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Application of on-line sample preconcentration by large-volume dual preconcentration by isotachophoresis and stacking (LDIS) on straight-channel microchips

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Abstract

In this study, large-volume dual preconcentration by isotachophoresis and stacking (LDIS) which is an on-line sample pre-concentration technique coupling large-volume sample stacking with an electroosmotic flow pump (LVSEP) with transient isotachophoresis (tITP) was applied to microchip electrophoresis (MCE) for improving both detection sensitivities and peak shapes. To realize LDIS in MCE, we investigated experimental procedures for injecting a short plug of a leading electrolyte (LE) solution into a straight microchannel without any sophisticated injector apparatus. We found that a short LE plug could be injected into a sample-filled straight-channel only by making the liquid level of the LE solution in an outlet reservoir higher than that in an inlet one. By applying a reversed-polarity voltage to the microchip, anionic analytes injected throughout the microchannel were first enriched by LVSEP, followed by tITP. Through the second preconcentration effect by tITP in LDIS, sensitivity enhancement factor (SEF) and asymmetry factor for a standard dye were improved from 878 and 0.62 to 1330 and 1.14, respectively, relative to those in conventional LVSEP. It should be noted that more viscous running buffer containing sieving polymers could be employed to the LDIS analysis, which was effective for improving the SEF and the separation efficiencies, especially for bio-polymeric compounds. Finally, LDIS was applied to the oligosaccharide and protein analyses in MCE, resulting in the SEFs of 1410 and *ca*. 50 for maltotriose and bovine milk casein, respectively.

Keywords Microchip electrophoresis · On-line sample preconcentration · LDIS · LVSEP · tITP

Abbreviations

LDIS	Large-volume dual preconcentration by isotachophoresis and stacking
LVSEP	Large-volume sample stacking with an electroosmotic flow pump
tITP	Transient isotachophoresis
LE	Leading electrolyte
TE	Terminating electrolyte
HPMC	Hydroxypropyl (methyl cellulose)
BGE	Background electrolyte
MCE	Microchip electrophoresis
SEF	Sensitivity enhancement factor

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Introduction

Analytical method for performing electrophoretic separations in narrow channels fabricated on a microchip is called microchip electrophoresis (MCE) [1–6]. Although MCE allows high-speed and high-performance separations with a small amount of samples, several issues have been still remained. One is insufficient sensitivity, the other is the problem generated from sample adsorptions onto the microchannel surfaces, and the third one is the need for complex voltage controls when injecting samples into the separation channel. To overcome these problems, in our research group, an on-line sample preconcentration by large-volume sample stacking with an electroosmotic flow (EOF) pump (LVSEP) [7] was employed to MCE [8–15]. In MCE-LVSEP, a sample solution is injected into an entire straight channel modified with poly(vinyl alcohol) (PVA), and then a single voltage is applied as with capillary electrophoresis (CE) [16], which allows the preconcentration and the separation of anionic analytes without the polarity switching during the run. Such simple experimental procedures can omit the complex

voltage program like a pinched injection technique [17], and the stacking of target analytes filled in the whole channel should improve the detectability. In LVSEP, additionally, the PVA modified channel is used to control the EOF during the stacking and the separation steps [8, 9], which suppress the analyte adsorption onto the channel surface, especially for biological samples. Although we have reported successful LVSEP enrichments of various samples in MCE [8–15], a serious problem has remained, i.e., a peak fronting is often observed in LVSEP.

To suppress the peak fronting in LVSEP, Kawai et al. has reported a combination of LVSEP with transient isotachophoresis (tITP) [18–20] to improve the peak shape and enrichment efficiencies in CE, which was named large-volume dual preconcentration by isotachophoresis and stacking (LDIS) [21, 22]. In the previous report, a focusing effect by LDIS gave good asymmetry factor (A_S) of 0.9 (almost no fronting/tailing), whereas those of conventional CE and LVSEP were 1.5 (tailing) and 0.6 (fronting) for a standard analyte [21]. It should be appreciated that the improvements of the peak shape and the enrichment efficiencies is expected by the application of LDIS to MCE. However, in MCE-LDIS, it is difficult that a short plug of leading electrolyte (LE) should be injected into the straight microchannel for the tITP preconcentration.

Figure 1 summarizes the principle of LDIS. First, anionic analytes dissolved in distilled water are loaded into the entire straight-microchannel, followed by the injection of a short plug of LE. Background electrolyte (BGE) solution containing terminal electrolyte (TE) is added to both reservoirs (Fig. 1a). When a voltage is applied, the analytes are concentrated due to the principle of field-amplified sample stacking. Since the linear channel is filled with the low-ionic strength sample solution, a faster EOF is generated in the stacking step [8]. Hence, the focused analyte zone is transported by EOF, and the vacant sample matrix goes out to the cathodic reservoir (Fig. 1b). As the vacant sample matrix is eliminated and the microchannel is filled with high-ionic strength BGE, the EOF is suppressed. When the electrophoretic velocity of the analyte (v_{ep}) exceeds the EOF velocity (v_{eo}), the direction of the movement of the stacked analyte zone is reversed. At this point, the analyte zone is sandwiched between LE and TE, which causes tITP enrichments of partially diluted analytes (Fig. 1c). Finally, the dilution of the LE zone by longitudinal diffusion results in the switching from tITP to zone electrophoretic mode, so that the concentrated analytes are separated during the migration towards the anodic reservoir (Fig. 1d). This two-step on-line sample preconcentration is expected to achieve highly sensitive MCE analyses with high efficiencies. To realize the LDIS in MCE, we investigated the procedure for injecting a short plug of LE into the microchannel, and the fundamental analytical performances of the LDIS were evaluated using
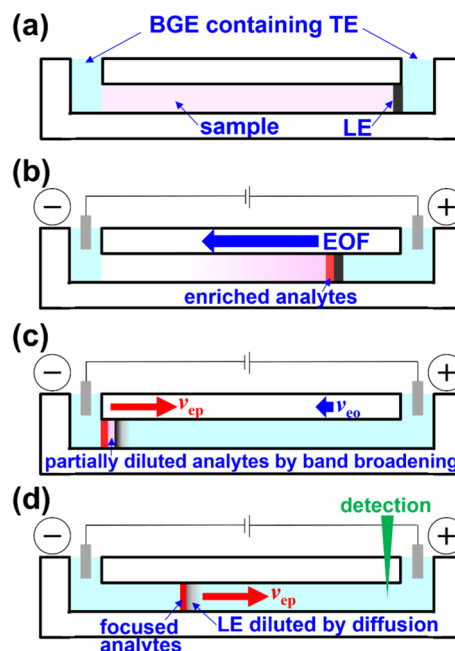

Fig. 1 Schematics of LDIS in MCE. **a** Sample, LE and BGE injection into the straight microchannel and reservoirs, **b** LVSEP preconcentration by applying dc voltage, **c** reversal of the migration direction of the focused analyte zone and the start of tITP, and **d** zone electrophoretic separation of the analytes after finishing tITP

fluorescein as a standard analyte. Furthermore, the MCE-LDIS technique was applied to the separation of oligosaccharides and proteins.

Experimental

Materials and chemicals

Poly(vinyl alcohol) (PVA, Mw 80,000, 88% hydrolyzed), hydroxypropyl(methyl cellulose) (HPMC, Mw 22,000), fluorescein isothiocyanate (FITC)-labeled casein from bovine milk and 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) was obtained from Sigma-Aldrich (Tokyo, Japan), fluorescein sodium salt, maltotriose (G3), maltopentaose (G5) and maltoheptaose (G7) from Tokyo Chemical Industry (Tokyo, Japan), HEPES from DOJINDO (Kumamoto, Japan), PDMS base and curing reagent (Sylgard 184) from Dow Corning Toray (Tokyo, Japan), SU8-50 from Micro-Chem (Newton, MA, USA), NaCl and sodium dodecyl sulfate (SDS) were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). All solutions were prepared with deionized water purified by Auto-Still (WG510, Yamato Scientific, Tokyo, Japan) and Simplicity-UV (Merck Millipore, Billerica, MA, USA) systems, and filtered through a 0.45 µm pore membrane filter prior to use. For fluorescence labeling,

0.1 mL of 20 mM G3, G5 and G7 was mixed with 0.01 mL of 20 mM aqueous APTS and 0.02 mL of 0.1 M NaBH$_3$CN in THF. The mixture was kept for 16 h at 37 °C. The obtained stock solution of 10 mM APTS-labeled G3 ~ G7 was stored at −20 °C. In the MCE analysis, the stock solution was diluted to an appropriate concentration with deionized water. In the LVSEP and LDIS measurements, 0.0 ~ 3.0% HPMC in 50 mM HEPES buffer (pH 8.0) was mainly used as the BGE. In LDIS, HEPES ions acted as TE, and 50 mM NaCl was injected as LE. Viscosity of the BGE containing HPMC was measured by VISCO (ATAGO, Tokyo, Japan).

A PDMS microchannel was fabricated by the conventional soft lithography technique. A straight-channel chip had a single channel (150 µm width × 100 µm depth) with a total separation channel length of 60 mm. After producing reservoir wells with a piercer, the microchannel plate was directly bonded onto the slide-glass lids (76.2 × 25.4 mm) via plasma treatment (YHS-R, SAKIGAKE-Semiconductor, Kyoto, Japan). Fabricated PDMS-glass hybrid microchannels were coated with PVA to suppress the EOF by the vacuum-drying method [9]. Briefly, a 10 mM SDS solution was manually introduced into the whole microchannel using a microsyringe to hydrophilize the PDMS surface. After removing the SDS solution, the microchannel was loaded with 0.1% PVA solution. The microchip was put into a vacuum-chamber under reduced-pressure (< ~ 1 Torr) with an oil rotary vacuum pump (G-50DA, ULVAC KIKO, Saito, Japan) for 10 min. The PVA-coated channel was washed with water, and finally conditioned with the BGE prior to use.

Apparatus and procedures

MCE experiments were performed on a fluorescence microscope (IX73, Olympus, Tokyo, Japan). A 130 W mercury lamp (SHI-1300L, Olympus) for excitation was introduced to the microscope, and irradiated to the microchannel through a dichroic filter (BP460-480GFP, Olympus) and an objective lens (LUCPLFLN 20 ×, Olympus). Fluorescence from analytes collected by the same objective lens was passed through a dichroic filter (U-FBWA, Olympus), and led to a multichannel photodetector (PMA-12, Hamamatsu Photonics, Hamamatsu, Japan). All the analytes were detected by the fluorescence detection scheme at the point 10.0 mm from the anodic end of the separation channel. To evaluate the enrichment efficiency in LDIS and LVSEP, the value of sensitive enhancement factor (SEF) was calculated by comparing the peak height obtained under the LDIS/LVSEP condition with the fluorescence intensity from the microchannel filled with a sample solution (no preconcentration) taking into account a dilution factor.

In the LDIS analysis on the straight-channel chip, a sample solution was introduced into the entire channel by manually using a syringe. After the injection of a short plug of LE (details shown in the next section), the anodic and cathodic reservoirs were filled with 10.0 µL of BGE. A voltage of −1.5 kV was applied with a power supply (HCZE-30PN0.25, Matsusada Precision, Kusatsu, Japan) through two platinum electrodes immersed in the two reservoirs. In the LVSEP measurements, the experimental procedures were same as those in LDIS without the LE injection.

Results and discussion

Application of LDIS to MCE

In the application of LDIS to the MCE analyses, we investigated an injection method for the LE solution into the microchannel as a short plug. First, the sample solution was manually injected into the entire channel using a microsyringe (Fig. S1a), and then the sample in the two reservoirs was removed using a micropipette (Fig. S1b). In Fig. S1c, 9.0 µL of the LE solution was added into the vacant anodic reservoir to produce the liquid level of 2.0 mm, which was left for 30 s to introduce of the short LE plug by pressure flow. After the LE injection, the LE solution in the anodic reservoir was aspirated using the micropipette, and then BGE containing TE was loaded into the two reservoirs as shown in Fig. S1d.

When a voltage was applied to the microchip prepared with the above procedure, a sharp peak of fluorescein was observed (Fig. 2a), demonstrating successful on-line sample preconcentration by LDIS in the microchannel. To verify the effectiveness of the LE injection, the SEF obtained in a conventional LVSEP analysis was compared with that in LDIS. In the normal LVSEP, i.e., no injection of the LE solution, the peak of fluorescein was significantly broader than that in LDIS as shown in Fig. 2b. In LVSEP, the peak width and the SEF were 6.2 s and 878, respectively, whereas in the LDIS analysis the width was narrowed to 3.1 s, and the SEF was increased to 1330 as summarized in Table 1. Hence, the combination of tITP and LVSEP in the LDIS technique was
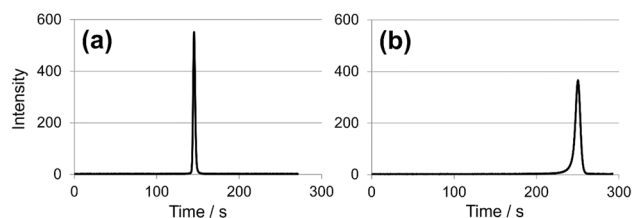
Fig. 2 a LDIS and **b** conventional LVSEP analyses of fluorescein on the straight-channel microchip. Sample, 50 ppb fluorescein dissolved in deionized water; BGE, 50 mM HEPES buffer (pH 8.0); LE, 50 mM NaCl; LE injection, 2.0 mm for 30 s; applied voltage, −1.5 kV

Table 1 Comparison of LDIS and conventional LVSEP analyses of fluorescein[a]

	Migration time	SEF	$w_{1/2}$[b]	A_S[c]
LDIS	145 s (6.4%)	1330 (1.1%)	3.1 s (10.5%)	1.14 (3.8%)
LVSEP	243 s (5.0%)	878 (1.7%)	6.2 s (17.2%)	0.62 (2.3%)

[a]Values in the parentheses are %RSD ($n = 3$)

[b]Peak width at half height

[c]Asymmetry factor calculated by $A_S = b/a$ where a and b are distances from the mid-point to the fronting and terminating edge of the peak at 10% of the peak height, respectively

effective for sharpening the enriched peak and improving the peak height, resulting in the increase of the SEF.

It should be emphasizing that, in LVSEP, the asymmetry factor (A_S) was 0.62 (fronting peak), while in LDIS that approached to unity, 1.14, indicating that the peak became closer to symmetrical by injecting LE. For the stacked analytes in LVSEP, the sample diffusion in the BGE zone freely occurred. In contrast, the concentrated analytes could not penetrate into the vacant sample matrix due to faster anodic migration in the enhanced electric field in the sample zone [21]. Such partial diffusion toward the anode caused partial peak broadening as shown in Fig. 1c. When the partially broadened analyte zone and the fully concentrated zone adjacently moved toward the anode without the tITP enrichments, the peak fronting was observed in LVSEP as shown in Fig. 2b. On the other hand, in LDIS, the diluted analyte zone should be focused by tITP, so that almost symmetry peak was obtained as shown in Fig. 2a. Thus, the second step of the preconcentration by tITP improved the peak shape in LDIS.

We also investigated the effect of the concentration and the injection time of the LE solution (data not shown). In the LDIS analyses using 20, 50 and 100 mM NaCl as LE, the best SEF was obtained at 50 mM since the low concentration gave lower tITP enrichments and the higher LE concentration caused band broadenings according to Joule heating. When the injection time was varied at 15, 30, 45, and 60 s at the liquid level of 2.0 mm in the LE injection, the SEFs of fluorescein were 960, 1330, 1210 and 940, respectively. It was verified that, therefore, the highest SEF was achieved at the injection time of 30 s. At the liquid level of 2.0 mm, which was equivalent to the applied pressure of 19.6 Pa, the

pressure flow velocity was calculated to be 0.20 mm/s from Hagen–Poiseuille equation. As a result, the injected length of the LE solution was estimated to be *ca.* 6.0 mm at the injection time of 30 s. From these results, in MCE-LDIS, it was found that further enrichments of the analytes by tITP were successfully attained by injecting the LE solution into approximately 10% of the total channel length from the anodic reservoir.

Effect of HPMC concentration of SEF in LDIS

In MCE, HPMC is often added to the BGE to separate complexed mixtures, especially for bio-polymeric compounds [23, 24]. Hence, we investigated the application of the BGE containing HPMC to the LDIS analyses. As a result, the peak heights of fluorescein were gradually increased upon increasing the HPMC concentration as shown in Fig. 3. When the concentrations of HPMC were increased from 0.0 to 3.5%, the SEFs of fluorescein were increased from 1330 to 3540, respectively, demonstrating that higher HPMC concentrations gave higher SEFs as summarized in Table 2. The improvement of the SEFs by HPMC would be due to the increased viscosity, which suppressed the band broadening caused by longitudinal diffusion and increased the peak intensity. As the HPMC concentrations were increased, on the other hand, the detection times were increased from 160 to 540 s, and consequently the peak widths were also broadened from 2.6 to 14.8 s, which was unfavorable for the separation. At 4.0% HPMC, the peak was not detected owing to too higher viscosity. Despite quite higher viscosity

Table 2 Effect of the concentration of HPMC on the analytical performances of LDIS analysis of fluorescein[a]

[HPMC]	Viscosity	Migration time	SEF	$w_{1/2}$[b]
1.0%	8.8 cP	163 s (12%)	1440 (34%)	2.6 s (35%)
1.5%	16 cP	189 s (2.1%)	1690 (56%)	3.4 s (68%)
2.0%	34 cP	271 s (23%)	1790 (21%)	4.8 s (30%)
2.5%	69 cP	420 s (17%)	1800 (34%)	8.8 s (51%)
3.0%	135 cP	504 s (20%)	3640 (14%)	10.6 s (51%)
3.5%	201 cP	540 s (15%)	3540 (25%)	14.8 s (12%)

[a]Values in the parentheses are %RSD ($n = 3$)

[b]Peak width at half height

Fig. 3 Effect of the concentration of HPMC in the LDIS analyses of fluorescein. **a** 1.0, **b** 2.0 and **c** 3.0% HPMC. Conditions are as in Fig. 2a
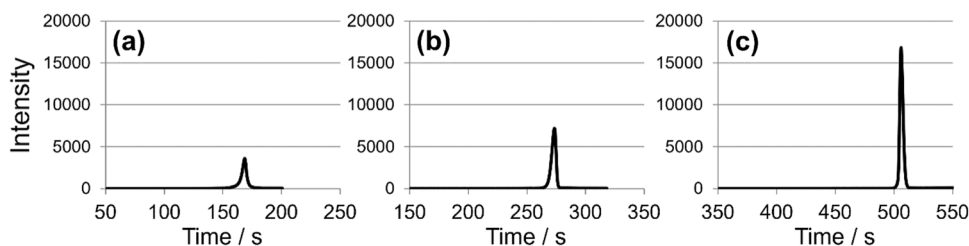
of 201 cP at the HPMC concentration of 3.5%, successful enrichments by LDIS were achieved, showing the SEF of 3540. In conventional LVSEP analysis, the peaks were not observed at the HPMC concentrations above 2.0%, while in LDIS it was found that the on-line preconcentration could be achieved even with high-viscosity BGEs. To verify the difference between LDIS and LVSEP, the below experiments shown in Fig. S2 were tested on the basis of the hypothesis that a pressure flow generated by the LE injection contributed to the increase in the upper limit of the HPMC concentration in LDIS.

In the LVSEP analysis, the stacked analyte zone moves toward the cathode due to a faster EOF (Fig. 1b). When a high-viscosity BGE is employed, however, the enriched zone is not detected since the BGE in the anodic reservoir is difficult to be drawn into the microchannel in LVSEP [10]. Thus, the BGE containing HPMC would be easily flowed into the channel from the anodic reservoir in LDIS. We investigated the possibility of a pressure flow generated during the LE and BGE injections as shown in Fig. S2. In the sample solution loaded microchip, the LE solution was added to the anodic reservoir (AR) to inject a short plug of LE by the pressure flow. Assuming that a weak flow occurred even after removing the LE solution from the AR, the residual pressure flow would assist the introduction of the high-viscosity BGE into the microchannel at the field-amplified stacking step.

To verify the hypothesis, we compared that the effect of the injection order of BGE containing 3.0% HPMC in LDIS, i.e., BGE was loaded into the AR and then into the CR (Fig. S2a), and BGE was added to the AR after the CR (Fig. S2b). When a voltage was applied, the results in Table S1 were obtained. Since the injection order in Figs. 2 and 3 was same with that in Fig. S2a (AR→CR), the result in Table S1 a was identical with Fig. 3c. In the microchip prepared with the reversal injection order (CR→AR), the migration time was increased from 504 to 692 s, and the SEF was decreased from 3640 to 2500 as summarized in Table S1. When the LE was first added to the AR, a faster pressure flow toward the CR would be generated as shown in Fig. S2a, which assisted the introduction of the high-viscosity BGE into the channel

during the stacking process. In the case of the former loading of the BGE to the CR, on the other hand, the generated weak flow toward the AR might be balanced the counter flow occurred by the LE injection, resulting in a possible weak flow as depicted in Fig. S2b. Weaker pressure flow toward the CR caused a slower transportation of the stacked zone, resulting in the increase in the detection time and the decrease in the SEF due to band broadening. Since the observed phenomena cannot be fully explained by the generation of the weak pressure flow alone, further investigations should be required. Anyway, it was revealed that a higher viscosity BGE can be applied to the LDIS analysis, which was effective for enhancing the SEF through suppressing the band broadening. In the present stage, however, the RSDs of the SEF were poor as demonstrated in Table 2. To achieve accurate and precise analyses, internal standards should be included in the sample solution to improve the RSDs.

Application to oligosaccharide and protein analyses

To demonstrate the utility of LDIS, oligosaccharides and proteins were analyzed in MCE. In the LDIS analysis of oligosaccharides, the mixture of APTS-labeled maltotriose, maltopentaose, maltoheptaose were employed, which will be denoted as G3, G5, and G7, respectively. For comparison, the LVSEP analysis of the oligosaccharide mixture is shown in Fig. 4a. Three separated peaks were observed, and the SEFs were ranging from 278 to 841 as summarized in Table 3. In LVSEP, the broader peaks resulted in the resolutions lower than 1.5, indicating insufficient separations. In contrast, sharper and higher peaks were obtained in the

Table 3 Comparison of LDIS and conventional LVSEP analyses of oligosaccharides[a]

	SEF$_{G3}$	SEF$_{G5}$	SEF$_{G7}$	$R_{S,G3-5}$	$R_{S,G5-7}$
LDIS	1410 (5.7%)	954 (5.5%)	448 (10.2%)	1.72 (4.4%)	1.29 (2.4%)
LVSEP	841 (9.0%)	578 (8.2%)	278 (7.1%)	1.42 (9.1%)	1.21 (5.4%)

a. Values in the parentheses are %RSD ($n = 3$)

Fig. 4 **a** Conventional LVSEP and **b** LDIS analyses of oligosaccharides. Sample, 4.0 µM APTS-G3, G5 and G7 dissolved in deionized water; BGE, 0.0% HPMC in 50 mM HEPES buffer (pH 8.0). Other conditions are as in Fig. 2
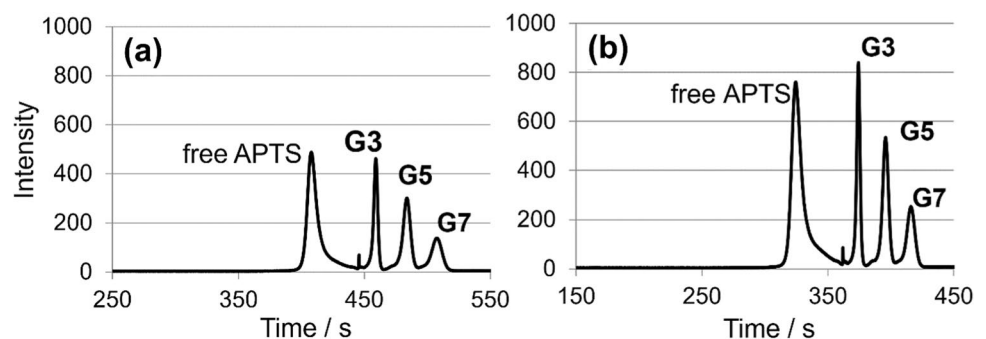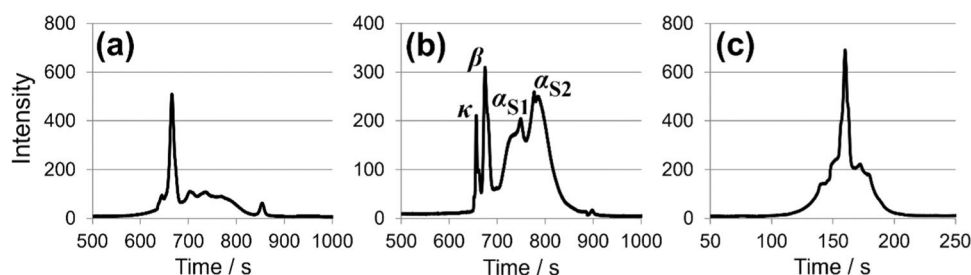
LDIS analysis of the oligosaccharides as shown in Fig. 4b. The SEFs in LDIS were ranging from 448 to 1410, which were higher than those in LVSEP. The sharper peaks led to the resolutions exceeding 1.5 between G3 and G5, achieving a baseline separation. Due to the dual focusing effect by LVSEP and tITP, the LDIS analysis gave superior resolutions in comparison with LVSEP. It should be emphasized that the addition of HPMC in the oligosaccharide analyses caused poor resolution, so that 0.0% HPMC solution was selected as the BGE in LDIS and LVSEP.

Finally, the application of LDIS to the analysis of FITC-labeled casein from bovine milk was investigated. In the LVSEP analysis using the BGE containing 1.0% HPMC, one sharp peak was overlapped with an unseparated broader zone as shown in Fig. 5a. In the LDIS analysis with 2.0% HPMC, on the other hand, two sharper peaks and two broader peaks were observed (Fig. 5b). Considering that bovine milk mainly contains four isozymes of caseins, i.e., α_{S1}, α_{S2}, β and κ-casein, peak assignments were done by comparing electrophoretic data from previous reports [25–28]. For comparison, the BGE without HPMC was employed to the LDIS analysis. As well as Fig. 5a, one sharp peak was overlapped with an unseparated broader zone in Fig. 5c. Therefore, the separation of casein isozymes was successfully achieved only with the LDIS analysis using the BGE containing higher concentration of HPMC. Considering that α-casein constitutes 55% of total casein in bovine milk [27], the approximate SEF was estimated to be about 50. Although the SEF was lower than that of fluorescein and oligosaccharide analyses, it was confirmed that MCE-LDIS can be applied to protein analysis.

Conclusion

In this study, LDIS was applied to MCE on the straight-channel microchip by injecting a short LE plug. In the conventional LVSEP, the SEF of the standard dye was 878, while LDIS gave 1330-fold increase in the sensitivity. This is first demonstration for the application of LDIS to the MCE analyses. Furthermore, the combination of LVSEP with tITP was effective for improving the peak shape, i.e., the peak became closer to symmetrical since A_S was varied from 0.62

to 1.14 by injecting LE. In LDIS, interestingly, the enriched peak was observed even with a highly viscous BGE containing 3.5% HPMC, whereas in normal LVSEP, any peak was not detected at the HPMC concentration above 2.0%. Since the injection order of viscous BGE to the two reservoirs strongly affected the SEF and the migration time in LDIS, a weak pressure flow generated during the LE and BGE injections might assist the introduction of the high-viscosity BGE into the channel during the first LVSEP step. In MCE-LDIS, oligosaccharides and proteins could be enriched and separated. Consequently, we found that MCE-LDIS was quite useful as the on-line sample preconcentration method using sieving gel matrices, so that the application to the analyses of closely resembled bio-polymeric compounds is expected in the near future.

Declarations

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