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Over 1000-fold protein preconcentration for microliter-volume samples at a pH junction using capillary electrophoresis

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Abstract

An effective protein preconcentration technique specifically designed for microliter-volume samples is presented. The preconcentration is based on the capturing of protein in its isoelectric point (p*I*) within an applied electric field, using a pH junction created by a discontinuous buffer system. The buffers were chosen to selectively preconcentrate proteins of neutral p*I*, myoglobin in this case, while removing other proteins with acidic or basic p*I*s. For the suppression of electro-osmotic flow (EOF) and protein adsorption, the capillary inner wall was modified with a zwitterionic phospholipid bilayer coating. A preconcentration factor of up to 1700 was obtained for a 1 μg/mL solution of myoglobin. The preconcentration was completed in approximately 20 min. Several sample introduction conditions were presented to accommodate sample volume from one to a few hundreds of microliters. The final volume of the preconcentrated sample band was estimated to be approximately 5 nL.

Keywords: Protein preconcentration; Nanoliter-scale sample preparation; Capillary electrophoresis; Isoelectric point; Zwitterionic surfactant

1. Introduction

A major limitation in the current approaches of proteome research lies in the sensitivity towards low abundant components, which are often the most interesting from a biological standpoint; e.g., early detection of diseases. Even though the current state-of-the-art mass spectrometry (MS) allows detection of proteins at the zepto mole level, the sample must be pre-purified and preconcentrated to nano- or even subnanoliter-volumes, in order to meet the concentration detection limit of MS [1–3]. Nevertheless, most conventional sample handling and separation techniques are designed to work only as low as microliter-volumes. In other words, one must be able to preconcentrate proteins from microliter- to nanoliter-volumes in order to fully benefit from the high sensitivity of MS.

Solid phase extraction (SPE) based on reversed phase or affinity chromatography (e.g., Zip Tips) has been the conventional method of peptide and protein enrichment [4–6]. While it is generally effective for preconcentrating peptides, it can suffer from significant sample loss for proteins due to poor sample recovery from the stationary phase. More importantly, the elution volume typically far exceeds the nanoliter-scale.

Alternatively, numerous approaches of nanoliter-scale sample preconcentration have been developed with capillary electrophoresis (CE). In the recent work reported by Swerdlow and coworkers [7,8], an electric field was applied to a small section of conductive membrane within a flow channel to capture proteins with opposite net charges. A capturing time of 45 min and a concentration factor up to 40 was obtained. Customized fabrication of the capture device was required in this case. Many other online preconcentration methods more readily applicable to commercial instruments have also been developed and reviewed by Osbourn et al. [9]. One of the more common approaches is on-line sample stacking. Stacking is based on a reduction of the analytes' electrophoretic mobilities at the junction of a discontinuous buffer system, which compresses the ions to a narrow band. In field amplified sample stacking, the analytes' mo-

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bilities are modified by a drop in electric field strength generated by the different conductivity in discontinuous buffers [10,11]. Alternately, for weakly acidic or basic analytes, the alteration of mobility can be more effectively achieved by controlling the pH. In such pH-mediated stacking, the sample is prepared in a buffer having a different pH from that of the background electrophoretic buffer. Large sample volumes up to half of the total capillary length are injected. Upon voltage application, the sample molecules are slowed down at the pH junction and become stacked. Once stacked, the molecules typically remain charged at the pH junction, and thus will migrate out of the junction into the background buffer and continue with the normal zone electrophoresis. Using pH junctions, preconcentrations of peptides [12,13] and other small biological molecules have been reported with concentration factors from several tens to hundreds [14–17]. The use of pH junction for protein preconcentration has also been performed by Chang and coworkers [18], with concentration factors up to one hundred. While the improvement is significant, higher concentration factors are needed to bridge the gap between the microliter- and nanoliter-scale.

In this report, we will investigate an alternate method of preconcentrating proteins using a pH junction. In the previous attempts described above, the preconcentration was combined with the electrophoretic separation into a single step procedure. Hence, the sample loading was restricted to less than half of the capillary volume, and thereby limiting the concentration factor. To overcome this limitation, we will focus on the sample preconcentration and use a discontinuous buffer system that can sustain the pH junction for long periods of preconcentration time. The idea is similar to the work recently reported by Shave and Vigh, in which isoelectric trapping of proteins took place in between two isoelectric membranes of pre-defined pH values [19,20]. In our work, the proteins will be trapped isoelectrically at the junction of two buffers that are at pH values higher/lower than the p*I* of the protein. The objective is to perform sample loading exceeding 100% of the capillary volume and thus achieving much higher preconcentration factors than those previously reported.

2. Experimental

2.1. Apparatus

All protein preconcentration experiments were performed on an Agilent 3D-CE Capillary Electrophoresis Instrument (Palo Alto, CA, USA) with direct UV absorbance detection at 200 nm. Data acquisition was obtained through the ChemStation software by Agilent. Uncoated capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The dimensions of the capillaries were 50 μm i.d., 360 μm o.d., 48.5 cm in total length and 40 cm to detector. The capillaries were thermostated at 25 °C.

2.2. Reagents and samples

All solutions were prepared using 18.2 MΩ deionized water from a Millipore Water Purification System (Bedford, MA, USA). Reagent grade sodium hydroxide, acetic acid, phosphoric acid, and ammonium hydroxide were purchased from EM Science (Gibbstown, NJ, USA). The zwitterionic phospholipid, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) (Sigma–Aldrich, St. Louis, MO, USA) was prepared as 0.1 mM solution in 20 mM Tris–HCl (Sigma–Aldrich) at pH 7.2 containing 20 mM of calcium chloride (Sigma–Aldrich), according to reference [21]. DLPC forms a semi-permanent bilayer coating on the silica capillary surface that is effective in suppressing the electroosmotic flow and minimizing protein adsorption onto the capillary wall [21,22]. Myoglobin (horse heart), amyloglucosidase (*Aspergillus niger*), cytochrome *c* (bovine heart), and carbonic anhydrase I (human erythrocyte) were used as received from Sigma–Aldrich.

2.3. Protein preconcentration

Prior to use, all new capillaries were flushed (1 bar) with 0.1 M sodium hydroxide for 5 min, deionized water for 5 min, and finally with DLPC for 20 min. In between runs, the capillary was re-coated with DLPC for 10 min (1 bar). The pH junction was formed by the discontinuous buffers of pH 9.25 ammonium and pH 4.75 acetate, both at 10 mM. The pH of the acetate buffer was adjusted with ammonium hydroxide, and likewise the pH of the ammonium buffer was adjusted with acetic acid. The proteins were prepared in either one of the ammonium or acetate buffer, or in both buffers. The typical volume of the sample solution placed in the reservoirs at the electrodes was 100 μL. The concentrations of the proteins were 0.001–0.1 mg/mL for myoglobin, 0.5 mg/mL for amyloglucosidase, 0.2 mg/mL for cytochrome *c*, and 0.9 mg/mL for carbonic anhydrase I. Application of constant voltage of 20 kV was used to perform the protein preconcentration.

3. Results and discussion

3.1. Protein preconcentration by discontinuous buffer

In our preconcentration technique, the pre-purified proteins were concentrated as zwitterions at the junction of discontinuous buffers, in which the pH was higher than the protein's p*I* on one side and lower than the protein's p*I* on the other. The protein sample was placed in either one or both of the two buffers, and all protein molecules acquired a net cationic or anionic charge. When the acidic end of the capillary was placed at the anode and the basic end was placed at the cathode, all protein ions electromigrated towards the junction. As they approached the junction, the pH change altered the protein's ionization. Since the p*I* of the protein coincided

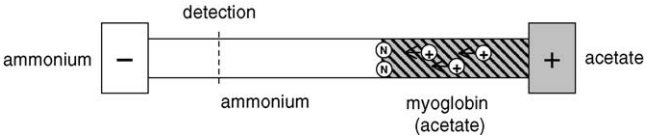

Fig. 1. Schematic representation of protein preconcentration at the pH junction. The cations represent myoglobin in acetate buffer and the neutral molecules represent zwitterionic proteins at the pH junction. The shaded sections are filled with acetate, and the unshaded sections are filled with ammonium. Crosshatch represents the presence of the proteins.

within the pH shift at the junction, all protein molecules were focused at the junction as zwitterions.

The effectiveness of this preconcentration technique was determined by the pH profile of the discontinuous buffers. Ideally, the proteins should be stacked as a very narrow band for high preconcentration factors, and hence a sharp pH drop is preferred at the junction. To achieve such a step-gradient pH profile, the two buffers should only provide significant buffering capacities at the acidic and basic regions but little or no buffering capacity at the junction (protein's p*I*). In this work, ammonium and acetate were selected for the discontinuous buffers of pH 9.25 and 4.75, respectively. This system allowed high buffering capacities above pH 9 and below pH 5, and little capacity at pH 7. Myoglobin was selected as a model protein to evaluate the preconcentration. It has a p*I* of 7.2, but also remains soluble in acidic and basic pH environments.

The progression of protein preconcentration upon voltage application was monitored by UV-absorbance detection. After pre-conditioning with DLPC, the capillary was filled with the ammonium buffer, and was then injected (30 mbar) with myoglobin in acetate to approximately one-third of the capillary length (Fig. 1). Voltage was applied to induce preconcentration at different time periods (0, 2, 4, 6 and 10 min), and the capillary content was then mobilized at low pressure (30 mbar) for detection. The results are shown in Fig. 2. Without the application of voltage, the expected long plug-shape signal was recorded for the original 0.1 mg/mL myoglobin

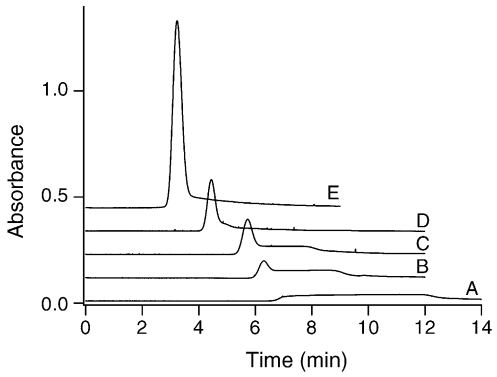

Fig. 2. Progression of myoglobin preconcentration under increasing periods of voltage application: (A) 0 min, (B) 2 min, (C) 4 min, (D) 6 min, and (E) 10 min. Experimental conditions: myoglobin concentration, 0.1 mg/mL; otherwise as shown in Fig. 1.

injection, and the absorbance intensity was approximately 0.030 (Fig. 2A). After 2 min of voltage application (Fig. 2B), peak formation was observed at the front of the sample plug, which corresponded to the ammonium/acetate junction. With longer periods of voltage application, the preconcentration proceeded to yield an intensifying peak (Fig. 2C and D). Finally after 10 min of voltage application, the peak shoulder/tail from the initial sample plug disappeared (Fig. 2E), suggesting the completion of preconcentration. A final peak height approaching an absorbance of 1 was recorded. Such high absorbance reading is rarely obtained in conventional capillary zone electrophoresis (CZE) since the peak height from a concentrated sample is usually limited by band broadening due to electrodispersion. However, in this case, the concentrating effect at the pH junction appeared to overcome the electrodispersion and resulted in the exceptionally intense signal. To prove that the observed peak formation was not an artifact arising from the pH junction, the same experiment was repeated in the absence of proteins. No intense peaks were recorded, and only a small absorbance shift of 0.005 in the baseline was observed at the position where the intense peak was anticipated (data not shown). This can be explained by the difference in background absorption between the ammonium and acetate buffers.

Based on the difference in peak height between Fig. 2A and E, the calculated preconcentration factor is approximately 30. It should be noted, however, that a nonlinear calibration is expected at an absorbance of 1 due to stray light. Hence the actual preconcentration factor may be much higher than 30. Furthermore, the peak height is also limited by band broadening during pressure mobilization and limited sample loading (one-third of capillary volume). The experimental conditions will be modified in a later section to address these issues and improve the concentration factor.

It should be noted from Fig. 2 that the elution time of the peaks was shifting ahead with longer periods of voltage application; i.e., the concentrated myoglobin band was moving forward during the preconcentration. To determine the cause of such movement, a short band of mesityl oxide was injected to mark the ammonium/acetate buffer junction. Although the mesityl oxide peak was seriously broadened by laminar flow, it appeared to overlap with the preconcentrated myoglobin peak (data not shown). Since mesityl oxide is neutral, its movement can be attributed to a residual EOF, which is known to exist in a DLPC treated capillary [21]. This allowed us to conclude that myoglobin remained trapped at the pH junction during preconcentration, and the EOF slowly moved the pH junction forward to the detector. Nevertheless, the magnitude of this residual EOF was low ($<10^{-4}$ cm^2/(V s)), and thus allowed sufficient time (10–20 min) to complete the preconcentration before moving the sample band pass the detector.

To illustrate the importance of the selection of the discontinuous buffer, an alternate buffer system of pH 6 and pH 8 phosphate was attempted (both at 10 mM, pH adjusted with sodium hydroxide). The same experiment as in Fig. 2 was repeated with the two phosphate buffers. In this case, the rect-

angular plug-shape signal did not undergo any sharpening, and was only broadened with increasing voltage application (data not shown). Increases in peak height were not observed even after 10 min of voltage application, which concluded that the preconcentration was unsuccessful. In contrast to the ammonium/acetate buffers, the phosphate/phosphate system provided a strong buffering capacity at the junction (~pH 7). Hence, it produced the pH profile of a gradual-gradient at the junction, as opposed to the step-gradient formed by the ammonium/acetate buffers. As a result, the protein molecules could not be enriched to a sharp band.

3.2. The selective nature of protein concentration based on pI

The selected buffer system of ammonium/acetate was designed to preconcentrate proteins with neutral p*I* such as myoglobin, and not other proteins with acidic or basic p*I* values. To illustrate such selective nature, similar experiments as in Fig. 2 were conducted with two different proteins, amyloglucosidase and cytochrome c. Since the proteins cannot be differentiated by the UV-absorption detection used in this work, the experiment was performed individually for each protein. Starting with amyloglucosidase, 20 kV of voltage was applied at increasing time intervals to induce sample preconcentration. A small degree of peak sharpening began to occur at the front of the band, but the increase in height was less than a factor of 2 (Fig. 3). Such minor sample stacking at the pH junction was similar to that previously reported [18]. During injection of amyloglucosidase in acetate, some of the amyloglucosidase molecules entered the ammonium buffer due to laminar flow mixing. Upon voltage application, these molecules initially had higher mobilities in ammonium, but slowed down when they returned to acetate, resulting in the observed stacking. However, this effect did not lead to the substantial sample preconcentration observed for myoglobin (Fig. 2). With a p*I* of 3.6, amyloglucosidase was anionic in the pH 4.75 acetate, and therefore was constantly migrating towards the inlet, away from the pH junction. This was shown in Fig. 3, where the onset of the signals was delayed with increasing voltage application time. The amyloglucosidase band eventually moved out of the capillary after 6 min (Fig. 3D). This phenomenon enables the removal of amyloglucosidase (an acidic p*I* protein) during the preconcentration of myoglobin (a neutral p*I* protein), when both proteins are present in a mixture.

Next, the same experiment was repeated for cytochrome c. The p*I* of this protein is 10.6 and therefore should be highly cationic at pH 4.75 acetate and slightly cationic at pH 9.25 ammonium. Under the experimental conditions of Fig. 2, the injected cytochrome *c* molecules in acetate would migrate forward towards the buffer junction, and would then experience a significant reduction in mobility when crossing the junction to the ammonium section, resulting in sample stacking. As predicted, a concentration factor up to 10 (from 0.030 to 0.300 in absorbance) was recorded for 0.2 mg/mL cytochrome *c* (data not shown). Although the concentration factor was lower than that of myoglobin, the concentrated cytochrome *c* band may overlap with the myoglobin band when both proteins are present. In order to isolate the proteins from one another, an alternate experiment was performed. In this case, the cytochrome *c* sample was prepared in the ammonium buffer, as illustrated in Fig. 4(upper panel). The position of the detector was relocated closer to the anode, and pressure from the cathodic end was used to mobilize the sample zone pass the detector after voltage application. The results are shown in Fig. 4(lower panel). Essentially, the cytochrome *c* plug was not preconcentrated upon voltage application. It turns out that cytochrome *c* was less soluble in the ammonium buffer, possibly due to its near-zero net charge at pH 9.25. As a result, adsorption onto the capillary wall became serious, as evident by the peak tailing behavior in Fig. 4A. Unfortunately, such protein adsorption made the data less reproducible and more difficult to interpret after voltage application (Fig. 4B–D). Nevertheless, it appeared that the cytochrome *c* plug remained in the capillary after voltage application, but significant preconcentration was not evident.

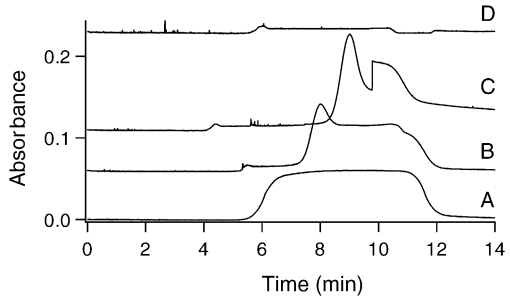

Fig. 3. Migration of amyloglucosidase in discontinuous ammonium/acetate buffers under various periods of voltage application: (A) 0 min, (B) 2 min, (C) 4 min, and (D) 6 min. Conditions: amyloglucosidase concentration, 0.5 mg/mL; otherwise as shown in Fig. 1.

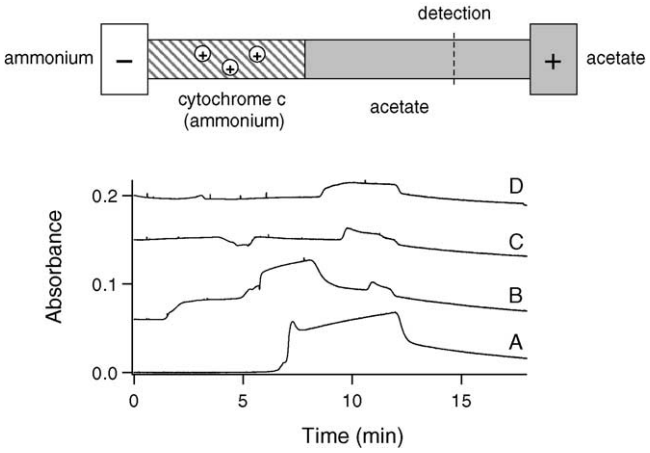

Fig. 4. Experimental setup (upper panel) and migration of cytochrome *c* in discontinuous ammonium/acetate buffers under various periods of voltage application (lower panel): (A) 0 min, (B) 2 min, (C) 4 min, and (D) 6 min. Experimental conditions: cytochrome *c* concentration, 0.2 mg/mL.

Hence, the cytochrome *c* plug should not overlap with the preconcentrated myoglobin peak when both are present in the sample, and therefore should result in a separation during the preconcentration.

3.3. Preconcentration under different sample introduction conditions

In the previous section, it was determined that a low EOF existed during the preconcentration of myoglobin. Such an EOF can be used to carry the enriched protein band towards the detector and eliminate the need of pressure mobilization. Since the direction of the EOF is cathodic, the pH junction should be initiated from the anode end of the capillary. This was achieved by first pre-filling the capillary with ammonium buffer, and then placing the anodic end of the capillary in the acetate reservoir. Preconcentration of 0.01 mg/mL myoglobin was performed using this setup, under three different conditions of sample introduction as illustrated in Fig. 5. In the first case (Fig. 5A), the capillary was pre-filled with myoglobin solution prepared in pH 9.25 ammonium. This provided a sample loading of a full capillary length or 0.95 μL for a 48.5 cm long capillary. In the second case (Fig. 5B), myoglobin was placed in the acetate reservoir (100 μL). Myoglobin migrated as cations and continuously entered the capillary during the preconcentration. The sample loading was more difficult to estimate in this case, as it depended on the relative mobilization between the protein and the pH junction (EOF). Depending on the protein's compatibility with acid or base, one of the two conditions above may be more suitable. In the third case (Fig. 5C), myoglobin was placed in both the ammonium and acetate reservoirs, as well as prefilling the capillary with myoglobin in ammonium. Although it was less convenient to prepare the proteins in both buffers, this setup allowed maximum and continuous sample loading from both capillary ends during the entire preconcentration period. While not all of the protein molecules in the vials were loaded in one run, the applied voltage was not destructive to the proteins, and the remaining sample could be used for additional preconcentration.

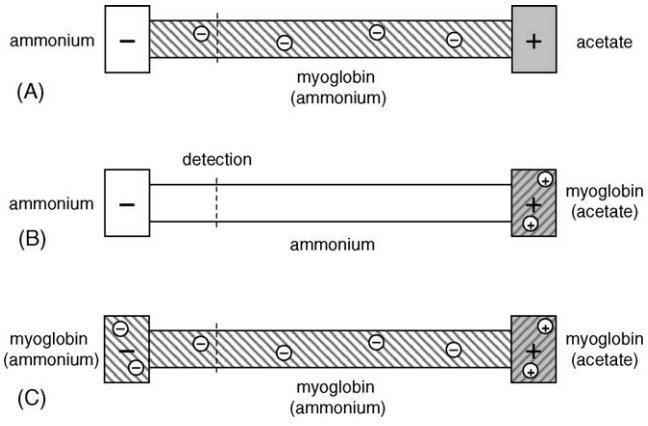

Fig. 5. Schematic depictions of the various sample introduction conditions used in the preconcentration of myglobin: (A) capillary pre-filled with myoglobin in ammonium, (B) myoglobin in the acetate reservoir at anode, and (C) myoglobin in both the ammonium and acetate reservoirs and in the capillary.

In all three conditions, a sharp peak was obtained for the preconcentrated myoglobin in each run. Under the conditions illustrated in Fig. 5A and B, the average absorbance was 0.65 and 0.91, resulting in high preconcentration factors of 220 and 300, respectively. The preconcentration factors were determined from the average peak height taken from at least three replicates and an initial (prior to preconcentration) absorbance of 0.003. The observed peak width at half-height ranged from 1 to 4 s, indicative of the highly compressed sample bands resulted from the step-gradient pH junction.

In the final sample introduction condition (Fig. 5C), the peak height reached an absorbance of 1.30, with a width of 7 s at half-height. Thus, the preconcentration factor was improved to 435. However, at such a high absorbance reading, stray light interference was very probable. The experiment was therefore repeated with myoglobin diluted by 10-fold to 1 μg/mL. A peak height of 0.51 in absorbance (preconcentration factor of 1700) and a width of 2 s was recorded (Fig. 6). Further dilutions were also performed. However, protein loss due to adsorption onto the vials became significant at those very low concentrations, and therefore greater preconcentration factors were not observed.

The migration time for the preconcentrated myoglobin peaks recorded in all cases were found to vary roughly from 10 to 20 min. This was determined to be caused by the changes in the EOF from day to day. Protein adsorption was responsible for this EOF variation, as a separate experiment monitoring the EOF in the absence of proteins showed very little change. Even though the DLPC bilayer has been proven to be effective in preventing protein adsorption in CZE [21,22], the amount of proteins exposed to the capillary surface is much greater in the preconcentration experiments. In CZE, protein is typically introduced as a small plug of a few nanoliters for each run. In contrast, for the protein preconcentration experiments, up to a few capillary volumes of protein solution were rinsed through the capillary in each run, and as a result, significantly more serious protein adsorption was probable. We were able to achieve stable EOF under 10^{-4} $cm^2/(V\,s)$ for 3–5 days before having to start with a new capillary.

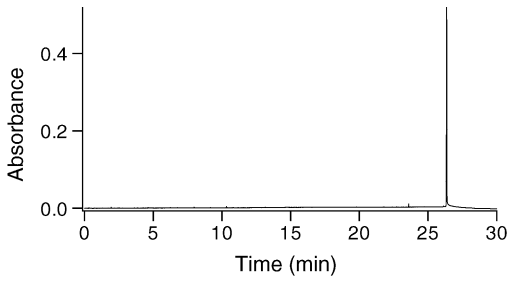

Fig. 6. Preconcentration of 1 μg/mL myoglobin. Conditions as in Fig. 5C.

To estimate the volume of the preconcentrated protein band, the peak widths were determined and they ranged from 3 to 9 s. Taking the migration times into account, the estimated volume of the preconcentrated peak was approximately 5 nL. As a comparison, the initial sample volume required in each run ranged from one capillary volume (1 μL) in Fig. 5A to the volume of two sample vials in Fig. 5C (100 μL each, but can be reduced to a few tens of microliters). Hence, our technique was capable of preconcentrating microliter-volume protein samples to the nanoliter-volume scale required for subsequent analyses such as mass spectrometry or capillary separation.

In addition to myoglobin, preconcentration of another protein of similar p*I*, carbonic anhydrase I (p*I* 6.60), was also attempted. Similar preconcentration behavior was observed (data not shown). When we performed preconcentration with both proteins present, a single peak similar to Fig. 6 was obtained, though the peak appeared wider in this case.

4. Conclusion

State-of-the-art analytical techniques, such as mass spectrometry and capillary-format separations, are down-sizing the optimal sample volume to nanoliters for improved sensitivity. Yet, most conventional protein preconcentration methods are designed to enrich samples from milliliters to microliters. To bridge the gap between the micro- and nano-scales, a pH junction from a discontinuous buffer system within a capillary was used to preconcentrate protein samples with microliter-volumes. Concentration factors exceeding 1000 was achieved, and the estimated volume of the preconcentrated sample was 5 nL.

It should be possible to further improve the preconcentration factor by using longer capillaries, although longer preconcentration times will also be required. Likewise, the volume of the preconcentrated sample band can be increased by using larger i.d. capillaries, though the magnitude of the applied voltage may be limited by joule heating.

With the selected buffer system of ammonium and acetate, one can simultaneously perform preconcentration of proteins with neutral p*I* and the removal of other proteins with acidic or basic p*I*s. This can be extremely useful in preconcentrating/purifying simple protein mixtures, such as fractions from a chromatographic separation. For more complex mixtures, subsequent separation will be required to resolve proteins of similar p*I*s. The coupling of preconcentration with conventional capillary zone electrophoresis is underway. More importantly, it should be noted that our protein preconcentration occurs at a liquid junction, and therefore one can expect a much more quantitative sample recovery compared to that from conventional sorption-based sample preconcentration.

Acknowledgements

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