Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 990 (2003) 169–178
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Free-flow electrophoresis in a microfabricated chamber with a micromodule fraction separator
Continuous separation of proteins

Hidesaburo Kobayashi[a,*], Katuyoshi Shimamura[a], Tomohiko Akaida[a], Kenji Sakano[a], Nobuyoshi Tajima[b], Jun Funazaki[b], Hirobumi Suzuki[b], Etsuo Shinohara[b]

[a]*Department of Chemistry, Josai University, Saiatama 350-0295, Japan*
[b]*Olympus Optical Co. Ltd., Hachioji 192-8512, Japan*

Abstract

Continuous free flow electrophoresis of proteins was carried out in a microfabricated free flow electrophoresis (mFFE) module with the 30-μm thick slit of the separation. The newly developed micromodule fraction separator (MFS) was attached to the down-stream end site of the separation chamber of mFFE. By using the MFS, electrolyte solution from the separation chamber was introduced to the peristaltic pump without disturbing the electrolyte solution flow at the bottom side of the chamber. The separation of protein mixture samples was achieved by a hydroxypropylmethylcellulose pretreatment coating of the separation chamber. The pretreatment of the sample chamber effectively suppressed electroosmotic flow. All fractionated samples were collected using the MFS after continuous elecrophoresis and analyzed by reversed-phase HPLC. From the results of HPLC analyses none of the cytochrome *c* fractions at the other ports revealed cross talk phenomena at adjacent ports. A similar result occurred for the myoglobin. This means that these proteins were completely separated from each other by continuous mFFE, and the MFS functioned efficiently during continuous electrophoresis.

Keywords: Free-flow electrophoresis; Electrophoresis; Instrumentation; Fraction separator; Proteins; Hydroxypropylmethylcellulose

1. Introduction

The technology of free flow electrophoresis (FFE) is introduced more than 40 years ago [1] and the field is now experiencing a new development through a microfabricated free flow electrophoresis (mFFE) module [2]. During these decades many valuable experiments of FFE were reported to separate biological materials such as proteins. In the last decade, Bauer [3] introduced a new mode of free flow zone electrophoresis, the interval FFE, for high resolution protein purification and Weber [4] achieved a higher stability of FFE sufficiently stable for overnight. At the same time, the theory of the correlation between preparative FFE separation and the capillary zone electrophoretic separation was developed and carried out in practical experiments by Kasicka [5]. Recently, Poggel and Melin [6] reported the throughput of protein up to 1.1 g/h. So far, studies in conventional size of FFE come to have almost all ripened and will apply to production of protein reagents but another approach also started by Hoffmann et al. [7] to use FFE for cell-mapping

*Corresponding author.
E-mail address: hkoba@josai.ac.jp (H. Kobayashi).

0021-9673/02/$ – see front matter
doi:10.1016/S0021-9673(02)01964-7

proteomics. For these objectives more wide range of scale of separation technology have to develop.

In 1994, Raymond et al. [8] were the first to apply microfabrication technology to the construction of a FFE module. The FFE module was composed of a glass–silicon structure and successfully separated rhodamine-B isothiocyanate labeled amino acids. Nevertheless the maximum voltage used in his work was only 50 V because the current was conducted through the silicon rather than the solution. It was, therefore, expected that such a device should perform more efficient separation if much higher voltages could be applied.

We have developed a new FFE module for protein separation [9]. The module is made from two Pyrex glass forms with very narrow gap between them to apply high voltages and fabricated on a 4-inch type glass wafer that is easily processed by the semiconductor fabrication technology (1 in.=2.54 cm). An Xylene cyanol dye sample was used first in a density change experiment. The sample flow characteristics were constant over a relatively large surface area while maintaining its laminar flow and holding the mixing of the dye and buffer solutions to a minimum. This effect also carried over to the velocity distribution experiment with an applied voltage of 5 kV and prevented the dye solution into the buffer solution from mixing. Without the voltage the sample flowed in a straight stream, nevertheless this high voltage caused electroosmotic flow perpendicular to the buffer flow. The dye, a negatively charged ion, moved, however, to the cathode because electroosmotic flow (EOF) was much stronger than electrophoretic migration in this module [9].

Although a microfabricated chamber of FFE is advantageous for making a laminar flow compared to a conventional one, the former chamber causes larger EOF effects on electrophoretic separation than the latter one. EOF in electrophoresis will cause complex phenomena for separation of proteins so that coating of Pyrex glass is a necessary to reduce EOF. In our experiment, hydroxypropylmethylcellulose (HPMC) coating solution was applied to the separation chamber to suppress EOF. HPMC was chosen because it is simply adsorbed dynamically onto the surface of Pyrex glass.

Another problem to miniaturize the thickness of the FFE chamber is in the fraction of the sample components separated in mFFE. Now few interface tools for microfabricated ware are developed. So we developed a new tool, the micromodule fraction separator (MFS) and attached it at the down stream end site of the separation chamber of mFFE. By doing this the electrolyte solution was easily introduced to the fraction tubes.

The separation of proteins has become very important for studying protein functions in the proteome and proteomics fields so that the separation technologies have to be developed for the native proteins but not for the denatured ones. Native protein purification or isolation could be allowed for subsequent analyses such as protein–protein, protein–biomolecules interactions to elucidate protein functions. Such technologies which use any surfactants in gel electrophoresis or any organic solvents in HPLC are not acceptable because almost all proteins will be denatured in those separation processes. The FFE is able to operate the separation procedure with neither denaturation reagents nor gel, such as in two dimensional gel electrophoresis, so that sample proteins keep the native conformation during separation processes.

In mFFE, laminar flow is stable [2], and the thin layer of electrolyte flow in the electrophoresis separation chamber has a low conductivity. Thus mFFE is able to operate under a lower electric power of low current and high voltage. Under these new conditions the efficiency of controlling the heat transfer with conventional air blow equipment is high. The temperature in mFFE is more suitable for preserving biological functions of proteins and other biopolymers.

Furthermore, an advantageous thing in the mFFE separation technologies is to operate in suitable sample volumes (from microliters to infinite volume) if one needs a larger scale of preparation of materials such as proteins. The small scale experiment was already attempted in the separation study of DNA and bovine serum albumin [2]. In this experiment the sample and the fractionated volume were 67 and 300 μl each, respectively. This small scale separation is mentioned since it is realizable only in mFFE and impossible by conventional FFE.

FFE also has a feature of continuous operation. If conditions of electrophoresis and flow of electrolytes are controlled, one can separate continuously pro-

teins in desired scale. This continuous process is only realizable in FFE and impossible by other separation technologies such as capillary electrophoresis or HPLC.

We have tested the continuous process of zone electrophoresis in a separation experiment when we have separated cytochrome *c* and myoglobin mixture samples by the mFFE–MFS system. To estimate the separation efficiency in cFFE mode we have analyzed the obtained mFFE fractions by HPLC.

2. Experimental

2.1. Equipment

2.1.1. mFFE module

The size of separation module was 66×70 mm in which a 56.5 (width)×35 (length) mm×30 μm (depth) separation bed on a Pyrex glass (∅ 100×2 mm) wafer was fabricated. The narrow banks (1 mm in width and 20 μm in thickness; residual gap: 10 μm) between the separation chamber and the both sides of electrode chambers were fabricated by using microfabrication technology. Seven inlets for buffer solution, three inlets for samples and 19 outlets were formed on the module. Two platinum electrodes were performed on the both edges parallel to its flow direction (Fig. 1) [10].

2.1.2. Operation system of mFFE

An illustration of continuous mFFE system is shown in Fig. 2. A reservoir indicated ''R1'' was used for electrophoresis buffer. And ''R2'' for 0.1 *M* NaOH–ethanol (50:50, v/v), ''R3'' for 0.01 *M* HCl and ''R4'' for 80% aqueous ethanol for use during washing cycle. By operating check valves those solutions were changed and poured into the buffer inlets port of the mFFE via a tandem connection of a dumper, a drain check valve and a high pressure fuse

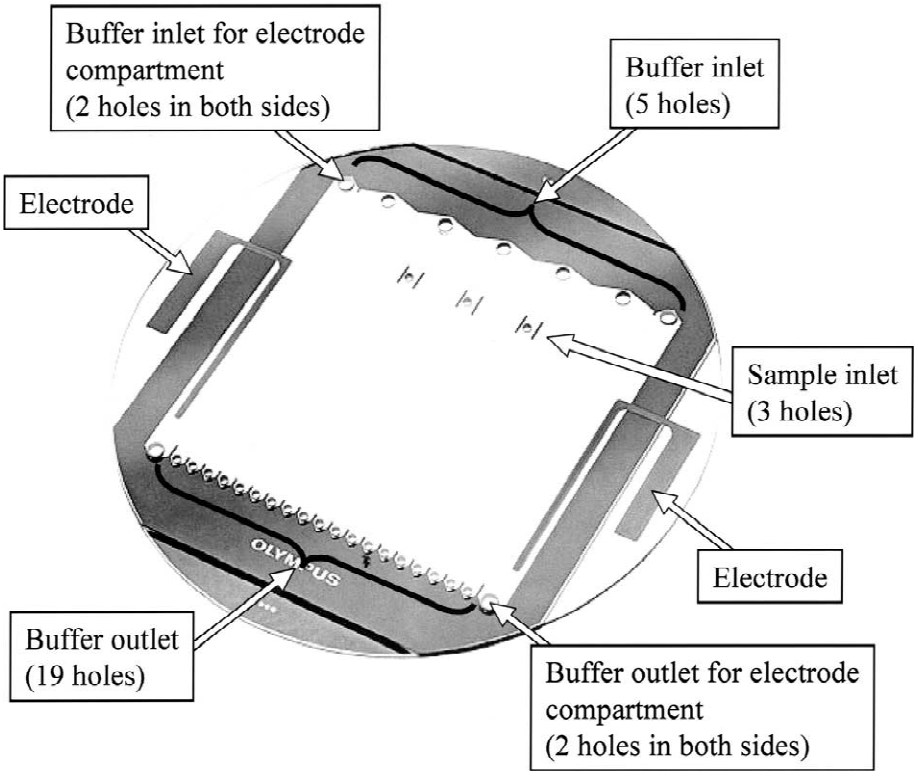

Fig. 1. A photograph of mFFE module. The size of separation module is 66×70 mm in which 56.5 (width)×35 (length) mm×30 μm (depth) separation bed on Pyrex glass (∅ 100×2 mm) wafer was fabricated.

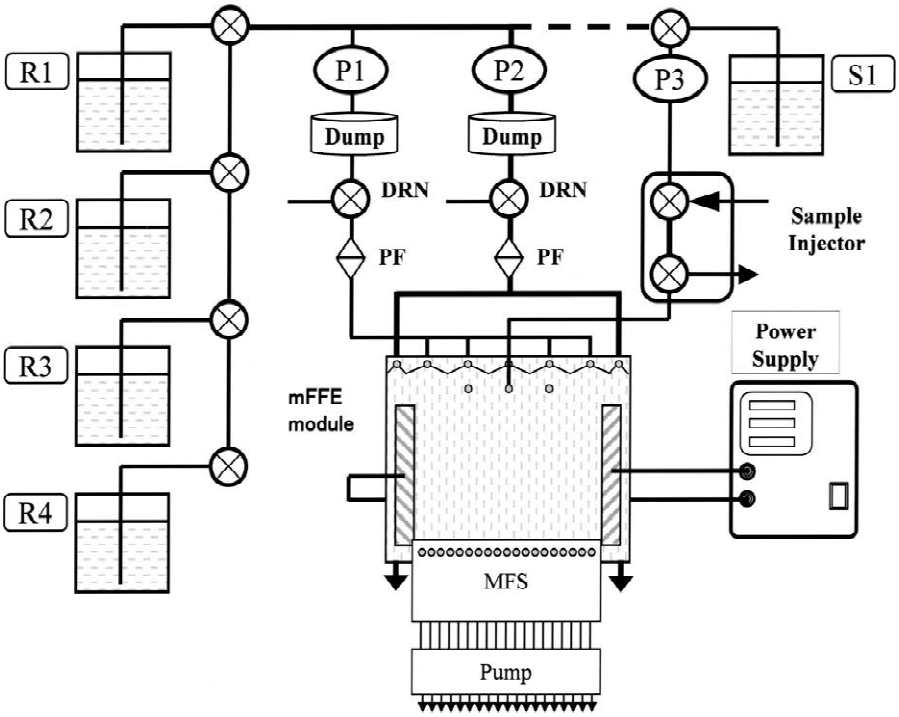

Fig. 2. An illustration of continuous mFFE system. A reservoir R1 is used for electrophoresis buffer, and at washing cycle, R2 for 0.1 *M* aqueous NaOH–ethanol (50:50, v/v), R3 for 0.01 *M* HCl and R4 for 80% aqueous ethanol, respectively. S1 is a sample reservoir. P1, P2 and P3 are peristaltic pumps for pouring the solutions into the separation chamber, the electrode chambers and the sample injection, respectively. D is a dumper, DRN a drain duct, PF a fuse unit for excess pressure, respectively. The function of the micromodule fraction separator, MFS, is explained in Fig. 3 and described in the text.

using a peristaltic pump (Eyela microtube pump MP-3, Tokyo Rikakikai, Tokyo, Japan). The same type pump was connected to the electrolyte chambers (P2 in Fig. 2) for feeding the solution. A sample solution was introduced to the sample inlet port of mFFE by a peristaltic pump (Gilson Minipuls-3, Gilson, F-95400 Villers Le Bel, France) indicated ''P3''. Electrophoresis power was supplied by constant voltage equipment (Atto Constapower 3500, Atto, Tokyo, Japan) and a current of electrophoresis was monitored using a multimeter (Keithley 2010, Keithley Instruments, Cleveland, OH, USA).

2.1.3. Micromodule fraction separator

The newly developed micromodule fraction separator (MFS) is shown in Fig. 3. The MFS is attached to the separation chamber down stream end site in continuous mFFE system (Fig. 2). This separator consists of 19 stainless steel tubes (0.8 mm I.D.×1.0 mm O.D.) connected to a multi-channel peristaltic pump (Ismatec IPC high precision multichannel dispenser ISM939 V4.00, Ismatec, Labortechnik-Analytik, Glattbrugg, Switzerland) with silicone tubes. The other ends of the stainless steel tubes make a firm and unassisted contact to the ports of the separation chamber end ports exhaust holes (∅ 1.5 mm). Although Fig. 3 is not to scale, it is shown to explain the function of three of 19 outlet ports. The two Pyrex forms with cross section of the chamber are shown at the top of Fig. 3. Between them the direction of the electrophoresis buffer is indicated by the bold arrows. An appropriate flow-rate of the peristaltic pump makes air plugs in the flow. By this configuration electrophoresis buffer containing sample components from the separation chamber is introduced to the peristaltic pump without disturbing the electrolyte solution flow at the bottom side of the chamber.

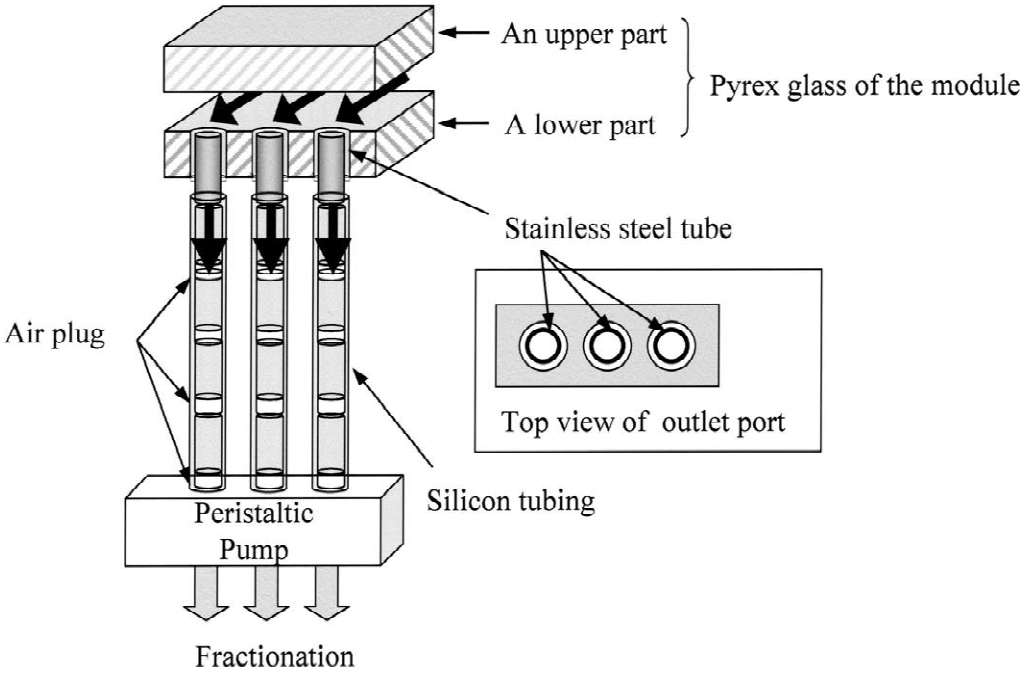

Fig. 3. The newly developed micromodule fraction separator (MFS). A cross section of the separation chamber's bottom with upper and lower parts of Pyrex glass. Details of the function are described in the text.

2.2. Materials

Horse heart cytochrome *c* and sperm myoglobin were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Cyan BCI-5C printer ink was purchased from Canon (Tokyo, Japan). HPMC and other chemical reagents were purchased from Sigma–Aldrich Japan and Wako (Osaka, Japan), respectively.

2.3. Methods

2.3.1. Washing and pretreatment with HPMC

Washing of the mFFE module was carried out in the following order: 0.1 *M* NaOH–ethanol (50:50, v/v), 0.01 *M* HCl, 80% aqueous ethanol and then 2 ml of a 0.1% HPMC solution was poured down from pump P1 in Fig. 2. Then 2 m*M* of Tris–HCl (pH 8.0) solution of electrophoresis buffer from seven buffer inlets with flow-rate 0.55 ml/min for 2 min equilibrated the separation chamber and concluded the pretreatments procedure.

2.3.2. Electrophoresis and fractionation

An electrophoresis buffer was poured into the separation chamber by a pump P1 and electrode chambers by a pump P2. A sample solution was introduced to the center inlet of the sample by pump P3 in Fig. 2. A small volume of sample was introduced via the sample injection port. When the sample stream observed in the separation chamber made stationary flow the voltage of 2 kV was applied to the chamber. Fractionation was started after steady state of electrophoretic sample separation was achieved as judged by the flow shape. The electrophoresis continued and the fractionated samples were introduced to the 19 microtubes via MSF.

2.3.3. Analysis of laminar flow and electrophoresis

The photos of the top view of the separation chamber plane showing the electropherograms were digitized using the IgorPro software (WaveMetrics, OR, USA). This was then used to estimate the chamber laminar flow and migration profiles of the sample lanes. We conducted a ''zebra flow'' made from a 0.2% bromophenol blue (BPB) dye in buffer

solution and without any dye buffer solution to observe laminar flow. This ''zebra zone'' makes a white-yellow zebra pattern on the separation chamber plane. The solution of Canon cyan (BCI-5C) dye was also used for observations of the sample streams. Flow rates in the separation chamber and in the electrode chambers were 0.55 and 1.1 ml/min, respectively, and the sample flow was 7.26 μl/min.

2.3.4. Analysis of proteins by HPLC

The component amounts of the cytochrome c and the myoglobin were estimated by HPLC analysis. The HPLC system consisted of two LC-9A pumps (Shimadzu, Kyoto, Japan), a Rheodyne 7125 injector (Rheodyne, Rohnert Park, CA, USA), an SPD-6AV detector (Shimadzu), and a data sampling system composed from a multidigital meter Sanwa PC5000 with a personal computer link (Sanwa Electric Instrument, Tokyo, Japan) and a computer IBM Aptiva (IBM Japan, Tokyo, Japan). The separation system consisted of Merck Chromolith performance RP-4e column (Merck, Darmstadt, Germany) and the gradient of mobile phase was made from 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA containing 60% acetonitrile (pH 2.0). The flow-rates was 1.0 ml/min. Analog signals from SPD-6AV detector were digitized by a multidigital meter PC5000 and then transferred to a computer IBM Aptiva by a personal computer link. The digitized data were treated with softwares PC Link, Microsoft Excel 2000 (Microsoft Japan, Tokyo, Japan) and Igor Pro for making chromatograms and for calculating chromatograph parameters. In this system the conversion parameter of output voltage to absorbance was 800 mV/AU.

3. Results and discussion

3.1. Laminar flow

Actual flow testing was carried out using BPB dye as a sample solution. BPB solution was introduced to number 2 and 4 of the buffer inlet ports and at the both inlet ports of the electrode chambers. And the remaining 1, 3, and 5 inlet ports were introduced with buffer solution so that the solution flow should make stripes on the separation chamber's plane. We call this stripe ''zebra flow''. A photo of the ''zebra flow'' is shown in Fig. 4a). The green ingredient of the archived color photo estimates the laminar flow behavior in the mFFE module (Fig. 4b). From calculation with the digitized data, the zones of BPB were estimated to remain in the shape of 0.5 mm broad boundary in the right-angled direction of the flow. This means satisfactory laminar flow in mFFE and compensates the reported results of laminar flow that the flow in the separation bed showed very stable and homogeneous velocity distribution over wide range by xylene cyanol dye experiment [10].

3.2. HPMC pretreatment

In order to check the pretreatment effect of HPMC, the experiment using a Canon cyan (BCI-5C) dye solution was conducted. The effects were estimated by digitized data according to extraction of the red ingredient of the color photos of electrophoresis. At zero voltage, the width of the sample stream at the bottom was the same as at the site of injection port (Fig. 5a) but when the voltage of 2 kV was applied under the same conditions, the zone of the dye had expanded as it flows down to the bottom side (Fig. 5b). After the separation chamber was pretreated with HPMC as described in Section 2.3, the spread of the dye zone was reduced to one sixth of the non treated case at 2 kV (Fig. 5c). The currents of the electrophoresis were maintained within around 0.7 mA in both cases. It is thought that this phenomenon is based on performance of the coating in reducing EOF. Although we did not measure EOF since a neutral dye with a molar absorption coefficient measurable under visible light was not purchased. Our temporary consideration is that pretreatment of the chamber with HPMC seems effective in reducing EOF.

3.3. Sample injection rate

After our pretreatment procedure, a Canon cyan (BCI-5C) dye solution was used to measure the amount of sample poured into the separation chamber. A sample injection speed for the mFFE module was changed from 3.63 to 18.15 μl/min (Fig. 6), and a spread of a zone was measured based on digitized data of photos as described above. The

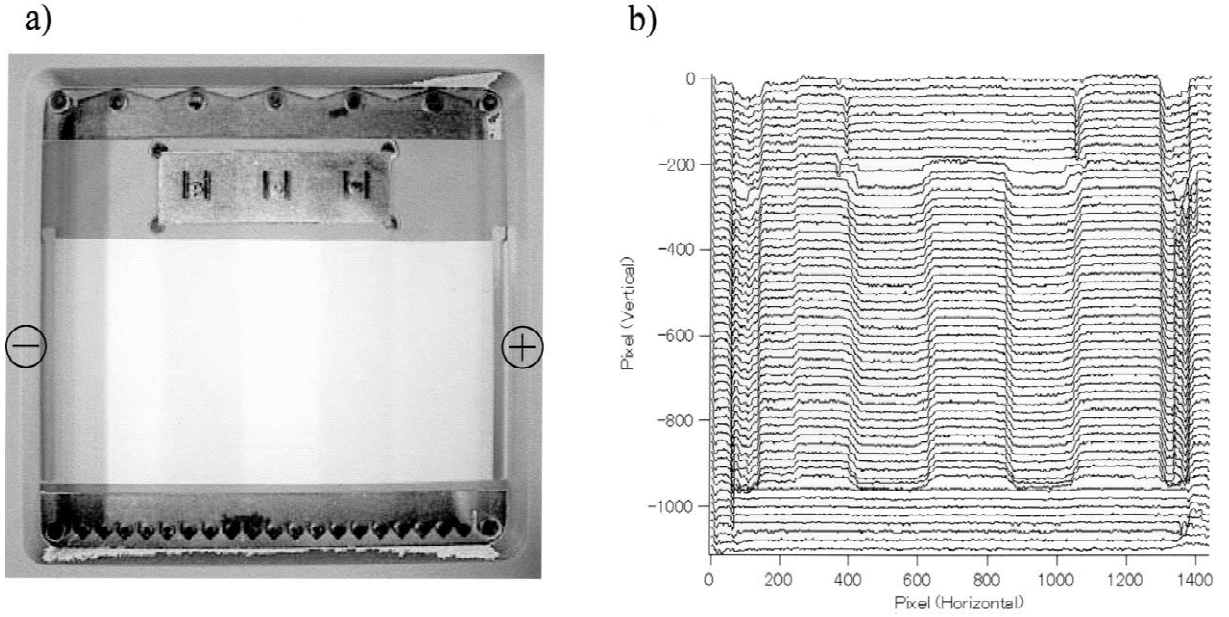

Fig. 4. A profile of the ''zebra flow'' demonstrated with BPB dye introduced into electric chambers and inlets no. 2 and 4. (a) A photograph taken from the upper part of the mFFE separation chamber. (b) A digitized profile of the extracted green ingredient of the color photograph of a. A vertical and horizontal axes are indicated in pixels of digitized data (100 pixels=4.25 mm).

tendency for the zone width at an injection port tended to spread as the flow velocity increased was observed in both upper and lower separation chamber parts. We selected the flow-rate of 7.26 μl/min because the maintained zone was relatively narrow and yet not too slow to investigate continuous electrophoresis.

3.4. Continuous free flow electrophoresis of proteins

Under the previously described conditions, continuous free flow electrophoresis of the protein sample by mFFE was carried out. For a preliminary experiment, the cytochrome *c* solution (1 mg/ml) was introduced to the center of the sample inlet port at the flow-rate of 7.26 μl/min. The photo of electrophoresis of cytochrome *c* is shown in Fig. 7. The zone width had kept constant both at the injection port and the bottom harvesting port when 2 kV was supplied. In the case of myoglobin (1 mg/ml) the contrast of the photo was not clear (data is not shown) but the protein zone showed similar stability. Good results were obtained in each experiment for proteins so that a protein mixture sample of cytochrome *c* and myoglobin (1 mg/ml of each) was applied to continuous electrophoresis. When the cytochrome *c* stream observed in the separation chamber made stationary flow, the voltage of 2 kV was applied. The electrophoresis continued and the fractionated samples were introduced to the 19 microtubes via MSF. The electrophoresis time for complete protein separation in the mFFE separation chamber was about 10 s. This fractionation took 25 min and the current of the electrophoresis was maintained within around 0.7 mA. All of the fractionated samples were kept in refrigeration (−80 °C) until the HPLC analysis was carried out.

3.5. Analysis of proteins of the fractionated samples

The fractionated samples were analyzed by reverse phase HPLC as described in Section 2.3. As shown in Fig. 8 none of the cytochrome *c* at fraction number 7 reveals cross talk phenomena at adjacent fractions. For the myoglobin a similar but somewhat broader separation result occurred at fraction num-

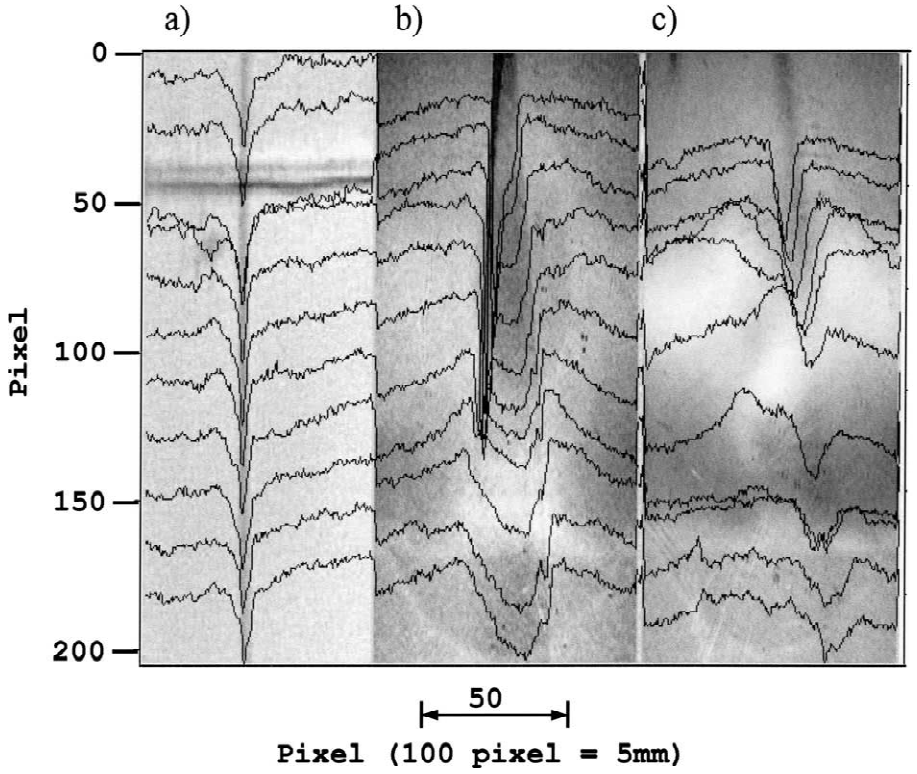

Fig. 5. The electrophoresis profiles of a cyan dye. (a) No voltage supplied. (b) At applied voltage 2 kV. (c) At applied voltage 2 kV after the pretreatment of the chamber with HPMC. Digitized data of the extracted red ingredient of the color photograph taken from the upper part of the mFFE separation chamber are piled up on each of photographs.

bers 8 and 10 in both sides of the main fraction 9. The weak signals in the other fractions at the same retention time to the myoglobin were thought that these signals originated from mobile phase buffer solution in HPLC. This electrophoresis result was in agreement with the following theoretical prediction. If the isoelectric point of an ampholyte is the same value as the electrophoresis buffer (pH 8.0), the component does not have an effective charge and will flow down into the center of the separation chamber, into fraction number 9, without migration. In the case of proteins as the isoelectric points (p*I*) of cytochrome *c* and myoglobin are 10.2 and 7.4, respectively, the net charges of the former is positive and the later slightly negative in the buffer so that the former migrates to the cathode and the later to the anode depending on the size of its charges. A little part of the myoglobin fraction in number 8 might result as migration is slight and thus shares its zone stream with fraction numbers 9. Although this result is considered as a part of separation capacity and as a limitation due to the manufacturing of the module configuration, it is possible, by replacing the electrophoresis buffer pH with a more suitable pH, to modify these results.

4. Conclusion

The efficiency of the banks' shape of the mFFE module was enough to prevent dispersion to the separation chamber of bubbles generated at the electrodes. Within a continuous mFFE–MFS system under 2 kV of electrophoresis separation of the protein mixture sample occurred after 25 min. As a result, cytochrome *c* and myoglobin were completely separated from each other by continuous mFFE, and the MFS functioned efficiently during ''continuous''

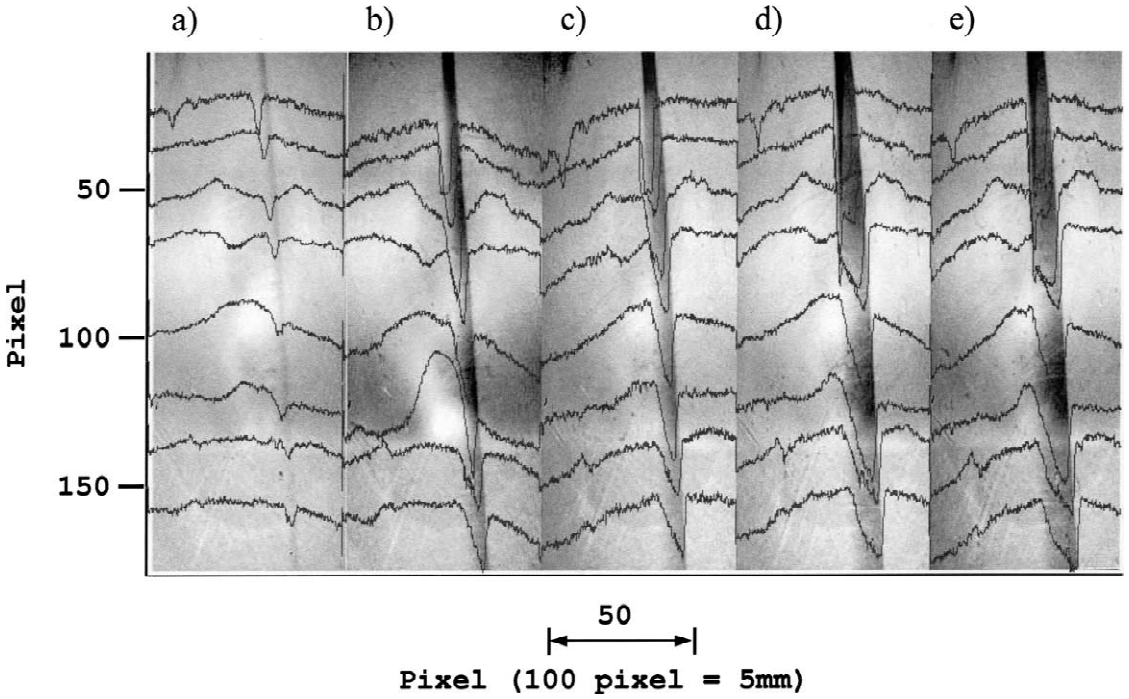

Fig. 6. The electrophoresis profiles of a cyan dye at 2 kV. The flow-rates of the samples were (a) 3.63, (b) 7.26, (c) 10.89, (d) 14.52, and (e) 18.15 μl/min. Digitized data of the extracted red ingredient of the color photograph taken from the upper part of the mFFE separation chamber are piled up on the each photographs.

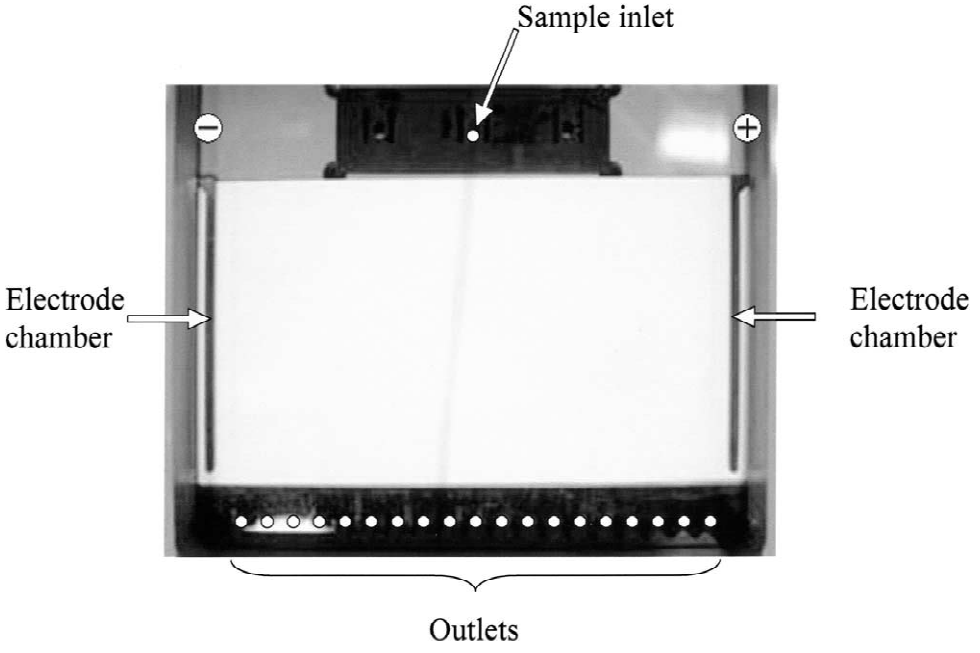

Fig. 7. A separation profile of the cytochrome *c* in mFFE chamber at 2 kV. A photograph taken from the upper part of the mFFE separation chamber.

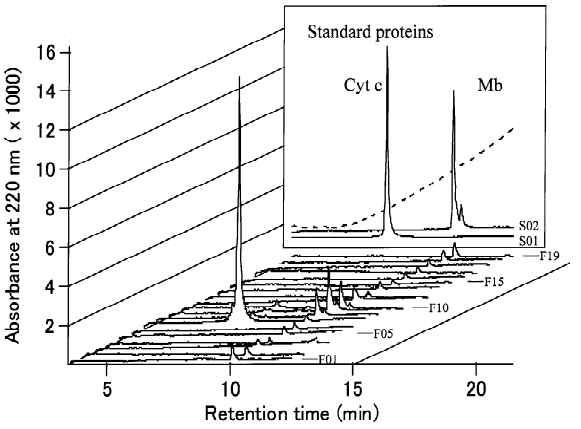

Fig. 8. HPLC separation profile of protein samples fractionated by using of MFS attached to mFFE. Analytical conditions for HPLC: column: Chromolith performance RP-4e 100×4.6 mm, gradient system: 0–60% acetonitrile in 0.1%TFA). S01 and S02 indicated in right side are the chromatograms of the standard samples, cytochrome *c* and myoglobin, respectively. The HPLC analyses of the separated fractions by mFFE are shown in 19 chromatograms from F01(cathode side) to F19 (anode side).

electrophoresis. Although the EOF in the mFFE system was suppressed by the HPMC pretreatments procedure, its efficiency was not enough for usage during more long term. Recently, we succeeded in chemical coating of the surface of the separation chamber of mFFE module and preliminary results of suppression experiments of EOF were satisfactory to compare with those of HPMC. By using this coated mFFE module, since this module is also easy to transfer the heat by using conventional air blow equipment, it will become more advantageous to separate biological materials such as proteins without losing biological activity, even if the size of sample is smaller or larger.

References

[1] K. Hannig, Z. Anal. Chem. 181 (1961) 244.
[2] N. Tajima, H. Suzuki, K. Kano, E. Shinohara, in: Proceedings of the 22nd International Symposium on Capillary Chromatography, Gifu, Japan, 1999, p. 2.
[3] J. Bauer, G. Weber, J. Disp. Sci. Technol. 19 (1998) 937.
[4] G. Weber, P. Bocek, Electrophoresis 19 (1998) 3094.
[5] V. Kasicka, Z. Prusik, P. Sazelova, J. Jiracek, T. Barthi, J. Chromatogr. A 796 (1998) 211.
[6] M. Poggel, T. Melin, Electrophoresis 6 (2001) 1008.
[7] P. Hoffmann, H. Ji, R.L. Moritz, L.M. Connolly, D.F. Frecklington, M.J. Layton, J.S. Eddes, R.J. Simpson, Proteomics 7 (2001) 807.
[8] D.E. Raymond, A. Manz, H.M. Widmer, Anal. Chem. 66 (1994) 2858.
[9] E. Shinohara, N. Tajima, H. Suzuki, J. Funazaki, Anal. Sci. Suppl. 17 (2001) i441.
[10] E. Shinohara, N. Tajima, J. Funazaki, K. Tashiro, S. Shoji, Y. Utsumi, T. Hattori, in: Proceedings of the μTAS 2001 Symposium, 2001, p. 51.