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Research Article

Highly sensitive double-fluorescent dye staining on microchip electrophoresis for analysis of milk proteins

We demonstrated a highly sensitive double-fluorescent dye staining in microchip electrophoresis (ME) for analysis of milk proteins. The detection sensitivity of ME was very limited so far and needed improvement. Our staining method consisted of two steps. First, in sample preparation before electrophoresis, protein was covalently bound to an amine-reactive fluorescent dye, Cy5. Then, the Cy5-attached protein was denatured with SDS and was further stained, during electrophoresis, with Agilent fluorescent dye, which was non-covalently attached to hydrophobic regions of the SDS-protein complexes. This double-fluorescent staining enhanced fluorescent intensity and lowered the detection limit to 200 pg of protein. This provided higher sensitivity than silver- or SYPRO Ruby-staining methods, which have previously given the highest sensitivity in protein staining. In addition, we applied our staining method to analysis of milk proteins and achieved their successful detection, whereas it was difficult to analyze them by the unimproved method.

Keywords:

Double-fluorescent dye staining / Microchip electrophoresis / Potent milk allergen / Size-based protein separation
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1 Introduction

Revealing functions and profiles of proteins has been an important issue in understanding the disease states in proteomics. Due to the multiplicity of proteins, many analytical tools are required for proteome analysis. For decades, the standard technique in proteome analysis has been a size-based protein separation (SBPS) such as SDS-PAGE. Shapiro *et al.* [1] first adopted this technique for analysis of polypeptide chains. Laemmli [2] improved it by using a discontinuous buffer system. It has been recently applied to microchip devices as microchip electrophoresis (ME) due to its promising potentials for fast analysis, small analyte consumption and good integration of analytical

procedures. It is hoped that microchip devices will become an alternative analytical tool not only for proteome research but also for clinical analysis [3]. Successful high-performance electrophoretic separations have been done on microchips. For example, Yao *et al.* [4] compared the performance of CE-dependent SBPS to that of glass-based ME and Bousse *et al.* [5] succeeded in separating 11 proteins from 6 to 200 kDa on a soda-lime glass microchip having channels filled with a low-viscosity separation medium, poly(dimethyl acrylamide), as a substitute for poly(acrylamide) gel.

Conventional SBPS devices such as slab gel electrophoresis devices require sample volumes at microliter levels, whereas the sample volumes handled in microchip devices are actually limited to the picoliter level [6], resulting in low detection sensitivity. To enhance the sensitivity, alternatives such as LIF detection instead of UV detection [7], on-line preconcentration [8], porous membrane [9] and a special detection method like refractive index detection [10] have been examined. However, they needed custom-made instruments and were labor intensive, and so have not been put to practical use.

Recently, as the only commercially available system of ME for SBPS, the Agilent 2100 Bioanalyzer with both Protein 200 Plus LabChip kit and Protein 200 Plus assay software has

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Abbreviations: α , α -lactalbumin; **AFD**, Agilent fluorescent dye; β , β -lactoglobulin; **BCA**, bicinchoninic acid; **β -GAL**, β -galactosidase; **Lys**, lysozyme; **ME**, microchip electrophoresis; **MW**, molecular weight; **SBPS**, size-based protein separation

been introduced [11]. It integrates sample preparation, labeling, gel loading, separation, detection by LIF, and data analysis. Several analyses of biological protein samples were demonstrated by the Agilent 2100 Bioanalyzer, which resulted in highly accurate protein sizing within 5% reproducibility [12, 13]; the Bioanalyzer even gave the limit for overall detection sensitivity.

The focus of this work was to enhance the sensitivity of microchip SBPS. We demonstrated a simple double-fluorescent dye staining method, composed of pre-separation and on-separation fluorescent dye staining for highly sensitive protein detection on the Agilent 2100 Bioanalyzer, and we successfully detected 200 pg (3.0 fmol) of protein. Finally, the double-staining method was applied to the analysis of milk proteins and provided their highly sensitive detection, which was not possible using the unimproved staining method.

2 Materials and methods

2.1 Sample preparation

Each protein, lysozyme (Lys; 14.4 kDa) from chicken egg white, carbonic anhydrase (CA) from bovine erythrocytes, BSA (68.0 kDa), and β -galactosidase (β -GAL; 116 kDa) from *Escherichia coli* (Sigma Chemical, St. Louis, MO), was used as a standard or a test protein and milk proteins extracted from commercially available products of bovine milk (Tokai Coop, Nagoya, Japan) were used as a real biological protein mixture.

The extraction of milk proteins partially followed the reported protocol [14]. Briefly, the bovine milk was diluted with a homogenization buffer (0.3 M NaCl and 0.2% w/w Triton-100) to 1:2 ratio (sample volume:buffer ratio). The mixture was shaken at room temperature for 20 min and then was centrifuged at 14 000 rpm for 50 min at 4°C in order to separate lipids in the milk. The top fat layer (lipids layer) was removed by a spatula and then filtered through a 0.45- μ m cartridge filter. Furthermore, the milk sample solution (1 mL) was acidified by decreasing its pH using 6 M HCl to the isoelectric pH (pI) of caseins (pI 4) and then, the caseins were precipitated. Most of the milk proteins are caseins and they often interfere with electrophoresis due to their large excess. Therefore, it is necessary to remove them before analysis.

2.2 Pre-separation fluorescent dye staining

Pre-separation fluorescent dye staining using Cy5 mono-reactive Dye (GE Healthcare Bio-Sciences, Piscataway, NJ) was performed according to the manufacturer's instruction. Briefly, each standard protein or the test protein was dissolved in 200 mM Na₂CO₃ solution (pH 9.3) to the desired concentration. For the pre-separation staining of milk pro-

teins, the refined product of milk proteins prepared as mentioned above was directly introduced to the Na₂CO₃ solution. An aliquot of 1 mL of each solution was transferred to a vial containing Cy5 (concentration not available) and the mixture was incubated for 30 min at a room temperature. The mixture was introduced to the Sephadex G-25 (GE Healthcare Bio-Sciences) column, which had already been swelled with water (Milli-Q apparatus, Millipore, Bedford, MA) to remove the free dye.

2.3 Microchip electrophoresis

All the processes of ME followed the protocols of the Protein 200 Plus LabChip kit (Agilent Technology, Waldbronn, Germany), which included sample buffer, sizing ladder, sieving matrix and a microchip. Briefly, the protein sample (non-Cy5-labeled or Cy5-labeled protein) was denatured with the sample buffer containing lower and upper markers (6 and 210 kDa) and then was introduced to the microchip. Separation and detection were carried out using the Agilent 2100 Bioanalyzer. Data collection and analysis were done with Protein 200 Plus assay software (Version A.02.12).

2.4 On-separation fluorescent dye staining

During electrophoresis, the protein sample (non-Cy5-labeled or Cy5-labeled protein) was stained with Agilent fluorescent dye (AFD, Agilent Technology) contained within the sieving matrix. Therefore, in the Bioanalyzer, conventionally, non-Cy5-labeled protein is just stained with AFD, which emits fluorescence after binding to the hydrophobic regions (long alkyl chains of dodecyl sulfate) of SDS-protein complexes [5]. Therefore, the single staining was performed. However, when Cy5-labeled protein was further stained with AFD during electrophoresis, this was described as the double staining (pre-separation fluorescent dye staining and on-separation fluorescent dye staining). Scheme 1 shows the steps in the double-staining method and the single-staining method. As mentioned above, the single- and double-staining methods were quite simple.

3 Results and discussion

3.1 Sensitivity of double-staining method

We compared the fluorescent intensity of the double-staining method with that of the single-staining method (Fig. 1). Clearly, the double-staining method (Fig. 1B) gave much higher fluorescent intensity for the same concentration of protein than when just stained by the single-staining method (Fig. 1A). Moreover, we evaluated the relationship between protein concentration and peak area using three standard proteins (Lys, BSA, and β -GAL) with quite different Cy5-labeling efficiency. In Fig. 2, graphs of the peak area (log₁₀)

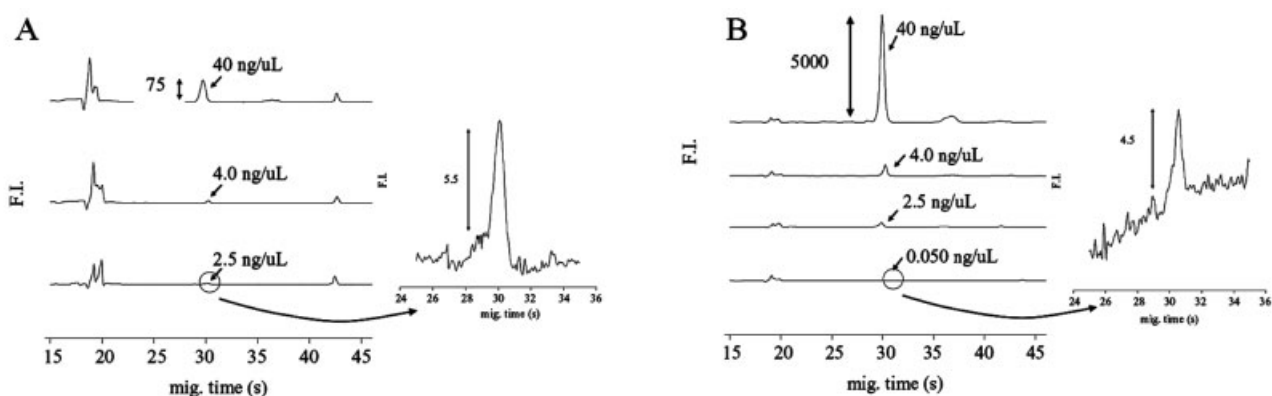
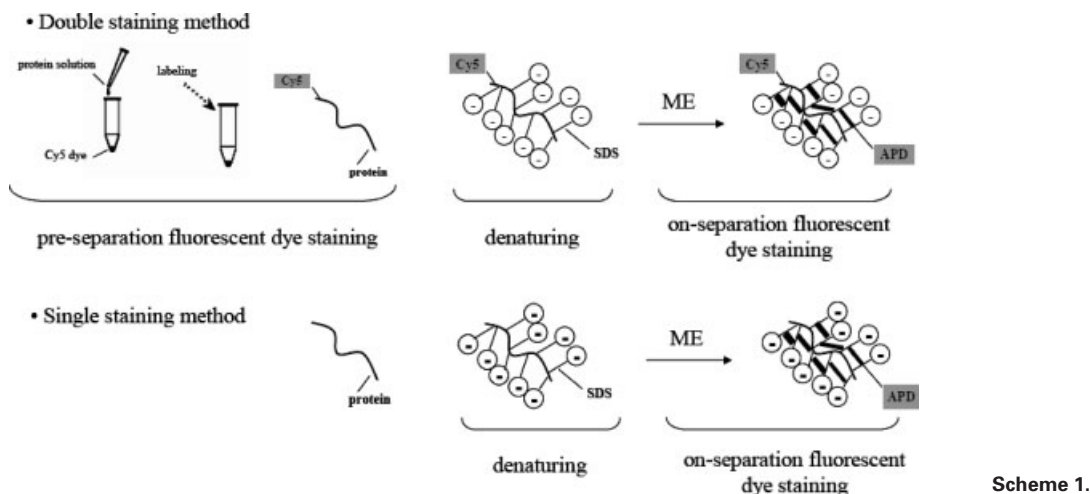


Figure 1. Comparison of fluorescent intensity of the single-staining method (A) and double-staining method (B). BSA (68 kDa) was used. Each arrow shows the concentration of BSA and insets (magnified electropherograms) are available for easy observation and even have detectable peaks ($S/N > 3$).

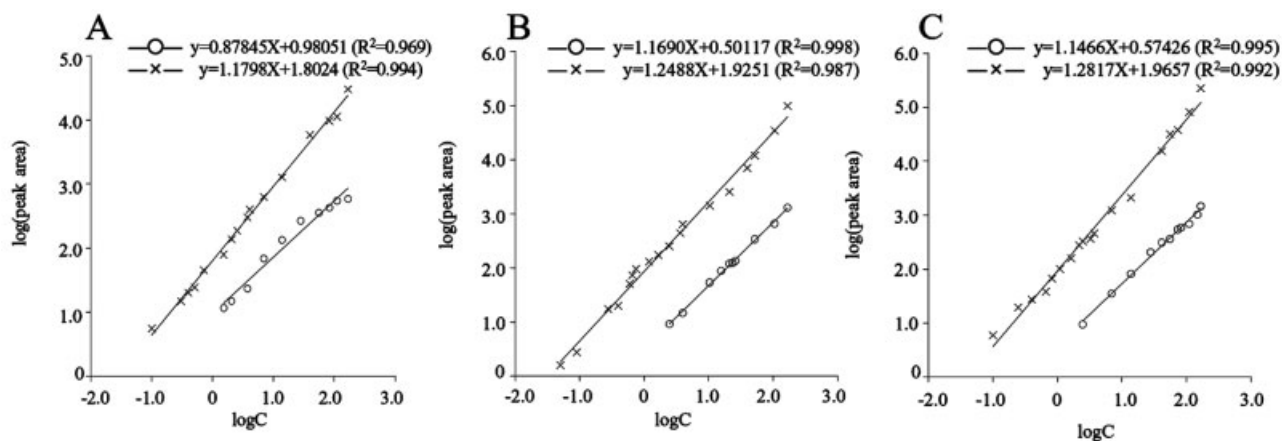


Figure 2. Linear responses between $\log(\text{peak area})$ and $\log C$ ($\log(\text{concentration})$) for (A) Lys, (B) BSA and (C) β -GAL. The —○— lines are for the single-staining method and the —×— lines are for the double-staining method. Concentration range: in (A), —○— and —×—, from 1.52 to 166.67 and from 0.10 to 166.67 ng/ μ L, respectively; in (B), —○— and —×—, from 2.5 to 166.67 and from 0.05 to 166.67 ng/ μ L, respectively; in (C), —○— and —×—, from 2.5 to 166.67 and from 0.10 to 166.67 ng/ μ L, respectively. The Cy5-labeling efficiency of each standard protein is shown in Table 1. Each plot was averaged from ten different measurements using ten different chips.

against protein concentration ($\log_{10}$) for the three standard proteins indicated a linear response spanning approximately a 100-fold range in protein concentration for the single-staining method and approximately 1000-fold for the double-staining method. We also showed the LOD for the three standard proteins (Table 1). In the single-staining method, LODs for all the standard proteins were almost the same and ranged from 6.08 to 10.0 ng, which corresponded to the LODs of the CBB-staining method [15]. On the other hand, in the double-staining method, LODs were improved by roughly 50 degrees and were from 0.200 to 0.400 ng, which were roughly five times more sensitive than the silver-staining method or roughly twice more sensitive than the SYPRO Ruby-staining method regardless of quite different Cy5-labeling efficiency of the standard proteins [15–17]. Silver-staining and SYPRO Ruby-staining methods are known to offer the best detection sensitivity, but LODs of these staining methods are, unfortunately, quite different among proteins. However, the double-staining method was superior to these staining methods, as it gave little variation (0.2 to 0.4 ng) for LODs among proteins.

As mentioned in Section 2, AFD emits fluorescence after noncovalently binding to the hydrophobic regions (long alkyl chains of dodecyl sulfate) of SDS-protein complexes. Unfortunately, AFD also binds to SDS micelles. The AFD attached to the micelles emits highly fluorescent background interfering with the detection of SDS-protein complexes. To overcome the problem of the background, dilution channels were added just before the detection point of the Bioanalyzer to decrease the concentration of the micelles below their critical concentration and then a dilution step suppressed the background [5]. In this dilution step, the fluorescent intensity of SDS-protein complexes as well as that of micelles was simultaneously reduced due to

the reduction of the hydrophobic regions (long alkyl chains of dodecyl sulfate of SDS-protein complexes or SDS micelles) that were AFD bound, which gave the LOD. On the other hand, in the double-staining method, Cy5 attached to SDS-protein complexes supplemented the reduction of fluorescent intensity of the SDS-protein complexes, as Cy5 covalently attached to the proteins *via* amide bonding, which is more difficult to remove by the dilution step than the non-covalent binding of AFD to the hydrophobic regions. Moreover, the excitation wavelength and maximum fluorescent emission of Cy5 (ex/em: 640/670 nm) were similar to those of AFD (ex/em: 630/670 nm), leading to the detected fluorescent intensity of the double-staining method to be increased in comparison with that of the single-staining method.

Furthermore, in the double-staining method, almost no variant LODs (0.2 to 0.4 ng) among standard proteins were found regardless of quite different Cy5-labeling efficiency of the standard proteins and the higher Cy5-labeling efficiency did not always give the better LODs. Namely, there was no correlation between Cy5-labeling efficiency and LODs. The reasons for the absence of correlation have not been understood until now. Ping *et al.* [18] mentioned the effect of SDS in the separation of lipoproteins. When the concentration of SDS in running buffer was lower than or even close to that in sample buffer, the peak intensity of lipoproteins was increased. In addition, the peak intensity was decreased with further increase of the concentration of SDS in running buffer. They discussed that the enhancement or the decrease of peak intensity was caused by field-amplified stacking or detachment of Cy5, respectively. We speculated that their discussion could be applied to our results. Each sample band might experience a different electric field due to the slight charge difference of each band and also some Cy5 incorporating with protein were detached with SDS during electrophoresis, which might be involved in the finding that there was no correlation between Cy5-labeling efficiency and LOD.

3.2 Calibration plot

The *R*-squared values of the three lines (— × —) in Fig. 2 clearly showed excellent linear behaviors for quantitation of protein concentration. We evaluated whether the lines for the double-staining method were suitable for calibration plots to determine concentration of an unknown protein. Figure 3 indicates the quantitation results for concentrations of a test protein (carbonic anhydrase, CA). The quantitation errors of the concentration of CA were within 40% using any calibration marker with different Cy5-labeling efficiency, and they were comparable to the biuret, Lowry-Peterson and bicinchoninic acid (BCA) assays, which are major protein quantitation methods [19]. Namely, the double-staining method indicated the same quantitative responses against the general quantitation methods. We noted that it was possible to obtain concentrations of any protein using the calibration plots of the double staining method even if

Table 1. LOD of single- and double-staining methods^{a)}

Standard protein	Single staining	Double staining	
	LOD ^{b)} (ng)	LOD ^{b)} (ng)	Cy5-labeling efficiency ^{c)}
Lys	6.08	0.400	0.426
BSA	10.0	0.200	2.33
β-GAL	10.0	0.400	6.25

a) The data for LOD were averaged from ten different measurements using ten different chips. The data for Cy5-labeling were averaged from ten different measurements.

b) The values are expressed in mass level (ng).

c) Cy5-labeling efficiency was calculated by the following equation: $D/P \text{ ratio} = \{ (\text{absorbance of Cy5-protein at } 650 \text{ nm}) / (\text{molar extinction coefficient of Cy5 at } 650 \text{ nm: } 250000) \} / \{ (\text{absorbance of Cy5-protein at } 280 \text{ nm}) / (\text{molar extinction coefficient of Cy5-protein at } 280 \text{ nm}) \}$. The molar extinction coefficients of the three Cy5-proteins: 38 467 for Lys; 32 250 for BSA; 219 650 for β-GAL. All the proteins were denatured and then their absorbance was measured at 40 ng/μL.

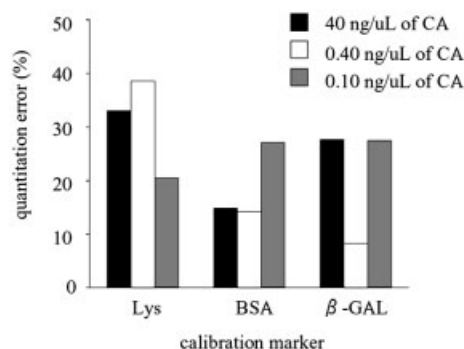


Figure 3. Quantitation results for concentrations of a Cy5-labeled test protein (carbonic anhydrase, CA) using the calibration plots (the — × — lines in Fig. 2) of the calibration markers (Cy5-labeled Lys, BSA and β-GAL). The Cy5-labeling efficiency of each standard protein is shown in Table 1.

the Cy5-labeling efficiency of the standard proteins (calibration markers) was significantly different.

3.3 Mobility shift after the double-staining method

The known constant stoichiometry between SDS and protein is 1.4 g SDS/1.0 g protein and each SDS-protein complex has almost the same charge to weight ratio and similar electrophoretic mobility [20]. Therefore, the SDS-protein complexes are separated based just on their own size [1, 2]. After the pre-separation staining, binding Cy5 to protein gave a slight change of molecular weight (MW) of protein as well as that of charge density since a protein labeled with a Cy5 molecule was supplemented with the MW of Cy5 (MW = 791.99) and the minus charge of Cy5 was added to the protein. Therefore, it seemed to be possible that the binding Cy5 slightly changed the charge to weight ratio, leading to different electrophoretic mobility. The difference in the mobility between the single-staining method and the double-staining method is shown by the bar graph of MW against their reciprocal electrophoretic migration time as a substitute for electrophoretic mobility (Fig. 4). The difference of the mobilities was within 3.5%. The mobility of each protein showed almost no change after the double staining even if Cy5-labeling efficiency was different. We found that the binding Cy5 was not affected by the electrophoretic mobility. The charge to the weight ratio of each protein was considered to be maintained and the mobility of each protein was not changed after the pre-separation staining.

3.4 Application of the double-staining method to the analysis of milk proteins

We applied the double-staining method to the analysis of a real biological protein mixture, bovine milk proteins, to confirm the utility of the double-staining method. Allergy to bovine milk proteins is the most common food allergy during infancy and bovine β-lactoglobulin (β) is the main allergen of bovine milk [14]. The allergenicity is generally char-

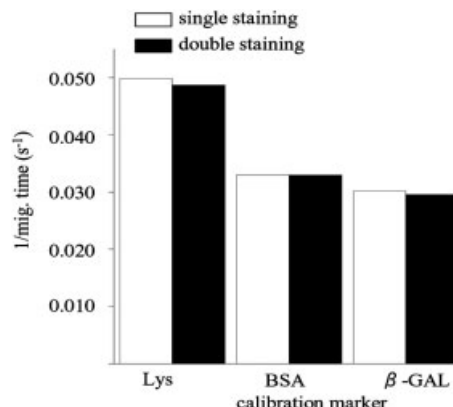


Figure 4. Difference of electrophoretic mobilities of the three standard proteins stained by the single-staining or the double-staining method. Electrophoretic mobility was calculated by the equation, $\mu = L/(t \cdot E)$; where μ is the electrophoretic mobility, L is the effective separation length, t is the migration time (s) of the sample protein and E is the electric field. Because L and E were not revealed by Agilent Technology, the μ of each standard protein could not be calculated. The reciprocal electrophoretic migration time of each standard protein was substituted for the μ values.

acterized by ELISA or radioallergosorbent test (RAST). In these assays, it is necessary to isolate β from bovine milk. However, loss of β is generally inevitable during the isolation, making its detection more difficult. That is why we turned to the double-staining method to monitor low volume of β in the isolation step. The comparison of electropherograms between the single- and the double-staining methods is shown in Fig. 5. The peak intensities for α-lactalbumin (α), β and caseins were increased using the double-staining method. As

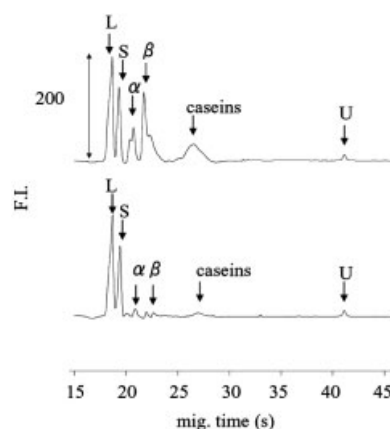


Figure 5. Analyses of milk proteins by the single- (low) and the double- (upper) staining methods. L, lower marker (6 kDa); S, system peak; α, α-lactalbumin (14.3 kDa); β, β-lactoglobulin (18.8 kDa); caseins (28–32 kDa); U, upper marker (210 kDa). Peak intensities of the L, S and U were almost the same between electropherograms of the single-staining and the double-staining methods, since L, S and U were included in the sample buffer, which also had denaturing solution (β-mercaptoethanol).

Table 2. Sizing and quantitation analysis of milk proteins^{a)}

	Sizing (kDa)		Quantitation (ng/ μ L)				
	Ave.	Error (%)	Lys ^{b)}	BSA ^{c)}	β -GAL ^{d)}	Ave. ^{e)}	Error ^{f)} (%)
α	14.3	0	1.49	1.16	1.08	1.24	± 19.9
β	18.9	3.27	4.51	3.31	2.98	3.60	± 25.2
Caseins	48.88	52.8–95.5 ^{g)}	2.97	2.23	2.03	2.41	± 23.2

a) The data were obtained by ten different measurement using ten different chips.

b) The quantitation results using the calibration plot of Lys (-x-) in Fig. 1.

c) The quantitation results using the calibration plot of BSA (-x-) in Fig. 1.

d) The quantitation results using the calibration plot of β -GAL (-x-) in Fig. 1.

e) The average of quantitation results using the three calibration markers.

f) The maximum error based on the average of the quantitation results.

g) Aggregations of caseins were formed by the precipitation step in sample preparation. The aggregations resulted in sizing errors. The proper MW of caseins are 25.0 to 32 kDa [21].

mentioned in Section 2.1, caseins should have been almost completely removed by acidic precipitation, but aggregations of caseins due to the acidic precipitation step were clearly distinguished in the double-staining method although they were hardly detected in the single-staining method. These results validated the utility of the double-staining method as a highly sensitive detection method. The sizing and quantitation data of these milk proteins are shown in Table 2. Except for casein aggregations, the sizing accuracy was within 3.5%, which was regarded as good in ME [4–10] and the quantitation results were also good compared to those of another report [19]. Consequently, these results suggested that our staining method was promising for analysis of real biological proteins.

4 Concluding remarks

We demonstrated the highly sensitive double-fluorescent staining for microchip electrophoresis of protein. The staining method coupled covalent fluorescent staining with non-covalent fluorescent staining to protein and did not require cumbersome sample preparation procedures. The detection sensitivity was 50–100 times better than that of the unimproved staining method and was most highly sensitive in staining methods of protein. Moreover, the double-staining method offered qualitative and quantitative analysis, which was validated by analysis of milk proteins. Our developed staining method is well suited for not only routine analysis in a laboratory but also clinical analysis.

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