to form two consecutive electric fields along the two capillaries. Motion phase I is from positive electrode (inlet) to zero electrode (the VG interface). Motion phase II is from zero electrode to negative electrode (outlet). The effective separation length is 22 cm. The field strengths of the two electric fields are able to be adjusted independently. The detection window is made by burning off about 3 mm of polyimide coatings on the capillary. The analytes are monitored at 214 nm by a UV detector (spectra 100, for CE). The data is collected with a N2000 chromatographic work station (Zhejiang University, Zhejiang, China).

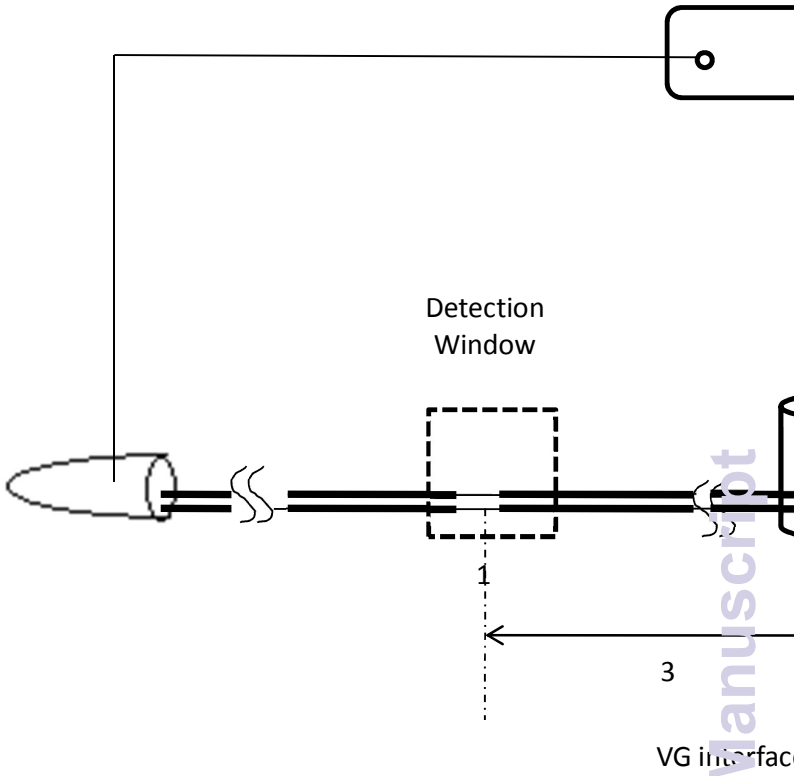

Fig. 1. Schematic diagram of VGCE set-up (not drawn to scale). (1) anode (positive electrode); (2); VG interface (zero electrode) (3) cathode (negative electrode); (4) detection window. The analytes migrate from 1 to 3. The capillary from (1) to (2) is in motion phase I, and the capillary from (2) to (3) is in motion phase II. Different field strength could be generated along the capillary channel by controlling the voltage applied in two motion phases. The separated analytes were monitored at detection window.

2.3 The manipulation of VGCE

The samples are injected into the capillary via gravitational effects. Sample injection is controlled by raising the inlet end the same height (9 cm) and the same time (6s) for each run. Before a new silica capillary was put into use, it is flushed with 1 M sodium hydroxide for 40min, followed by deionized water for 10 min and background electrolyte (BGE) for 10 min. Between two runs, the capillary is flushed with sodium hydroxide, deionized water and BGE for 10 min, 5 min and 5 min, respectively.

As soon as the sample is injected, the field strengths of two motion phases are adjusted as $E_1 = E_2 = 200$ V/cm. The proteins are sequenced in the sample zone according to their mass-to-charge ratios. VGCE is then implemented. Two steps are included in VGCE: Step 1, E_1 is adjusted to zero and E_2 remains unchanged. The protein owning the smallest mass-to-charge ratio will migrate in motion phase II. The other proteins remain stationary in motion phase I. In step 2, both the potentials of two motion phases are adjusted to 200 V/cm ($E_1 = E_2 = 200$ V/cm). The remaining proteins in

motion phase I continue to move into motion phase II. By repeating this two-step procedure, the sample zone can be fractionated into a series of peaks.

2.4 Theoretical basis of VGCE in fractionating intact proteins

The samples are injected into the capillary via gravitational effects (Fig. 2A). When an electric field is applied along the separation channel, all intact proteins migrate from anode to the VG interface because they are positively charged at pH 2.5. The proteins are sequenced in the sample zone according to their mass-to-charge ratios (Fig. 2B). VGCE is then implemented. Two steps are included in VGCE: Step 1 functions as a "knife" to cut the sample zone into small pieces, which is realized by adjusting E_1 to zero and keeping E_2 unchanged. The protein samples which migrate faster will enter in motion phase II. Whilst the other proteins stay in motion phase I. By means of this way, the sample zone is "cut" into small pieces according to the mass-to-charge ratios of proteins. The duration of this step is named as t_1. The bigger t_1 is, the longer the distance between two adjoining peaks becomes. Step 2 functions as a "conveyor" to send remaining sample to the "knife", which is realized by adjusting $E_1 = E_2 = 200$ V/cm. The duration of this step is named as t_2. The shorter t_2 is, the less sample molecules enter into motion phase II. By repeating this two-step procedure, the sample zone can be fractionated into a series of peaks. Since the sample zone is reordered according to the mass-to-charge ratios of the proteins, the complexity of each peak is reduced (Fig. 2C).

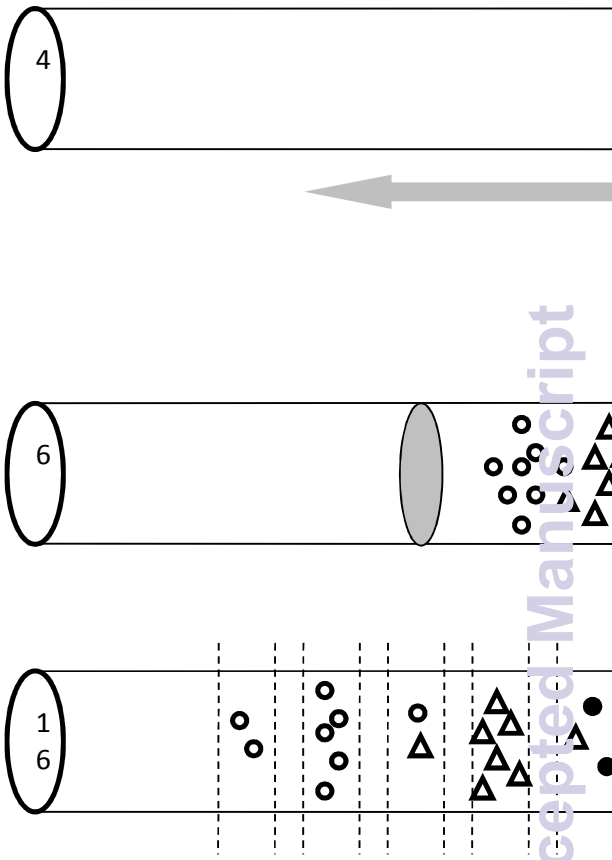

Fig. 2. Schematic diagram of the working principle of VGCE in fractionating proteins (not drawn to scale).The VG interface divides the entire capillary into motion phase Ⅰ and motion phase Ⅱ, of which the field strength is E_1 and E_2, respectively. (A) Injection of protein mixture. (B) Proteins reorder according to their mass-to-charge ratios. (C) VGCE fractionates the protein mixture into a series of subunits.

on the inner surface of the capillary was suppressed. The results were consistent with the previous research.[24,25]

3. Results and discussion

3.1. Suppressing EOF

As we mentioned in the manipulation of VGCE, the analytes in motion phase I should stop moving when $E_I = 0$. However, EOF may ruin VGCE by driving the analytes in motion phase I to motion phase II. As a result, EOF should be suppressed in VGCE. As we know, EOF is dependent on the zeta potential of the inner surface of the capillary, which is caused by the dissociation of silanol group. The strategy adopted in this experiment is to suppress the dissociation of silanol group. Therefore, acidic buffer (pH 2.50) is used as the BGE. To test the EOF, electrophoresis of neutral maker DMSO (1%, v/v) was performed. The experimental conditions: 100 mM formic acid-ammonium acetate, pH 2.50, 200 V/cm. The capillary was 50 μm ID and 22 cm effective length. The analytes were monitored at 214 nm by a UV detector. As we expected, no signal was detected in 150 min (data not shown). The result indicated that EOF was dramatically suppressed using low pH BGE, which was consistent with previous discovery.[22,23]

3.2. Study of reference Proteins in CE

Eight reference proteins, namely, cytochrome C, lysozyme, BSA, β-lactoglobulin, ribonuclease A, conulbumin, carbonic anhydrase and hemoglobin, were studied in CE, respectively (Table 1). It was found that protein samples were easily adsorbed on the inner surface of pristine capillary such that none or irregular peaks were observed (data not shown). To suppress unspecific adsorption of proteins, the capillaries were flushed with 1M sodium hydroxide solution for 40min, following by deionized water for 10 min and BGE for 10 min. In addition, 20% (v/v) acetonitrile was added into the BGE to reduce the analyte-wall interaction. As is shown in table 1, the RSDs were less than 3%, indicating that the reproducibility of migration time was good and the reversible adsorption of reference proteins

Table 1. The list of reference proteins and the repeatability of their mobilities

Protein	pI	MW (kDa)	RSD (%) [a]
cytochrome C	10.7	12.4	2.90
lysozyme	11.0	14.0	2.89
BSA	4.7	68.0	1.68
β-lactoglobulin	5.2	18.4	2.41
ribonuclease A	9.5	13.7	1.81
conulbumin	6.1	77.7	2.20
carbonic anhydrase	5.9	30.0	1.82
hemoglobin	6.8	67.0	2.11

[a] n=5

Experimental conditions: 100 mM formic acid-ammonium acetate, pH 2.50, acetonitrile 20% (v/v), 200 V/cm, 15 cm/22 cm of effective length/total length and detection wavelength 214 nm.

3.3. The effect of VGCE on fractionation of the mixture of two reference proteins

The mixture of BSA and lysozyme was used as the sample to prove the effect of VGCE on fractionation. Two proteins were partly resolved by conventional CE (Fig. 3A). Under the same experimental conditions, the mixture was then separated by VGCE (Fig. 3B-C). VGCE included two steps, i.e., step 1, $E_1 = 0$ and $E_2 = 200$ V/cm; step 2, $E_1 = E_2 = 200$ V/cm. Step 1 functioned as a cutting knife, pushing the component in motion phase II away from the components in motion phase I. Step 2 functioned as a conveyor, sending the components in motion phase I to motion phase II. By means of the two-step procedure, the sample zone was cut into subunits. The distance between two subunits was proportional to t_1 and the width of subunits was proportional to t_2, respectively. In Fig. 2B, the two-step procedure was carried out for 2 times, i.e., $t_1 = 100$ s, $t_2 = 49$ s and $t'_1 = 128$ s, $t'_2 = 44$ s. The sample zone was cut into three sections at the VG interface, i.e., pure lysozyme section, mixed section and pure BSA section. As we expected, two proteins were baseline resolved during three sections migrated from the VG interface to the detection window. The whole sample zone was fractionated into two pure lysozyme zones and two pure BSA zones. The result indicated that small amount of analyte led to higher resolution due to less dispersion. To further confirm this point, the two-step procedure was repeated for 7 times. For each procedure, $t_1 = 14$ s, $t_2 = 15$ s. The whole sample zone was fractionated into 8 subunits. Among them, three subunits were pure lysozyme zones, and 5 subunits were pure BSA zones (Fig. 3C).

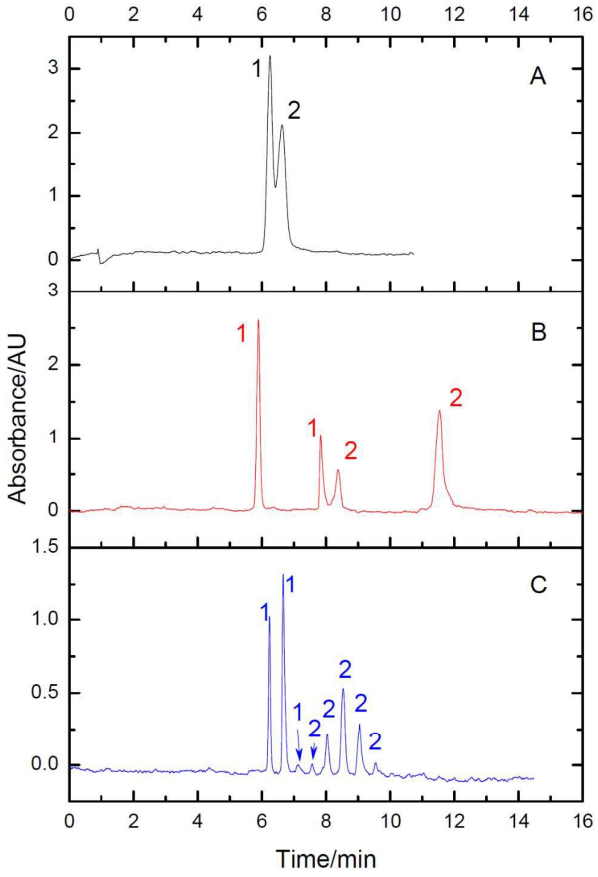

Fig. 3. The effect of VGCE on fractionating two reference proteins. Sample: 1-lysozyme and 2-BSA, 0.5 mg mL^{-1} for each. The separation was first performed in CE (A) and in VGCE (B-C). The two-procedure was carried out for 2 times. The sample was fractionated into two pure lysozyme peaks and two pure BSA peaks (B). The two-procedure was repeated for 7 times. The sample was fractionated into three pure lysozyme peaks and five pure BSA peaks (C). Experimental conditions: 100 mM formic acid-ammonium acetate, pH 2.50, acetonitrile 20% (v/v), 200 V/cm, 22 cm/30 cm of effective length/total length and detection wavelength 214 nm.

3.4. Fractionation of proteins in low abundance with VGCE

Many disease biomarkers in the serum are usually present at relatively low concentration [17]. They are easy to be submerged by the high-abundance proteins. Therefore, detection and identification of low-abundance proteins is a challenging task. Proteins in high-abundance are usually deleted from the sample for further analysis. But sample loss may company the deletion process. Based on the fact that VGCE can substantially reduce sample complexity, this problem can be easily solved via fractionating proteins in low-abundance from the proteins mixture. To confirm this point, lysozyme and BSA was mixed at the ratio of 1:150 to simulate low-abundance and high-abundance proteins. The peak of lysozyme was thoroughly subsumed by that of BSA and only mixture peak was observed in conventional CE (Fig. 4A). The mixture was then analyzed by VGCE. The two-step procedure was carried out for one time, t_1 = 202 s, t_2=49 s. The whole sample zone was fractionated into single pure lysozyme zone and two pure BSA zones (Fig. 4B). Enhanced resolution was achieved due to less dispersion. The result was consistent with our previous work.[26] Two advantages of VGCE should be mentioned in the analysis of the protein in low abundance. VGCE fractionated the protein in low abundance from the protein in high abundance to avoid the ionization suppression in MS. The other advantage was that a higher resolution could be obtained.

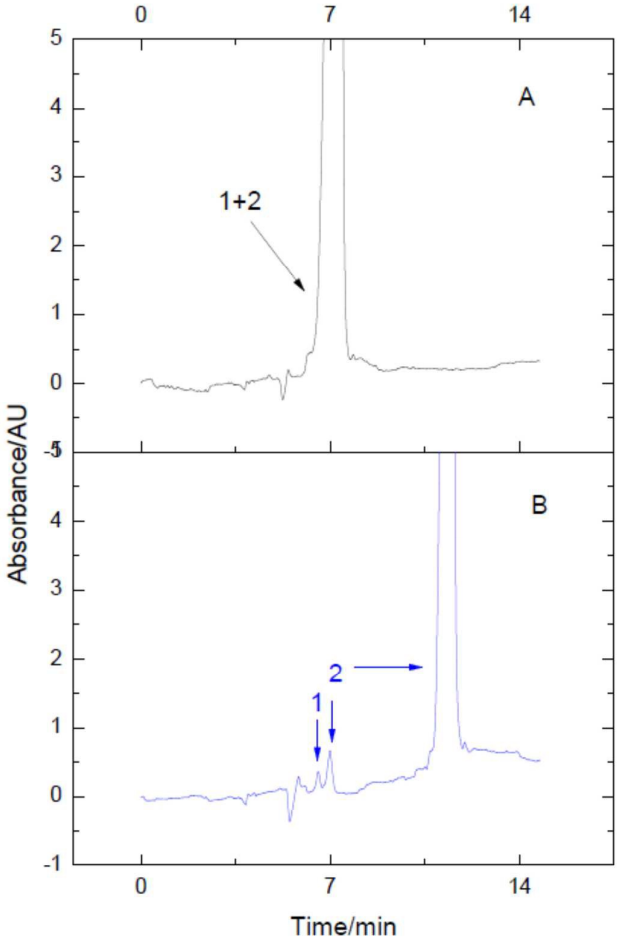

Fig. 4. Fractionating low-abundance protein from the mixture using VGCE. The mixture was composed of 1-lysozyme and 2-BSA. The concentrations of lysozyme and BSA were 0.19 mg mL^{-1} and 3.81 mg mL^{-1}. The separation was performed in CE (A). The separation was performed in VGCE and the two-step procedure was repeated for 1 time (B). The experimental conditions were the same as those in Fig. 3.

3.5. Enlarging the resolutions by VGCE

Various restrictions limit attempts to improve the resolution in CE, including high voltage, Joule heat, and effective separation length, etc. However, VGCE is able to enlarge the resolutions by simply adjusting t_1. The bigger t_1 was, the higher the resolution became. Separation of a protein mixture was carried out as an example. The mixture was composed of lysozyme, β-lactoglobulin and ribonuclease A. As shown in Fig. 5A, these three proteins were baseline separated in CE. The resolution between lysozyme and β-lactoglobulin was 1.91 (Rs_1=1.91). The resolution between β-lactoglobulin and ribonuclease A was 2.24 (Rs_2=2.24). When separations were carried out in VGCE, the resolutions were controllable. Adjusting t_1 = 1.5 min, the resolutions $R's_1$ = 5.70 and $R's_2$ = 7.30 (Fig. 5B). Adjusting t_1 = 5 min, the resolutions $R"s_1$ = 17.97 and $R"s_2$ = 25.54 (Fig. 5C). It was possible to further increase the resolutions if necessary. The enlarged resolutions may be useful when the separated components were analyzed by MS using off-line connection.

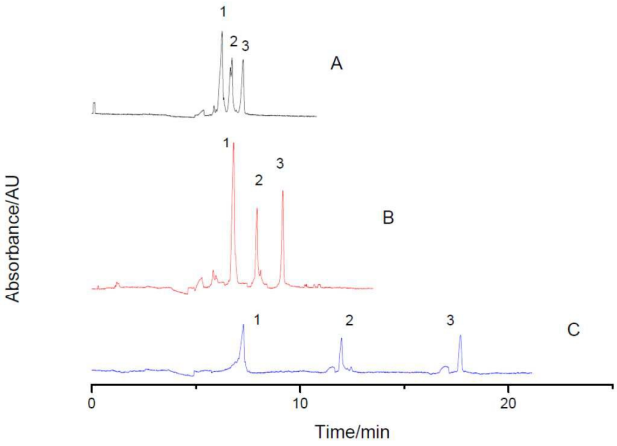

Fig. 5. Enlarging the resolution of two reference proteins in VGCE. Sample mixture was composed of 1- lysozyme, 2-β-lactoglobuline and 3-ribonuclease A. The final concentrations of all proteins were 0.5 mg mL^{-1}. The separation was carried out in CE as a reference (A). The separations were carried out in VGCE as t_1=1.5min (B) and t_1=5min (C). The two-procedure was repeated for 2 times. The experimental conditions were the same as those in Fig. 3.

3.6. Fractionating the mixture of 8 reference proteins using VGCE

Proteome samples are generally composed of many proteins. To test the feasibility of VGCE in the fractionation of proteomes, a mixture of eight reference proteins was separated as an example. The reference proteins had a wide range of isoelectric points, ranging from 4.6 to 11.0, and molecular weight ranging from 12.4 kDa to 77.7 kDa (Table 1). All reference proteins were positively charged in the BGE with pH 2.5. They migrated from anode to cathode in the electric field. For comparison, the separation of the mixture was first carried out in CE (Fig. 6A). According to different charge-to-mass ratios, the reference proteins sequenced in the sample zone. The sample zone was then fractionated into peaks by VGCE (Fig. 6B). The distance between two adjacent peaks was proportional to t_1, whilst the width of each peak was inversely proportional to t_2. In principle, the more pieces of the sample zone were cut, the more purified protein of each peak was obtained (It was proved in Fig 3C). The number of peaks was correlated with the repeating times of two-step procedure in VGCE. In this experiment, the two-step procedure was repeated 53 times and 54 peaks were obtained. In each two-step procedure, t_1 = 25 s and t_2 = 4 s. The number of the fractionated peaks was more than the number of the reference proteins because one reference protein zone may be cut into several pieces by VGCE.

It was worth noting that the identification of each peak would depend on mass spectrometry (MS). Our future work will focus on VGCE tandem MS to analyse proteome. Since VGCE can tremendously reduce sample complexity, it will undoubtedly facilitate identification work.

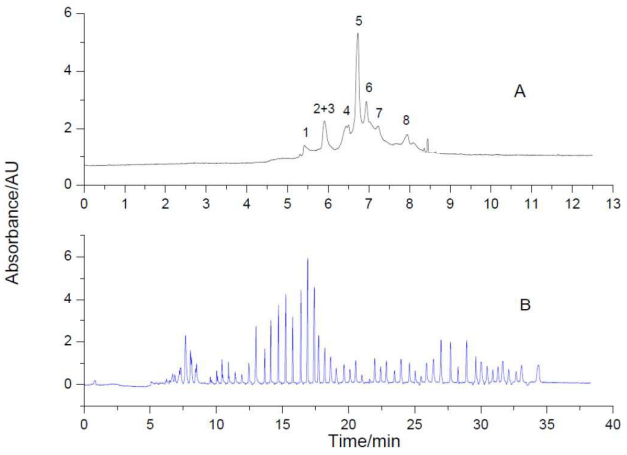

Fig. 6. Fractionation of 8 reference proteins using VGCE. Reference proteins were 1-cytochrome C, 2-lysozyme, 3-BSA, 4-β-lactoglobulin, 5-ribonuclease A, 6-conulbumin, 7-carbonic anhydrase, and 8-hemoglobin. The concentration of each protein was 0.5 mg mL^{-1}. The separation of the protein mixture was carried out in CE as a reference (A). The separation of the same sample was carried out in VGCE (B). The two-step procedure of VGCE was repeated for 53 times. The experimental conditions were the same as Fig. 3.

4. Conclusions

A new approach, VGCE, is proposed to fractionate the protein mixture. In addition to the advantage of high resolution, VGCE offers three extra advantages. The first is that VGCE is able to reduce the complexity of the protein mixture. Small difference of charge-to-mass ratio of two proteins leads to their different migration speed. Consequently, the different proteins are arranged in order during the separation procedure. When the protein mixture is fractionated into a series of subunits, the complexity of each subunit is reduced. The second is that VGCE offers a promising strategy in the analysis of low-abundance proteins in the proteomics. The third is that VGCE is a 1D separation technique, such that sample loss can be avoided, especially for precious proteins. The new approach will be run in tandem MS for top-down analysis of intact proteins in our future work.

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