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[Full Paper]

Miniaturized two-dimensional gel electrophoresis of high-molecular-weight proteins using low-concentration multifilament-supporting gel for isoelectric focusing

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SUMMARY

A miniaturized two-dimensional gel electrophoresis (2-DE) system was constructed with a multifilament-supporting (MFS) gel as a first-dimensional gel for isoelectric focusing (IEF). The MFS gel was 1 mm in diameter, had a multifilament yarn of acrylic as a gel-support, and could provide low-concentration (2.5%T) polyacrylamide with sufficient mechanical strength, resulting in fast IEF, and clear 2-D spots due to the relatively large pore and improved transfer efficiency from the first-dimensional gel to the second-dimensional one of especially high-molecular-weight (HMW) proteins. In this study, by using the MFS gel of 4 cm in length as a miniaturized IEF gel, three colored proteins were separated in 8 min, which was about twice faster than using the MFS gel of standard length (7 cm). The second-dimensional gel was also miniaturized to be 55 mm wide, 40 mm long and 0.75 mm thick. The low-concentration MFS gel after IEF was easily transferred onto the top of the miniaturized second-dimensional gel with a tweezers. By using the miniaturized 2-DE system, the HMW size marker was separated in 30 min by IEF and in 25 min by native-polyacrylamide gel electrophoresis (Native-PAGE), which was at least twice faster than by using the standard size 2-DE system using MFS gel. This miniaturized 2-DE system could be expected as a useful separation method for fast protein diagnosis and screening.

Key words: two-dimensional gel electrophoresis, high-molecular-weight protein, miniaturization, polyacrylamide, multifilament yarn

INTRODUCTION

Two-dimensional gel electrophoresis (2-DE) is a powerful separation method, where proteins can be separated according to their isoelectric point in the first isoelectric focusing (IEF) and their size in the second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the conventional 2-DE needs handling skills, and long analysis time, at least two days. A commercially available immobilized pH gradient (IPG) dry strip gel is easier to handle. However, it takes overnight to rehydrate the dry IPG strip gel. Also, the concentration of the IPG strip gel is 4%T and is not suited for fast separation of high-molecular-

weight (HMW) proteins. Some of the HMW proteins are considered to have relation with disease. For example, myosin heavy chain and thyroglobulin were known to have close relation with heart disease and disturbed thyroid function, respectively¹⁾. Hirabayashi demonstrated a 2-DE system with agarose gel in the first dimensional IEF could separate myosin heavy chain²⁾. Oh-Ishi et al. showed that a 2-DE method with an agarose IEF gel in the first dimension produced significant improvements in 2-DE separation of HMW proteins larger than 150 kDa and up to 500 kDa^{1,3,4)}. Due to the relatively large pore of agarose gel, much more proteins including HMW proteins are considered to enter agarose gel than polyacrylamide gel. However, as the 2-DE

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system using agarose gel for IEF needs technical skills so far, desired is a new 2-DE system that can provide fast separation of proteins including HMW species without need of such technical skills.

We have proposed as a new IEF gel for 2-DE a low concentration polyacrylamide gel supported by a loose multifilament string⁵. The multifilament-supporting (MFS) gel was 1 mm in diameter, and by using a multifilament yarn as an IEF gel support, the total acrylamide concentration of the gel could be reduced to 2.5%T with sufficient mechanical strength, and the gel was easily handled by picking up with tweezers. Due to the large pore of the low-concentration (2.5%T) polyacrylamide gel, proteins were focused faster than by using the 4%T gel. Also, proteins, especially in the HMW region, were observed more clearly on the silver-stained 2-D map than those focused in the 4.0%T gel, being comparable to the separation using the agarose IEF gel.

To speed up electrophoretic separation, miniaturization of the separation medium itself is also effective. Zheng et al. constructed miniaturized slab gel electrophoresis system, which was applied to fast genetic diagnosis^{6,7}. Micro 2-DE is also used as rapid separation method of small amount of protein samples using 4%T polyacrylamide IEF gel and second dimensional polyacrylamide gradient slab gels⁸⁻¹³. For example, Manabe et al. used multiplex system of 35 mm long, 1.3 mm I.D. capillary gel columns for IEF, and 38 mm wide, 38 mm long and 1 mm thick gradient polyacrylamide slab gels for second-dimensional electrophoresis with total separation time 140 min¹³.

In the present study we miniaturized the 2-DE system with the MFS gel. The miniaturized IEF system with the MFS gel was evaluated compared with the standard-sized (7 cm long) IEF system with the MFS gel. Furthermore, using HMW Native Marker Proteins as a sample, separation performance of the miniaturized 2-DE system for the MFS gel was evaluated in comparison with the result using the standard size of the 2-DE system with an IPG gel in the first dimension.

MATERIALS AND METHODS

Preparing the MFS gels

The MFS gels were made according to the literature⁵ with some modification. An apparatus for preparing the MFS gels is shown in Fig. 1. Each acrylic yarn (75 denier, 40 filaments) was set in each groove on one of the two acrylic boards. The two acrylic boards were assembled with two clamps. An acrylamide stock solution of 30%T, 5.4%C was diluted with water to 2.5%T or 4.0%T, 5.4%C. A 30 μ l of 10% ammonium persulfate (APS) and a 20 μ l *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added to a 10 ml of the diluted acrylamide solution. The mixed solution was immediately introduced into the 20 channels in the MFS gel maker using a syringe. After polymerization, the MFS gels of 9 cm in length and 1 mm in diameter were taken carefully out of the grooves. The MFS gel had an adequate flexibility as shown in Fig. 2A, and was not easily broken. Although multifilament yarns were found a bit localized in the lateral distribution as shown in Fig. 2B, IEF separation performance was not affected so far. A major reason for this should be that the multifilament yarns occupied below 1% of the MFS gel. The MFS gel was soaked in water for desalting, and stored in the refrigerator until use.

Miniaturized 2-DE system

The miniaturized 2-DE system for the MFS gel of 4 cm is shown in Fig. 3. The IEF plate was made of acrylic or

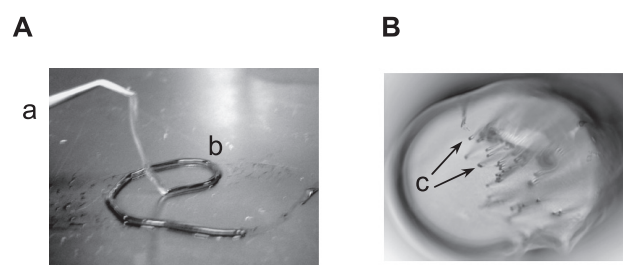


Fig. 2. Photos of the MFS gel (A) and a cross-sectional view of the MFS gel (B).

a, Tweezers; b, MFS gel; c, Multifilament yarns.

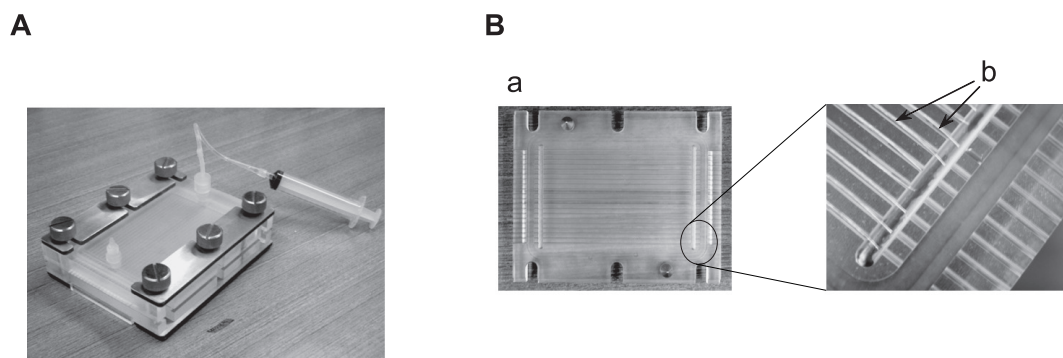


Fig. 1. Apparatus for preparing MFS gels (A) and acrylic board with 20 grooves (B).

a, Acrylic board; b, Multifilament yarns.

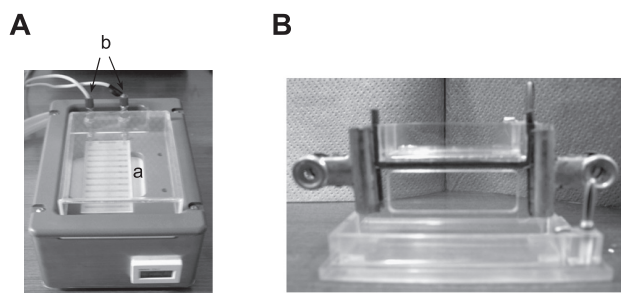


Fig. 3. Miniaturized IEF system (A) and second-dimensional electrophoresis apparatus (B).

a, Miniaturized IEF plate; b, Terminals.

ceramic, and had ten semicylindrical grooves (44 mm long and 1 mm in diameter) for the miniaturized MFS gels. The apparatus for the IEF system for the miniaturized MFS gel was 16 cm wide, 23 cm long and 11 cm high, and was equipped with a water-cooling unit. The second-dimensional gel was also miniaturized to be 55 mm wide, 40 mm long and 0.75 mm thick.

IEF using MFS gel

The MFS gel was soaked in 5 ml of Ampholine solution for at least one hour before it was applied to IEF. Filter paper was cut into 3 mm wide and 3 mm long, set in the groove, about 1 cm from the cathode end, and got to absorb 1–3 μ l of sample solution. The MFS gel was cut into appropriate length and then put in the groove of the IEF plate. The MFS gel could contact with the electrodes in the groove and electrode solutions were not used. Silicon oil was overlaid to prevent the MFS gel from drying. After the plate was covered and set on the IEF apparatus equipped with a water-cooling unit, separation voltage was applied, and the sample solution in the filter paper moved into the MFS gel. Applied voltage was raised at the rate of 10 V per 4 s or 8 s. Maximum voltage was set at 1.71 kV.

IEF using IPG gel

IPG gel (Immobiline DryStrip pH 4–7, 7 cm, GE Healthcare) was first rehydrated overnight, and applied to the first dimensional IEF (Multiphor II, GE Healthcare).

Native-PAGE

The MFS gel after IEF was put on the top of the second-dimensional gel with agarose sealing. The slab gel was composed of stacking gel that consisted of 4.0%T, 2.6%C polyacrylamide and the resolving gel of 7.5%T, 2.6%C. The second electrophoresis buffer of 25 mM Tris and 192 mM glycine (pH 8.3) was used. Separation condition was 15 mA/gel for the miniaturized system and, 30 mA/gel for the standard size system.

Silver staining was used to visualize proteins on the 2-D gel.

Sample preparation

To visualize the IEF separation performance, three colored proteins, bovine blood hemoglobin (MW 67 kDa, pI 7.2), bovine heart cytochrome *c* (MW 13 kDa, pI 9.6), spirulina C-phycocyanin (MW 264 kDa, pI 4.3) were used. They were dissolved in an Ampholine solution to be 15 μ g/ μ l, 6 μ g/ μ l, and 6 μ g/ μ l, respectively.

As a HMW protein sample, 1 vial (250 μ g protein) of HMW Native Marker Kit was dissolved in 100 μ l of Ampholine solution.

Materials and reagents

Acrylic yarn was provided by Mr. K. Onda of Textile Research Institute of Gunma. Acrylamide, *N,N'*-methylenebisacrylamide, APS, Ampholine pH 3.5–10.0, Ampholine pH 5.0–8.0, Ampholine pH 4.0–6.5, β -mercaptoethanol, HMW Native Marker Kit, and Silver Staining Kit for Proteins were purchased from GE Healthcare. TEMED, glycine, agarose, glycerol, Tris base, sodium dodecyl sulphate, polyoxyethylene (9) octylphenyl ether (NP-40), urea, potassium iodide, and *N*^α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) were from Wako Pure Chemicals (Osaka, Japan). Thiourea, bovine blood hemoglobin, and bovine heart cytochrome *c* were from Sigma (St. Louis, MO). Spirulina C-phycocyanin was from Tokai Sangyo Ltd. (Tokyo, Japan). All the reagents for IEF were ultrapure or specially manufactured for the use in gel electrophoresis since IEF is very sensitive to impurities.

RESULTS AND DISCUSSION

pH gradient of the miniaturized MFS gel

First we investigated on the pH gradient formed on the miniaturized MFS gel. Ampholine solution used was composed of 2.5% Ampholine (pH 3.5–10), 2.5% Ampholine (pH 5–8), 1.0% glycerol, and 2.0% NP-40. The miniaturized MFS gel was soaked in the Ampholine solution and set on the IEF plate. The voltage was applied for 30 min, and it was cut into pieces of 5 mm long. Each piece was soaked in a 25 μ l of water for 2.5 hours, and pH value of the solution was measured, and plotted as shown in Fig. 4. It was found that the pH gradient was almost linearly formed along the miniaturized MFS gel.

Fast IEF using the low-concentration miniaturized MFS gel

The standard-size MFS gel is 7 cm long. Compared were the MFS gels of 7 cm long and 4 cm long on IEF separation speed of the three colored proteins, C-phycocyanin, hemoglobin, and cytochrome *c*. Photos of the MFS gels were taken every 2 min to monitor the mobility of the three colored proteins. As shown in Fig. 5A, using the 7 cm MFS gel, the three colored proteins were separated in 14 min. While, using the 4 cm MFS gel, the proteins were separated in 8 min (Fig. 5B), which was nearly twice as fast as in

the case of using the 7 cm MFS gel.

By using multifilament yarn as a gel support, a low-concentration (2.5%T, 5.4%C) MFS gel was realized to be utilized. Because a low-concentration gel is considered to

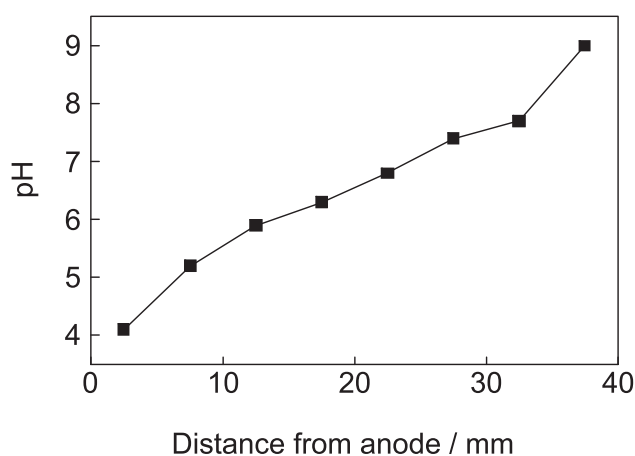


Fig. 4. The pH gradient along the miniaturized MFS gel.

have a larger pore, the MFS gel was expected to provide faster IEF separation of proteins. IEF separation performance was compared between 4.0%T, 5.4%C MFS gel and 2.5%T, 5.4%C MFS gel. Mixture of the three colored proteins, C-phycocyanin, hemoglobin, and cytochrome *c*, was used as a sample. As shown in Fig. 5C, using the 4%T MFS gel, the three proteins were separated in 14 min. While, using the 2.5%T MFS gel (Fig. 5B), the proteins were separated in 8 min. The low-concentration MFS gel was proved effective for fast IEF separation due to the large pore.

Fast 2-DE separation of HMW native marker proteins

Proteins of a HMW Native Marker Kit were separated as shown in Fig. 6A and 6B, using the standard size 2-DE system with an IPG gel as a first dimensional gel, and using the miniaturized 2-DE system, respectively. Comparing the two results, in the case using the miniaturized MFS gel, a half an hour seemed to be enough to perform the first dimensional IEF, and it took only 25 min in the second

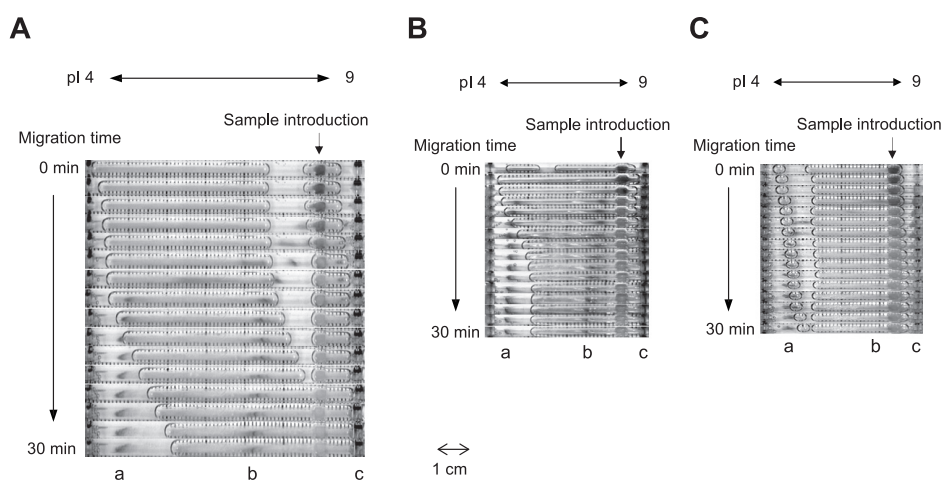


Fig. 5. Separation of three colored proteins using low-concentration (2.5%T, 5.4%C) MFS gels of standard length (7 cm) (A), miniaturized size (4 cm) (B), and standard concentration (4.0%T, 5.4%C) MFS gel of miniaturized (C).

Separation voltage was raised at the rate of 10 V per 4 s. Maximum voltage was set at 2.25 kV (A), and 1.71 kV (B and C). a, C-phycocyanin; b, Hemoglobin; c, Cytochrome *c*.

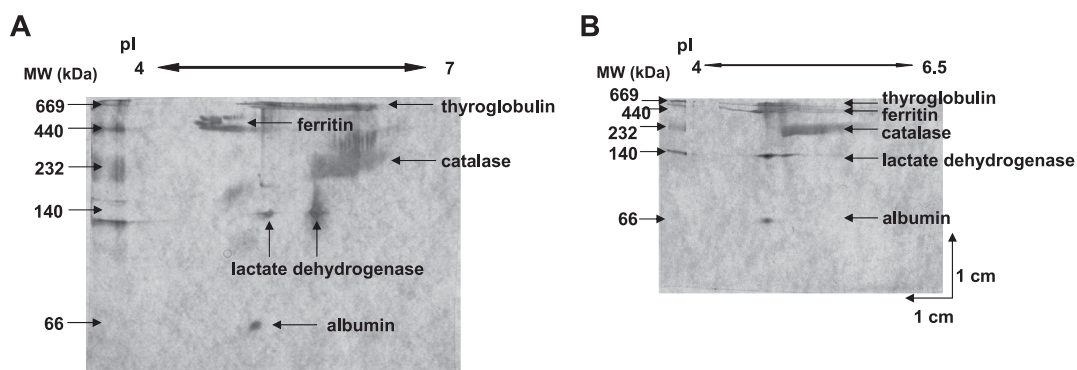


Fig. 6. 2-DE separation of HMW marker proteins using the standard size 2-DE system with an IPG gel (A), and the miniaturized 2-DE system with an MFS gel (B).

IEF: 300 V for 30 min, 300–1000 V for 30 min, 1000–3500 V for 50 min, and at 3500 V for 60 min (A); voltage was raised at the rate of 10 V per 8 s (max. 2.25 kV) (B). Native-PAGE: 30 mA/gel for 60 min (A), and 15 mA/gel for 25 min (B).

dimensional Native-PAGE. Therefore, an hour was enough to carry out 2-DE separation using the miniaturized system.

Miniaturized 2-DE system using MFS gel as an IEF gel was constructed, and its basic performance was evaluated. Proteins of the HMW Native Marker Kit was applied to fast 2-DE separation using the miniaturized 2-DE system, and proteins of HMW region above 200 kDa up to 669 kDa (thyroglobulin) were successfully separated and visualized on the 2-D map. This system can be expected as a fast protein separation method for disease diagnosis and screening.

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ABBREVIATIONS

2-DE, two-dimensional gel electrophoresis; MFS, multifilament-supporting; IEF, isoelectric focusing; HMW, high-molecular-weight; Native-PAGE, native-polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; APS, ammonium persulfate; TEMED, *N,N,N,N*-tetramethylethylenediamine; NP-40, polyoxyethylene (9) octylphenyl ether

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