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Regulation of electroosmotic flow and electrophoretic mobility of proteins for concentration without desalting

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Abstract

Proteins were concentrated and separated in 0.6% poly(ethylene oxide) (PEO) solution using a capillary filled with Tris–borate (TB) buffer prior to analysis and detected by laser-induced native fluorescence using a pulsed Nd:YAG laser. During the concentration and separation, PEO solution entered the capillary by electroosmotic flow. When proteins dissolved in high salts (phosphate-buffered saline) were separated using 0.6% PEO solution prepared in 200 m*M* TB buffer, pH 9.0, the limits of detection (LODs) at signal-to noise ratios=3 for carbonic anhydrase (CA) and α-lactalbumin (α-lac) were on the levels of sub μ*M* and μ*M*, respectively. The LOD values compared to those obtained in 38 m*M* TB buffer were relatively high, which is likely due to salt quenching, Joule heating and poor stacking. To improve sensitivity for analysis of proteins in high-conductivity media, two on-line concentration approaches without desalting were developed. When using a capillary filled with 1.5 *M* TB buffer, pH 10.0, and PEO solution prepared in 800 m*M* TB buffer, pH 9.0, the LOD values for CA and α-lac were 13.8 n*M* and 126.0 n*M*, respectively, which were about 4.7 and 11.2-fold sensitivity enhancements compared to those obtained by a conventional hydrodynamic injection (30 cm height for 10 s), respectively. The sensitivity was further improved by injecting a short plug of low pH buffer after protein injection using a capillary filled with 1.5 *M* TB buffer, pH 10.0, and PEO solution prepared in 400 m*M* TB buffer, pH 9.0. A linear relationship between the peak height and the injection volume up to 0.81 μl was obtained and the LOD values for CA and α-lac were down to 4.7 and 37.8 n*M*.

Keywords: Electrophoretic mobility; Electroosmotic flow; Proteins; Poly(ethylene oxide)

1. Introduction

There has been a long-standing interest in elucidation gene products more directly on a protein level [1,2]. The identification of proteins on cellular or subcellular levels is important for understanding which cellular compartment the protein exerts its function in [3]. Protein expression profiles can be analyzed using electrophoresis, such as capillary electrophoresis (CE) [4,5] and two-dimensional sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [6,7], in conjunction with mass spectrometry [8] or silver staining [9]. Typically, desalting, preconcentration, and derivitization are performed for optimum sensitivity and resolution prior to analysis by these techniques. However not only are these methods tedious and highly costly, but also denaturation, loss of samples and contamination may occur. Thus, methods allowing direct analyses of biological samples are highly demanded.

Over the past decade, CE in combination with laser-induced fluorescence (LIF) has been popular for protein analysis because of its high-resolving power, sensitivity, and rapidity [10–12]. In CE, a deactivated capillary is commonly used to minimize

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interactions with proteins and variations of electroosmotic flow (EOF) [13–16]. To further improve resolution for separating proteins with different sizes, deactivated capillaries filled with gel matrices or polymer solutions have been employed [17,18]. For analysis of trace proteins in biological samples such as plasma, CE with LIF is superior over with UV absorbance detection that is more universal but less sensitive. To perform CE–LIF, proteins must be labeled with suitable fluorephores such as fluorescein and its derivatives or contain tryptophan, tyrosine and phenylalanine residues [19–21]. Generally, a limit of detection (LOD) at the n*M* level is easily achieved under mild conditions, such as low ionic strengths [22].

Although CE is powerful for analysis of biological samples, a technique permits analysis of trace amounts of analytes such as retinol binding proteins in plasma is still needed [23]. To increase sensitivity, several concentration techniques such as isotachophoresis [24–27] and field amplification [28] have been developed in CE. However matrices cause a number of problems, including irreproducibility, poor resolution, poor stacking, and a short lifetime of capillaries [29–32]. Salts were also detrimental to fluorescence intensity when CE–LIF was applied to protein analysis. For example, we have found that the LOD for human serum albumin (HSA) in urine or phosphate-buffered saline (PBS) is only at sub μM levels, which is at least one order higher than that in 50 m*M* Tris–borate (TB) buffer, pH 9.0 [33]. To minimize problems associated with matrix, samples are generally subject to desalting by membrane filtration [34], chromatographic separation [35] and so on prior to analysis. In addition to tedious processes and high cost, loss of analytes and contamination may occur during pretreatment. Recently, on-line concentration techniques using membrane or hollow fiber have been developed [36,37]. These methods provide a high concentration factor and less matrix problems, while suffering from limits to large size or hydrophobic proteins and no ease for automation.

Recently, we have developed a method for on-line protein concentration using bare fused-silica capillary and a low-cost Nd:YAG laser [33]. During concentration and separation, poly(ethylene oxide) (PEO) solution enters the capillary filled with buffer by EOF. With negative charges, proteins traverse the boundary between the sample zone and the PEO solution during concentration and separation. Consequently, proteins stack mostly due to retardation by PEO matrices. When migrating against EOF, proteins with low electrophoretic mobility (μ_{ep}) are detected earlier in the cathode end. The LOD for CA is about 31 p*M* when injected at 15 kV for 600 s, which is about three orders of magnitude of sensitivity enhancement compared to that injected at 1 kV for 10 s. Although this new method provides advantages over most conventional methods [24–28] in CE, including no need to fill the capillary with polymer solution prior to analysis; high stacking efficiency; less susceptibility to matrices; and simplicity, proteins in high salts did not stack effectively. Consequently, the maximum volume injected without loss of resolution was only 9.6 nl and the LOD values for most proteins were only at sub–μM levels. In an attempt to overcome problems associated with matrices, we have developed two methods for protein analysis without tedious desalting processes. In order to enhance stacking efficiency (sensitivity), proteins prepared in high-salt media were injected into a capillary filled with 1.5*M* TB buffer and separated using PEO solution prepared in 800 m*M* TB buffer. The second strategy is that we injected a plug of low pH buffer after sample injection to optimize sensitivity. In this report, the results from these two new methods were compared with respects to maximum sample loading, stacking, migration time, and LOD.

2. Materials and methods

2.1. Equipment

The basic design of the separation system has been previously described [33]. Briefly, a high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) was used to drive electrophoresis. The entire detection system was enclosed in a black box with an high-voltage interlock. The high-voltage end of the separation system was housed in a Plexiglass box for safety. A Nd:YAG laser with 266 nm, 3000 Hz output from Continuum (model: EPO-5000, Santa Clara, CA, USA) was used for excitation. One UG 5 filter from Melles Griot

(Irvine, CA, USA) was used to block 532-nm light from the laser. The emission light was collected with a 10× objective (numerical aperture=0.25). One UG 1 filter from Edmund (Barrington, NJ, USA) was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The amplified current was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 5 Hz (Borwin™, JMBS Developments, Le Fontanil, France) and stored in a personal computer. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D.×365 μm O.D. were used for protein separations without any coating process.

2.2. Chemicals

All chemicals for preparing buffers were obtained from Sigma (St Louis, MO, USA), except that PEO (M_r 8 000 000) was from Aldrich (Milwaukee, WI, USA). TB buffers prepared from tris(hydroxymethyl)aminomethane (Tris) were adjusted with boric acid to pH 10.0 and 9.0. Unless otherwise noted, *X* m*M* TB buffer at different pH values means *X* m*M* Tris solution adjusted with a suitable amount of boric acid. PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KH_2PO_4, 0.46 g of Na_2HPO_4, and 0.2 g of KCl in 1l of H_2O, pH 7.4. PEO (0.6 g) was added to 100 ml of the TB buffers. Carbonic anhydrase (CA), β-casein, α-lactalbumin (α-lac), and trypsin inhibitor were dissolved in water and diluted to suitable concentrations with PBS prior to analysis.

2.3. Polymer solution

Increasing amounts of PEO were gradually added to the above buffers, respectively, in a water bath at 85–90°C. During the addition of PEO, a magnetic stirring rod was used to produce a homogeneous suspension. After adding the PEO, the solution was stirred for a further hour. PEO solutions were degassed with a vacuum system in an ultrasonic tank. The PEO solutions stored in a refrigerator at 4°C were usable for 4 days.

2.4. On-line concentration and separation

Capillaries were treated with 0.5 *M* NaOH overnight prior to use. Before each run, capillaries were washed with 0.5 *M* NaOH at 1.0 kV for 10 min to remove PEO solution and refresh the capillary wall. Hydrodynamic injections were performed at 30 cm height for times over 10–520 s. The end of the capillary was then immersed in PEO solution. During the analysis, PEO solution entered the capillary by EOF.

3. Results and discussion

3.1. Effect of TB buffer filled in capillaries

When injecting a long plug of proteins dissolved in high-conductivity media (e.g. PBS) into a capillary filled with low concentrations of TB buffer (<400 m*M*), band broadening occurs due adjusting the sample to the omega function inside the capillary [38], which causes poor resolution, loss of sensitivity and irreproducibility. When using a capillary filled with low concentration of TB buffer, irreproducibility and long separation time are also problematic because PEO adsorption on the capillary wall is marked that results in small EOF [33,39]. Another problem associated with high-conductivity samples is a long separation time because a lower voltage is generally applied to minimize Joule heating. To achieve reasonable resolution and sensitivity, it is necessary to refocus the bands during separations. It however is not as easy as that in low-conductivity media because of relatively low μ_{ep} of proteins in high salts, wherein proteins spend much longer times entering PEO solution from the sample zone. As a result shown in our previous study, the maximum sample loading volume without loss of resolution is much smaller (9.6 nl) and the LOD for CA was 0.18 μ*M* when injecting high-conductivity samples containing CA, β-casein, α-lac, and trypsin inhibitor [33].

If our reasoning above is right, concentration should be optimized if the differences in conductivities among TB buffer, sample zone, and PEO are minimized and the electrophoretic mobility of protein in PEO solution reduced. To test this hypothesis, using a capillary filled with high concentrations of TB buffer and/or separating proteins in PEO solution prepared in high concentrations of TB buffer should be worthy trying. Table 1 shows the results of

Table 1
Effect of the concentration of free buffer and PEO solution on the migration time, peak height and peak width when injection volumes were 60 nl and 280 nl respectively

TB buffer[a] (*M*)	TB buffer[b] (m*M*)	V_{inj} (nL)	Migration time (min)		Peak height (μV)		Peak width (min)	
			CA	β-Casein	CA	β-Casein	CA	β-Casein
2.5	200	60[c]	14.41	15.66	278	297	0.12	0.17
2.0	200	60[c]	14.82	16.13	318	317	0.13	0.21
1.5	200	60[c]	15.25	16.60	351	362	0.16	0.27
0.4	200	60[c]	N.D.[d]	N.D.	N.D.	N.D.	N.D.	N.D.
0.4	800	60[c]	34.25	35.66	112	79	0.34	0.21
1.5	200	280[e]	11.07	12.50	163	N.R.[f]	0.07	N.R.
1.5	400	280[e]	18.78	19.92	332	259	0.15	0.18
1.5	800	280[e]	21.43	22.84	334	321	0.14	0.18

[a] pH 10, used to fill the capillary before separation.
[b] pH 9.0, used to prepare PEO solution.
[c] [CA]=0.5 μ*M*, [β-casein]=10 μ*M*.
[d] Not detected.
[e] [CA]=0.25 μ*M*, [β-casein]=5 μ*M*.
[f] Not resolved from the peak corresponding to α-lac.

injecting 60-nL samples when using different TB buffers to fill the capillary and to prepare PEO solutions, respectively. When using 400 and 200 m*M* TB buffers to fill the capillary and to prepare PEO solution, the peaks corresponding to CA and β-casein were not detected, implying poor stacking. Although they were detected using 800 m*M* TB to prepare PEO solution, separation times were long (migration time for CA was 34.25 min) and protein did not stack effectively (relatively low intensities and broad peaks). For example, the maximum injecting volume was about 120 nl and the LOD for CA was about 28.4 n*M*. Herein we defined that the maximum injection volume was the one to obtain the maximum fluorescence intensity without dramatic loss of resolution (R_s>1.25) under certain conditions, such as ionic strength, pH, capillary length, Joule heating, and PEO solution. When comparing the results using a capillary filled with 1.5, 2.0, and 2.5 *M* TB buffers, respectively, and PEO solution prepared in 200 m*M* TB buffer, pH 9.0, peak heights decreased with increasing TB concentration, presumably because of the salt dependence of the quantum yield. To the end, we suggested that the capillary filled with 1.5 *M* TB buffer was superior.

3.2. Effect of PEO solution

To further explore the effect of PEO solution on stacking, resolution and migration time, we compared the results obtained from using 200, 400 and 800 m*M* TB buffers to prepare PEO solutions, respectively. As expected, Fig. 1 shows that peak height and migration times both increased with increasing TB concentration when injecting 280 nl of the sample. In 1.5 *M* TB buffer, PEO adsorption on the capillary wall was not profound [39]. In other words, PEO adsorption did not play a significant role in determining EOF. On the other hand, in addition to viscosity, the ionic strength of PEO solution was another major factor for determining EOF. Please note that β-casein and α-lac were not resolved when using 200 m*M* TB to prepare PEO solution. To conveniently compare the effects of PEO solution on protein analysis, the limits of detection (LOD) at signal-to-noise ratios=3, concentration factor (the ratio of the LOD for proteins injected at 30 cm height for 10 s to that for 180 s), and plate number were tabulated in Table 2. Although the separation time was comparatively shorter, PEO solution prepared in 200 m*M* TB buffer was not suitable, with respect to resolution and stacking. For CA, β-casein, and α-lac, use of PEO solution prepared in 800 m*M* TB provided better sensitivity and higher concentration factor (except that for CA). The LOD for carbonic anhydrase was 13.8 n*M*, which was a 4.7–fold enhancement in sensitivity compared to that at 30 cm height for 10 s. On the other hand, for trypsin

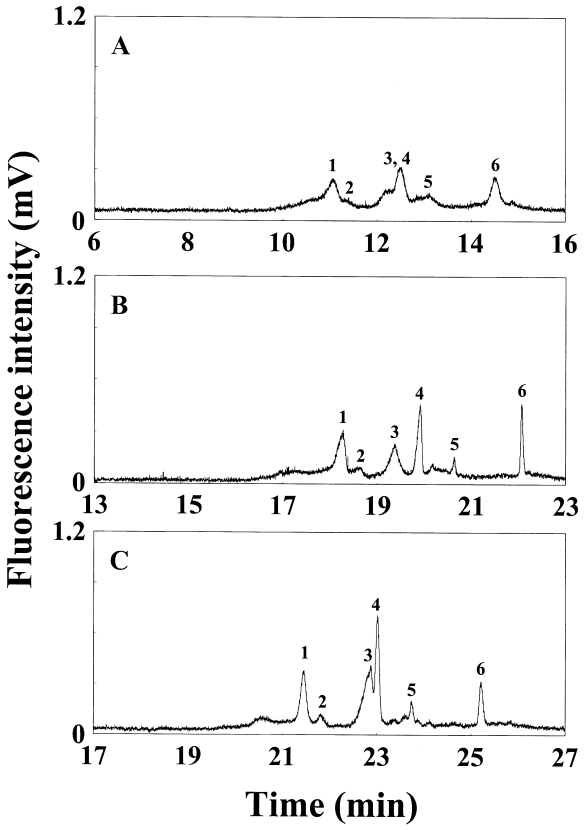

Fig. 1. Effect of PEO solution on on-line concentration and separation of sample proteins at 15 kV. The 60-cm capillary (50 cm in effective length) was filled with 1.5 *M* TB buffer, pH 10.0. Buffer vials contained 0.6% PEO solutions prepared in 200 m*M* (A), 400 m*M* (B) or 800 m*M* (C) TB buffer, pH 9.0. Hydrodynamic injections were performed at 30 cm height for 180 s. Peak identity: 1 and 2 for CA (0.25 μ*M*); 3 for β-casein (5 μ*M*); 4 and 5 for α-lac (2.5 μ*M*); 6 for trypsin inhibitor (1 μ*M*).

inhibitor, PEO solution prepared in 400 m*M* TB buffer were more suitable. The LOD for trypsin inhibitor was 45.3 n*M*, which was a 12.5-fold enhancement in sensitivity compared to that at 30 cm height for 10 s. The fact that the concentration factor was relatively less than the ratio of the injection volumes was due to Joule heating, which was supported by the fact that a poor sensitivity and stacking efficiency occurred for trypsin inhibitor in the PEO solution prepared in 800 m*M* TB buffer. We should also mention that the quantum yields of these proteins decreased with increasing temperature. It is also interesting to note that resolution between two peaks corresponding to CA increased with increasing TB concentrations, while the best condition for resolving β-casein and α-lac was achieved using PEO solution prepared in 400 m*M* TB buffer. The efficiency shown here did not agree with the general rule in CE that separation is generally optimized using low-conductivity media in CE. Some facts contributed to the result, including significant differences in migration time (diffusion), stacking efficiency, quantum yield (peak height), and Joule heating at different ionic strengths, and the solvent dependency of the electrophoretic mobility of proteins.

3.3. Effect of a plug of low pH buffer

Although the stacking efficiency of proteins in PBS was improved using high concentrations of TB buffer, the improvement result was not satisfied. To further improve sensitivity, we used PEO solution prepared in 400 m*M* TB buffer and applied a short

Table 2
Effect of PEO solution on LOD, concentration factor (CF), and plate number

	TB (m*M*) [a]											
	CA			β-Casein			α-lac			Trypsin inhibitor		
	200	400	800	200	400	800	200	400	800	200	400	800
LOD (n*M*) (*S*/*N*=3)	28.2	18.8	13.8	N.R.[b]	257.6	143.6	N.R.[b]	228.3	126.0	109.1	45.3	70.7
CF	2.16	5.26	4.70	N.R.	4.90	5.09	N.R	7.41	11.17	3.88	12.52	7.05
Plate number ($\times 10^5$)	2.54	1.45	2.32	N.R.	1.15	0.55	N.R.	3.64	6.02	0.60	15.92	7.83

[a] Used to prepare PEO solutions, other conditions as shown in Fig. 1.
[b] β-casein and α-lac were not completely resolved.

plug of low pH buffer (<pH 5.0) after injecting a long plug of proteins (18.4 cm). Because hydronium ions migrated much faster than proteins towards the cathode end, the proteins close to the injection end were affected by hydronium ions quickly and profoundly, leading to decreasing the μ_{ep} due to decreases in negatively charged density. Consequently, proteins close to the injection side migrated with a greater mobility towards the cathode end and thus stacked at the beginning of the concentration process. On the other hand, because the plug of low pH buffer was relatively short (0.4 cm) compared to the sample plug, the proteins close to the side of the TB buffer were affected quickly and profoundly by hydroxide ions, leading to a small migration mobility towards the cathode end. As a result, proteins stacked more effectively at the boundary between the TB buffer and PEO solution. Because pH changes did not cause significant effect on mobility of salts, proteins migrated further apart from sodium and chloride ions. Apparently, this method should allow one to inject a longer sample plug. When injecting a 10-s plug of low pH buffer, the maximum injection volume was increased to 810 nl, which was about 2.8-fold compared to that without applying this short plug. Important to note that the resolution between two peaks corresponding to CA increased in presence of a plug of low pH buffer. To further test the effect of the length of the plug on concentration and separation, the low pH buffer were injected for 20 and 30 s, respectively. As the plug length was increased, migration times shown in Fig. 2 became longer due to marked PEO adsorption at low pH [33,39]. Although the sensitivity for CA was improved, resolution between β-casein and α-lac decreased when injecting a 30 s plug of the low pH buffer. In terms of speed, resolution and sensitivity, the optimum length of the plug was 10 s. To further support our suggestion, we performed analysis with/without applying a 10-s plug of the pH buffer after injecting 280-nl samples. The LOD values for β-casein were 95.3 n*M* and 257.6 n*M*, respectively.

To obtain quantitative performance, we injected samples (prepared in PBS) containing 50 n*M* CA, 1 μ*M* β-casein, 500 n*M* α-lac, and 200 n*M* trypsin inhibitor into a capillary filled with 1.5 *M* TB buffer, pH 10.0, at 30 cm height for times over 10-520 s and then a 10-s plug of low pH buffer using 0.6% PEO

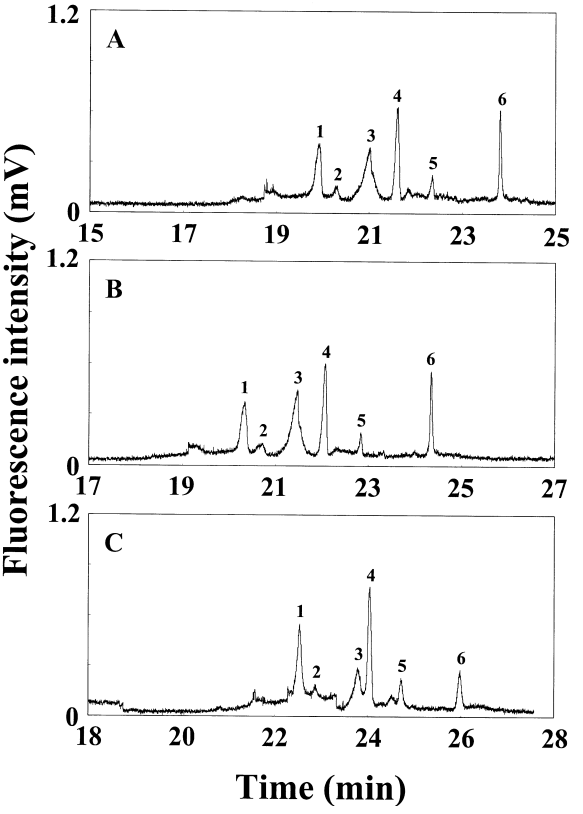

Fig. 2. Effect of the plug length of low pH buffer on on-line concentration and separation of sample proteins at 15 kV. Buffer vials contained 0.6% PEO solution prepared in 400 m*M* TB, pH 9.0. Low pH buffer was injected at 30 cm height for 10 s (A), 20 s (B), and 30 s for (C). Other conditions were the same as in Fig. 1.

solution prepared in 400 m*M* TB buffer, pH 9.0. Fig. 3A shows no peak detected when a hydrodynamic injection was performed for 10 s. The peaks corresponding to the four sample proteins only apparently visible when injecting for 360 s (0.56 μl) and Fig. 3B shows the electropherogram when injecting for 520 s (0.81 μl). The peaks were narrow (peak widths at half height range from 5 to 13 s) even an 18-cm sample plug was injected, indicating proteins stacked well. Table 3 shows good reproducibility in migration times (RSD values <1.0%) even when injecting 0.81 μl sample proteins. The separation time was longer (33 min) due to a small bulk EOF when injecting 0.81 μl of the sample. A linear relationship between the fluorescence intensity and the injection time occurred over the range from 360 to 520 s. The

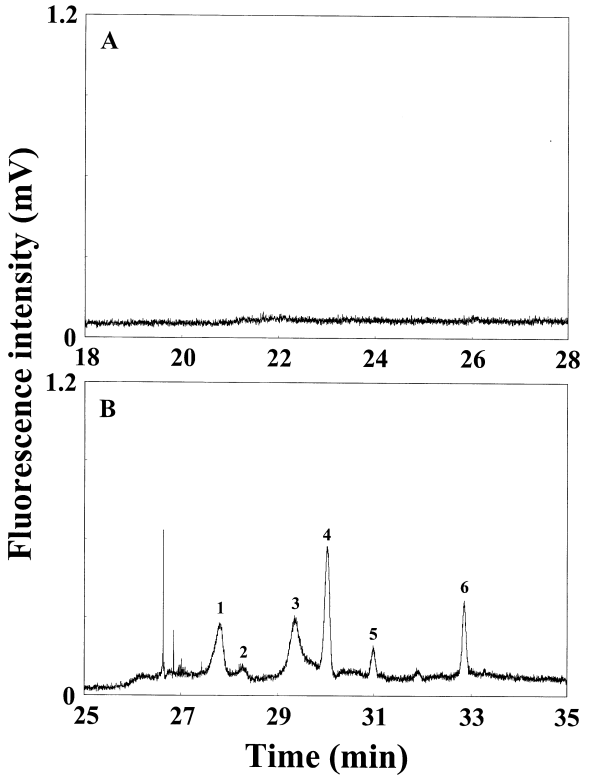

Fig. 3. Separation of low-concentration sample proteins without and with on-line concentration. Hydrodynamic injections were performed at 30 cm height for 10 s in (A) and 520 s in (B). Peak identity: 1 and 2 for CA (50 n*M*); 3 for β-casein (1 μ*M*); 4 and 5 for α-lac (500 n*M*); 6 for trypsin inhibitor (200 n*M*). Conditions were the same as in Fig. 2.

LOD values for the four proteins were in the range from n*M* to sub-n*M*, leading to 21.2 to 44.7-fold enhancements in sensitivity compared to those injected for 10 s. A relatively large deviation in the linearity for carbonic anhydrase compared to the others was due to poor stacking (broader peak width) because it, with a small μ_{ep}, spent a longer time entering PEO solution form the sample zone.

3.4. Comparison of concentration methods

Table 4 shows comparisons of results obtained from this study and our previous one [33], with respect to maximum sample loading, migration time and LOD values. In our previous study, we illustrated the maximum sample loading for proteins dissolved in 38 m*M* TB buffer, pH 9.0, was 1.40 μl when using a 60 cm capillary filled with 400 m*M* TB buffer, pH 10.0, and PEO solution prepared in 200 m*M* TB buffer, pH 9.0. The LOD values for CA and α-lac were 0.79 and 8.8 n*M*, respectively. The reason for using 38 m*M* TB buffer to dissolve proteins is to minimize PEO adsorption (long separation time) and allow a long injection length. As mentioned above, proteins dissolved in PBS did not stack effectively and the quantum yields were low under the same conditions. As a result, the LOD for CA was 1.8 μ*M*. Without a 10-s plug of low pH buffer, the maximum sample loading without loss of resolution was 0.28 μl. The LOD values for CA and α-lac were 18.8 and 228.3 n*M*, respectively. On the other hand, the

Table 3
On-line concentration and separation of four sample proteins dissolved in PBS in 0.6% PEO solution prepared in 400 m*M* TB, pH 9, using a 60-cm capillary filled with 1.5 *M* TB buffer, pH 10.0

	CA	β-Casein	α-lac	Trypsin inhibitor
Linear regression[a]	$y=0.84x-252.6$	$y=1.05x-330.6$	$y=1.76x-480.3$	$y=1.25x-381.0$
Linear regression coefficient (r^2)	0.941	0.972	0.998	0.987
LOD (μ*M*)[b]	0.10	1.26	1.69	0.57
LOD (n*M*)[c]	4.7	40.6	37.8	13.2
CF	21.3	31.0	44.7	43.2
Migration time (min)(RSD)[c, d]	27.71 (0.71%)	29.22 (0.74%)	29.88 (0.75%)	32.62 (0.82%)
Plate number ($\times 10^5$)	2.69	1.15	6.48	5.45

[a] y is the peak height (mV), x is the injection time (s).
[b] Injection volume was about 10 nl.
[c] Injection volume was about 810 nl.
[d] $n=3$.

Table 4
Effect of matrix and separation buffer on concentration performance using 60 cm capillaries

TB buffer[a]	Matrix	$V_{inj,\ max}$ (μl)	Migration time (min)		CF[b]		LOD (n*M*)		Ref.
			CA	α-lac	CA	α-lac	CA	α-lac	
0.4 M	38 m*M* TB	1.4	30.25 (0.68)[c]	31.27 (0.88)	148.3	35.1	0.79	8.8	33
	PBS[d]	0.0096	14.33 (0.85)	15.58 (1.20)	ND[e]	ND	180.7	512.8	This work
	PBS[f]	0.12	31.86 (0.43)	33.18 (0.72)	6.1	11.2	28.4	66.2	This work
1.5 M	PBS[g]	0.28	18.78 (1.35)	22.82 (1.33)	5.3	12.5	18.8	228.3	This work
	PBS[h]	0.81	27.71 (0.71)	29.88 (0.75)	21.3	44.0	4.7	37.8	This work

[a] Buffer used to fill the capillaries prior to analysis.
[b] Concentration factor, the LOD obtained compared to that obtained when injecting at 1 kV for 10 s in Ref. [33], and to that obtained when hydrodynamically injecting at 30 cm height for 10 s in this work.
[c] % RSD ($n=3$).
[d] the buffer used to prepare PEO solution was 200 m*M* TB, pH 9.0.
[e] Not determined due to poor stacking.
[f] The buffer used to prepare PEO solution was 800 m*M* TB, pH 9.0.
[g] The buffer used to prepare PEO solution was 400 m*M* TB, pH 9.0.
[h] The buffer used to prepare PEO solution was 400 m*M* TB, pH 9.0, low pH buffer was injected at 30 cm height for 10 s after sample injection.

maximum sample loading was increased to 0.81 μl when injecting a 10-s plug of lower pH buffer after sample injection. The LOD values for CA and α-lac were 4.7 and 37.8 n*M*, respectively. These results apparently show the potential for analysis of proteins in biological samples. Compared to other conventional concentration methods such as ITP [24–27], the proposed new method shows advantages, including less susceptibility to matrix effects, simplicity, reproducibility, and high sensitivity.

4. Conclusion

Using a capillary filled with 1.5 *M* TB buffer and 0.6% PEO solution prepared in 800 m*M* TB buffer, proteins dissolved in PBS stacked without desalting. When injecting a 10-s plug of low pH buffer after protein injection, the stacking efficiency was further improved in PEO solutions prepared in 400 m*M* TB buffer. We have shown that the maximum sample loading was 0.81 μl and the LOD for CA was 4.7 n*M*. On the basis of these and our previous results [33], we infer the potential of this new proposed method for analysis of proteins in biological samples such as urine and plasma. One of the advantages of these proposed methods over other on-line concentration methods [24–28] must be emphasized is that this method is robust, which can be applied to on-line concentration of small (in preparation) and macromolecules in high and low-conductivity media. In the future, we will focus on testing the possibility of using this method for analysis of peptides in biological samples, among which many, with concentration in the sub to n*M* range, have been known as important hormones. As our research of interest has currently been switched to proteomics, we would like to extend this method to protein screening.

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