New Detection Method After Cellulose Acetate Membrane Isoelectric Focusing: Activity Staining for Lactate Dehydrogenase, Detection for Sugar Chains Using Lectin and Simple Western Blotting

Shiro Iijima,^{1*} Miyako Kimura,¹ and Kiyoko Shiba²

¹*Division of Toxicology, Kyoritsu College of Pharmacy, Tokyo, Japan*

²*School of Allied Health Science, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo, Japan*

To extend the clinical applications for cellulose acetate (CA) membrane isoelectric focusing, we designed two detection methods and two blotting methods that improve the characteristics of the CA membrane; that is, detection of the lactate dehydrogenase (LD) isozyme on the CA membrane, detection of the sugar chain in glycoprotein on the CA membrane, and simple and rapid methods for western blotting. In the detection of the LD isozyme, when 50% saturated ammonium sulfate solution was used as a fixing solution, five main LD bands and seven sub LD bands were detected clearly. By treating the CA membrane with 5% sulfosalicylic acid, glycoprotein was fixed on

the CA prior to detection of the sugar chain of glycoprotein using Lens Culinaris (LCA), Phascolus Vulgaris-L₄ (PHA-L₄), Maackia Amurensis (MAM), and Sambucus Siedoliana (SSA) lectins. Using this procedure, clear electrophoretic patterns were obtained. As simple and rapid methods for blotting proteins on polyvinylidene difluoride membranes, we designed natural contact blotting and suction blotting techniques. The natural contact blotting method did not need special apparatus. The suction blotting method completed protein blotting for 10 minutes. By both methods, clear electrophoretic patterns were obtained. *J. Clin. Lab. Anal.* 13:234–240, 1999.

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INTRODUCTION

Even proteins with slight side chain differences can be separated by isoelectric focusing (IEF). Therefore, the detection of protein microheterogeneity was facilitated by IEF. Since protein microheterogeneity may provide useful information about analysis of pathologic conditions in various diseases, this has been the focus of our attention. Although agarose and polyacrylamide gels are widely used as supporting medium for IEF, preparation and handling of these gels are troublesome. In contrast, the cellulose acetate (CA) membrane is easy to handle and frequently used as a support medium in many clinical laboratories. Using the CA membrane as a supporting medium, we previously developed an IEF apparatus and reported optimal conditions for clearly detecting serum proteins as well as glycoproteins and specific proteins (1–4).

Furthermore, when we performed an IEF using six layers of CA membranes simultaneously, the levels of proteins detected were smaller at lower layers of the CA membranes. However, it was demonstrated that electrophoretic patterns were identical in each CA membrane. Thus, we have devel-

oped an IEF method that simultaneously provides six different information by single electrophoresis (5). However, three issues are unsolved at this time. First, Toda et al. reported that there were differences in protein distribution to each membrane based on differences in molecular weights when protein distributions in each CA membrane of six layers were compared after electrophoresis (6). Second, only two detection methods have been developed for proteins on the CA membrane; staining with Coomassie brilliant blue G-250 (CBB) and glycoprotein staining with Schiff reagent, in spite of six electrophoretic patterns, could be obtained. Third, a Western blotting method for the CA membrane IEF has not yet been developed. To further extend the application of this method, we investigated these points.

*Correspondence to: Shiro Iijima, Division of Toxicology, Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo, 105-8512, Japan. E-mail: iijima-sr@kyoritsu-ph.ac.jp

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MATERIALS AND METHODS

Samples

Patient sera were collected from Tokyo Medical and Dental University Hospital. Control sera were collected from normal volunteers.

Chemicals

Separax EF (CA membrane) and Sepaline 3.5–10 (carrier-ampholyte) were from Fuji Photo Film (Tokyo, Japan). SERVALYT 4–6, SERVALYT 9–11 (carrier-ampholyte) were from Boehringer Ingelheim (Heidelberg, Germany). Coomassie brilliant blue G-250 and tween-20 were from Sigma Chemical (St. Louis, MO). Gel Filtration Calibration Kit containing ovalbumin and bovine serum albumin (BSA) and HMW Gel Filtration Calibration Kit containing catalase and thyroglobulin were from Amersham Pharmacia Biotech (Arlington Heights, IL). PBS was from MBL (Nogoya, Japan). β -Nicotinamide adenine dinucleotide was from Kohjin (Tokyo, Japan). 1-Methoxy-5-methylphenazinium methylsulfate was from Dotite (Kumamoto, Japan). Rabbit antihuman transferrin antibody, peroxidase-conjugated streptavidin, and peroxidase-conjugated goat antirabbit immunoglobulin antibody were obtained from Dako (Glostrup, Denmark). Blockace (blocking solution) was from Dainihon Pharmaceutical (Osaka, Japan). Peroxidase-labeled Phasaelus Vulgaris-L₄ lectin (PHA-L₄), peroxidase-labeled Lens Culinaris lectin (LCA), biotin-labeled Maackia Amurensis lectin (MAM), biotin-labeled Sambucus Siedoldiana lectin (SSA), nitro blue tetrazolium (NBT), and other chemicals of reagent grade were from Wako Pure Chemicals (Osaka, Japan).

IEF Procedures

The conditions for IEF, CBB staining, transferrin detection, and analysis of electrophoretic pattern were carried out according to our method described (1,4,5).

LD Activity Staining on CA Membrane

Applied serum volume was according to the lactate dehydrogenase (LD) activity as follows: 3 μ l at 300–500 IU, 2 μ l at 501–1,000 IU, and 1 μ l at 1,001–1,500 IU. After IEF, the CA membrane was immersed in fixing solution (50% saturated ammonium sulfate, 450 mmol/l diethanolamine-HCl buffer, pH 8.8, prepared before use) for 15 minutes. After fixing, 2 ml of staining solution (60 mmol/l lithium L-lactate, 6 mmol/l β -nicotinamide adenine dinucleotide, 8.12 mmol/l NBT, 0.09 mmol/l 1-methoxy-5-methylphenazinium methylsulfate, 300 mmol/l diethanolamine-HCl buffer, pH 8.8, prepared before use) for LD-activity staining was overlaid on the CA membrane, and the membrane was incubated for 1 hour at 37°C. After staining, the CA membrane was immersed in a 70% ethanol solution containing 5% acetic acid for 3 minutes,

then in distilled water for 5 minutes. Before drying, the CA membrane was immersed in dry solution (7% glycerol, 5% acetic acid, 2% polyvinyl alcohol solution) for 15 minutes.

Detection of Sugar Chain Using Lectin on CA Membrane

After IEF, the CA membrane was immersed in 5% sulfosalicylic acid for 5 minutes, and washed 2 times in T-PBS (0.05% tween-20 in PBS) for 5 minutes each time. Peroxidase-labeled PHA-L₄ and LCA lectin were diluted to 1 μ g/ml with T-PBS, respectively. Biotin-labeled MAM and SSA lectin, and peroxidase-conjugated streptavidin were diluted to 3 μ g/ml with T-PBS, respectively. The CA membrane was immersed in each lectin solution for 3 hours at room temperature, and was washed 3 times in T-PBS for 5 minutes each time. The CA membrane treated with MAM or SSA lectin solution were immersed in 3 μ g/ml peroxidase-conjugated streptavidin solution for 1 hour and was washed 3 times in T-PBS for 5 minutes each time. The development solution containing of 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.006% H₂O₂ was prepared using T-PBS before use. The CA membrane was immersed in the development solution for 5 minutes.

Natural Contact Blotting

PVDF membrane (Immobilon, Millipore Corp., Bedford, MA) and filter paper (3MM Chr, Whatmann, Hillsboro, OR) were wetted with blotting buffer (25 mM Tris, 192 mM glycine, 5% methanol, 0.05% SDS). After IEF, the CA membrane was placed on a glass plate, and overlaid with a PVDF membrane and filter paper. To remove excess solution in PVDF membrane, CA membrane and filter paper, a hand roller was used for rolling over the layers. Then the layers on the glass plate was allowed to stand overnight in a moisture box.

Suction Blotting

The apparatus for suction blotting was made by improving a commercially available gel dryer (400H, Anatech, Japan). Figure 1 shows the diagram of our suction blotting apparatus. The PVDF membrane (Immobilon-P^{8Q}) (Millipore) was wetted with blotting buffer. PVDF membrane was placed on the porous plate, and overlaid with CA membrane. After 10 ml of T-PBS was poured onto the CA membrane, the solution was sucked through the membrane with a pressure of 100 mmHg for 1 minute.

RESULTS

Distribution of Proteins With Different Molecular Weights Among the Six CA Membrane Layers

Using this method, six layers of CA membranes can be simultaneously electrophoresed. However, the volumes of

Fig. 1. Diagram of suction blotting apparatus for CA membrane. **a**, top view; **b**, side view. 1, silicon rubber (thickness: 1mm); 2, porous plate; 3, suction hole; 4, PVDF membrane; 5, CA membrane.

proteins detected were smaller in lower layers of CA membranes (5). Using thyroglobulin (MW: 669,000), catalase (MW: 232,000), BSA (MW: 76,000) and ovalbumin (MW: 43,000), we evaluated whether protein distribution to each CA membrane differed depending on protein molecular sizes when layered CA membranes were simultaneously electrophoresed. After electrophoresis, proteins on all CA membranes were stained with CBB, and protein concentrations in each membrane were measured using the NIH image program (developed at the U.S. National Institutes of Health) to obtain the rate of protein distribution of each CA membrane. As shown in Figure 2, since detection rates of all proteins with different molecular sizes were almost identical in each CA membrane, all proteins were found to be equally distributed in each CA membrane regardless of differences in molecular sizes.

LD Activity Staining on CA Membrane

To investigate an optimal method for fixing LD on CA membrane, we compared several fixing solutions including 5% glutaraldehyde, 5% sulfosalicylic acid, and 50% saturated ammonium sulfate solutions. As shown in Figure 3, LD was not detected when 5% sulfosalicylic acid solution was used as the fixing solution. When 5% glutaraldehyde solution was used as the fixing solution, only the periphery of LD was

Fig. 2. Distribution of thyroglobulin, catalase, BSA, ovalbumin and serum protein among 6 layers of CA membrane. After electrophoresis, all CA membranes were stained with CBB. The relative ratios of each protein against its total amount in six membranes were measured using NIH image program. Each value represented the mean $\pm$ 1 SD of the calculated measurement value of 5 preparations.

stained. However, when 50% saturated ammonium sulfate solution was used as the fixing solution, LD was satisfactorily stained on CA membrane. Based on these results, 50% saturated ammonium sulfate solution was used as the fixing solution. When we attempted to analyze LD isozymes, bands of LD₁ through LD₅ were clearly detected by this method (Fig. 4). Isoelectric points of each LD isozyme were found to be as follows: 4.8 in LD₁, 5.1 in LD₂, 5.6 in LD₃, 6.8 in LD₄, and 8.6 in LD₅. Although color development was weak, seven sub bands were also detected.

Detection of Sugar Chains in Glycoproteins on CA Membrane Using Lectins

We evaluated whether sugar chains in glycoproteins can be directly detected on the CA membrane after IEF. The sugar chain in glycoprotein could not detect without fixing solution. Then we evaluated the concentration of sulfosalicylic

Fig. 3. Comparison of fixing solutions on LD activity staining. **a**, fixed with 5% sulfosalicylic acid; **b**, 5% fixed with glutaraldehyde; **c**, fixed with 50% saturated ammonium sulfate. One μ l of serum sample was applied to CA membranes.

Fig. 4. Isoelectric patterns of serum LD obtained from 8 healthy subjects.

acid, between 0.5 and 20%. The most satisfactory result was obtained when 5% sulfosalicylic acid solution was used as the fixing solution. Electrophoretic patterns of serum glycoproteins detected with PHA-L₄, LCA, MAM, and SSA without blocking procedure are shown in Figure 5. Serum samples were serially obtained postoperatively. Postoperative changes in sugar chains contained in glycoproteins were satisfactorily evaluated.

Natural Contact Blotting

We closely contacted the CA membrane to the PVDF membrane, and evaluated whether proteins on the CA membrane can be transferred to the PVDF membrane. Buffer solution for natural contact blotting basically comprised 25 mM Tris and 192 mM glycine. However, concentrations of methanol and SDS were examined. When we examined the methanol concentration at 0, 2, 3, 4, 5, and 10%, the rate of color development was increased with the methanol concentration by 3%. However, the rate of color development did not differ with the methanol concentration at 4, 5, and 10%. Based on these results, we established the methanol concentration at 5%. Moreover, we varied the SDS concentration at 0, 0.01, 0.05, 0.075, 0.1 and 0.5% for evaluation of the blotting buffer solution. The numbers of clear protein bands were increased when the SDS concentration was increased from 0 to 0.05%. However, when the concentration of SDS was higher than 0.075%, protein bands were detected even at the reverse side

Fig. 5. Isoelectric patterns for sugar chain of serum glycoprotein obtained by lectin on CA membrane. **a**, PHA-L₄ detection; **b**, LCA detection; **c**, MAM detection; **d**, SSA detection. Sample sera were ob-

tained preoperatively (-1 day) and postoperatively (0-10 days) from a patient with esophageal carcinoma.

of PVDF membrane. Therefore, the SDS concentration was established at 0.05%. Based on these results, a buffer solution containing 25 mM Tris, 192 mM glycine, 5% methanol, and 0.05% SDS was used for natural contact blotting. After an IEF, proteins on the CA membrane were transferred to the PVDF membrane using natural contact blotting to detect transferrin microheterogeneity. As a result, clear bands of proteins were detected (Fig. 6a).

Suction Blotting

Using an apparatus developed for suction blotting, we attempted to transfer proteins from the CA membrane to the PVDF membrane. To determine the appropriate pressure for suction blotting, we evaluated suction pressure between 50 and 300 mmHg. As a result, in both 5 μ g/ml and 0.5 μ g/ml of BSA, highest integrate densities on the PVDF membrane were obtained at a suction pressure of 100 mmHg (Fig. 7). Although clear BSA spots could be detected, 13.9% of applied BSA (5 μ g/ml) or 2.2% of applied BSA (0.5 μ g/ml) remained on CA membranes after suction blotting. As shown in Figure 6b,

Fig. 6. Comparison with blotting methods. Transferrin isoelectric patterns obtained by natural contact blotting (a) and suction blotting (b). Sample sera were obtained preoperatively (-1 day) and postoperatively (0–2 days) from a patient with esophageal carcinoma.

Fig. 7. Effects of suction pressure on amounts of transferred and residual protein. BSA solution was applied on the CA membrane, and transferred to PVDF membrane using suction blotting. The CA membrane and PVDF membrane were stained by the CBB method. All spots were quantified using an NIH image program. Open circles indicate 5mg/ml BSA spots, closed circles indicate 0.5 mg/ml BSA spots. Each value represented the mean $\pm$ 1 SD of the calculated measurement value of 5 preparations.

transferrin microheterogeneity pattern was detected using the suction blotting method clearly.

DISCUSSION

The characteristic of the CA membrane IEF method is that 1–6 layers of CA membranes can be electrophoresed and the migration of all protein bands are exactly the same between all layers. Therefore, it is possible to compare protein bands with very close isoelectric points to each other, because different staining techniques could be used at once on each CA membrane having exactly same electrophoretic pattern. Although protein distribution was smaller at lower layers of CA membranes compared to that at upper layers of CA membranes, we previously demonstrated that the reproducibility of protein distribution to each CA membrane was satisfactory (5). In this study, we further clarified that the molecular size of protein did not affect the protein distribution to each CA membrane. Toda et al. reported that there were differences in protein distribution to each membrane due to molecular size (6). The reason why our result did not agree with this finding was that Toda et al. detected the respective proteins using different staining; we detected all proteins using only the CBB method.

Moreover, since the pore size of the CA membrane was larger than that of any of the protein molecules, a molecular sieving effect can not be expected in CA membrane. Therefore, these results were considered to support our observations, although the protein distribution to the fifth and sixth sheets of CA membrane is very small as shown in Figure 2. A sufficient amount of the protein is kept for detection of proteins such as IgA and IgG that exist in the serum in the high concentration (5).

In this IEF method, multiple electrophoretic patterns can

be obtained at one time, and different staining methods can be performed to each membrane. To date, we have evaluated protein staining with CBB, glycoprotein staining with Schiff reagent, and specific protein detection by Western blotting (3). To extend the scope of application of this method, we developed the detection methods after CA membrane IEF.

First, using LD, we evaluated whether enzyme activity can be stained on CA membrane directly. LD activity is quite frequently analyzed in many clinical laboratories. Since optimal pH for LD was 8.8, the pH gradient (pH 3–10) formed during IEF should be equilibrated to pH 8.8 for LD activity staining. Using cellulose acetate gel membrane as a support medium, Ambler previously reported that 50% saturated ammonium sulfate solution was useful for fixing LD isozymes after electrophoresis (7). Since the CA membrane used in this experiment was thinner than cellulose acetate gel membrane, we attempted to fix proteins on CA membrane with 50% saturated ammonium sulfate solution after CA membrane IEF. As a result, even LD₁ and LD₂ were detected clearly, both of which having the pI in the acidic region. Therefore, it was found that the pH gradient on the CA membrane could be equilibrated to pH 8.8 together with a rapid protein fixation. Moreover, pIs of LD isozymes detected after IEF were almost identical to those reported by Saravia et al. (8).

Sugar chains in glycoprotein, except *N*-acetylneuraminic acid located at the end of sugar chain, are relatively stable under acidic conditions. Then, sulfosalicylic acid solution was used to fix proteins on the CA membrane. After fix, we tried to detect sugar chains in glycoproteins by lectins. As a result, the bands could be detected clearly using every lectin. The bands detected were the same as those detected using PVDF membrane (data not shown). SSA and MAM lectins detect *N*-acetylneuraminic acids that bind to mannose by α 2-6 and α 2-3 forms, respectively. The results revealed that the *N*-acetylneuraminic acid located at the end of the sugar chain is not eliminated by 5% sulfosalicylic acid, suggesting that sugar chains in glycoproteins can be easily detected on the CA membrane without transfer of the glycoproteins to the PVDF membrane. In recent years, it has been indicated that sugar chain structures in glycoproteins are related to the pathologic conditions of various diseases (9,10). Therefore, this method was considered useful for analyzing the pathologic conditions of diseases.

Since protein blotting to support medium such as PVDF membrane is essential for detecting specific proteins using antibodies, we developed simple and rapid blotting methods. Electrical semidry blotting is generally used to transfer proteins from polyacrylamide gel or agarose gel to PVDF membranes, because protein molecules stay in the gel meshes after electrophoresis (11). Since we used the thin CA membrane as the support medium, we speculated that the proteins in the CA membrane could be easily transferred to PVDF membrane without semidry blotting apparatus. Therefore, we ex-

amined whether proteins in the CA membrane could be transferred to the PVDF membrane simply by contacting the two membranes closely. As a result, proteins in the CA membrane were satisfactorily transferred to the PVDF membrane. When using the natural contact blotting method, Western blotting can be performed without any special blotting apparatus.

The suction blotting method is used to transfer DNA or RNA (12). We applied the principle and developed a suction blotting method for rapid transfer of proteins from the CA membrane to the PVDF membrane. As a result, transfer of proteins was completed in about 10 minutes, which facilitated rapid protein analysis. Although it is not shown in the results, when we used PVDF membrane with a pore size of 0.45 μ m, as generally used for protein blotting, the reverse side of the membrane was slightly stained. Therefore, we used the PVDF membrane with a pore size of 0.22 μ m and satisfactory results were obtained.

The detection of protein microheterogeneity was facilitated by the IEF having high resolution, and protein microheterogeneity has been reported to provide useful information for analysis of pathologic conditions in various diseases, such as alcoholism (13), malignant tumors (14), rheumatoid arthritis (15), and multiple myeloma (16). Changes in microheterogeneity depend on the variation of sugar chains binding to amino acid side chains and on variation of the amino acid array of protein. It is necessary to examine the IEF pattern in detail by using an antibody with a different antigen-recognition site and various lectins for those pathologic conditions analysis.

In conclusion, use of the CA membrane as a support medium for IEF gave some characteristics compared to other methods using polyacrylamide or agarose gels. That is, multiple and different data sets can be rapidly and simultaneously obtained in this method, suggesting that the method is expected to become useful for analyzing the pathologic conditions of various diseases in the future.

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