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Production of dye-binding esterase reactor after separation and detection using combined native isoelectric focusing and blue native electrophoresis

Youji Shimazaki^{1, 2*} and Hikari Nakamaru²

¹ Department of Chemistry and Biology, Graduate School of Science and Engineering, Ehime University, Matsuyama, Japan

² Faculty of Science, Ehime University, Matsuyama, Japan

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SUMMARY

When cytosolic proteins from mouse liver were separated by a combined method of native isoelectric focusing (IEF) and blue native electrophoresis, spots at *pI* 6.7/180,000 Da, *pI* 6.5/100,000 Da and *pI* 6.4/70,000 Da were detected by Coomassie Brilliant Blue. After separation and detection, the native enzymes were extracted by native electrophoresis and immobilized on the reverse-phase chromatography media ZipTip to produce an enzyme reactor. The hydrolysis activity of 4-methylumbelliferyl acetate by the spots at *pI* 6.7/180,000 Da and *pI* 6.4/70,000 Da was 3.0 and 2.4 times, respectively, greater than that with no enzyme. The method can be applied to systematically produce biological reactors after separation and detection of enzymes by the combined method of native IEF and blue native electrophoresis.

Key words: enzyme reactor, immobilization, Reverse-phase chromatography media, biological samples, CBB

INTRODUCTION

Many types of enzymes exist in biological samples such as liver. In order to produce enzyme reactors from biological samples, systematic processes are necessary to separate, extract and immobilize the native enzymes. Two-dimensional electrophoresis (2DE) combined with isoelectric focusing (IEF) and size separation is one of the techniques used to separate proteins with high-resolution¹⁾. In addition, native proteins of biological samples can be separated by nondenaturing 2DE combined with native IEF and size separation^{2, 3)}. Separated proteins retain their biological functions such as enzyme activity. However, after separation by 2DE, acetic acid and methanol are used for protein detection using dyes such as Coomassie Brilliant Blue (CBB), amido black, and direct blue. These agents denature proteins within the 2DE gel and provide an acidic environment that enhances interactions with the dyes⁴⁾. Thus, protein functions are thought to be lost after dye staining. However, accumulating evidence indicates that blue native polyacrylamide gel electrophoresis is a useful analytical method for the separation of protein and protein

complexes into their native and function forms^{5, 6)}. However, the resolution of blue native electrophoresis is limited, as proteins and/or protein complexes with similar molecular sizes cannot be resolved⁷⁾. Thus, a combined method of native IEF and blue native electrophoresis is suggested to improve the separation resolution. In addition, since proteins and protein complexes are gently bound to an anionic CBB dye during blue native electrophoresis⁸⁾, they can be easily encouraged to migrate towards an anode. Since it has been reported that proteins and protein complexes within the polyacrylamide gel can be extracted by native electrophoresis⁹⁾, the CBB bound proteins and protein complexes are thought to be extractable using native electrophoresis. Proteins within solutions are immobilized on media such as reverse-phase chromatography media within tips^{10–13)}. Thus, CBB bound proteins and protein complexes can be immobilized on the media after separation and extraction by native electrophoresis.

In the present study, a hybrid method of native IEF and blue native electrophoresis was applied to separate and detect enzymes that retain their activity. After separation and detection, CBB bound enzymes were extracted by native

* Corresponding author: Youji Shimazaki; Department of Chemistry and Biology, Graduate School of Science and Engineering (Science), Ehime University, Matsuyama, Ehime 790-8577, Japan
E-mail: shimazaki.yoji.my@ehime-u.ac.jp
Tel: +81 89 927 9617
Fax: +81 89 927 9590

electrophoresis and immobilized on reverse-phase chromatography media in order to produce enzyme reactors. These methods can be applied in the systematic production of enzyme reactors after separation and detection of the proteins by native IEF and blue native electrophoresis.

MATERIALS AND METHODS

Chemicals and sample preparation

Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10) and polyclonal anti-esterase (rabbit) antibody were purchased from Daiichi Pure Chemicals Co. Ltd. (Osaka, Japan), GE healthcare (Uppsala, Sweden) and Rockland Inc. (Gilbertsville, PA), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Osaka, Japan). After mouse liver was homogenized in 0.1 M Tris-HCl solution (pH 7.2), and the homogenate was centrifuged for 30 min at $10,000\times g$ to obtain the cytosol fraction.

Gel isoelectric focusing

For separation of cytosolic proteins in mouse liver by native IEF using a previously reported method^{2,3}. Sucrose was added to the cytosol fraction at a final concentration of 40% w/v. For separation of cytosolic proteins in mouse liver, IEF was done on gel column (30 mm length $\times$ 1.3 mm id) within a glass capillary (45 mm length $\times$ 1.3 mm id). A mixed solution of 4 or 8% w/v acrylamide (0.2% or 0.4% w/v *N,N'*-methylenebisacrylamide (Bis), respectively) containing 5% v/v pharmalyte (pH 3–10), 0.05% w/v ammonium persulfate and 0.029% v/v *N, N', N', N'*-tetramethylethylenediamine (TEMED) was used. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). The protein samples (100–250 μ g) were applied to cathode or

anode end of the IEF gel. Constant current of 0.1 mA/capillary was applied to reach a voltage 300 V. And, then the constant voltage at 300 V was applied for 40 min. To determine *pI* within the native IEF gel, the pH gradient was measured. For this purpose, the gel was cut into 3-mm segments and each segment was put into a 0.5 mL microtube. 100 μ L aliquot of water was added to the microtube and incubated for 2 h. Then, the pH of the solution was measured. For determination of molecular mass in the second dimension, the cytosolic proteins were mixed with human plasma proteins, and the mixture was separated.

Combination of native IEF and blue native electrophoresis

After native IEF gel electrophoresis, the IEF gel was placed on top of the second-dimension slab gel containing 8.5% w/v acrylamide (0.45% w/v Bis), and then the stacking slab gel, containing 0.1% w/v CBB G-250 and 5.1% w/v acrylamide (0.26% w/v Bis), was placed on the native IEF gel, as shown in Fig. 1. The electrode buffer contained 0.05 M Tris and 0.38 M glycine (pH 8.3) and a constant current of 10 mA/slab gel was applied. After the proteins were separated using the combinational method, the 2DE gel was incubated in 10 mL 0.2 M phosphate buffer (pH 7.1) containing 0.2 mL 1% w/v α -naphthyl acetate and 4 mg Fast garnet GBC. For CBB R-250 staining, the gel was stained with 0.1% w/v CBB R-250, 7% v/v acetic acid and 20% v/v methanol for 15 min and destained in 20% v/v methanol and 7% v/v acetic acid for 2 h.

Immunoblotting

After proteins were separated by the combinational methods of non-denaturing IEF and blue native electrophoresis,

Fig. 1 Schematic diagram showing a combined method of native IEF and blue native electrophoresis. The IEF gel was placed on top of the second-dimension slab gel and then a stacking slab gel containing 0.1% (w/v) CBB G-250 was placed on the native IEF gel (a). The gel containing the dye-binding enzyme was excised after electrophoresis. Following extraction of the enzyme from the solution, the enzyme was immobilized on ZipTip media (b).

they were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% w/v nonfat milk in 5 mM Tris-buffered saline comprising 0.05% v/v Tween-20 (TBST) and incubated with for 1 h. The TBST containing anti-Esterase antibody (1/1000 dilution) was added to the membrane, and the membrane was incubated at 25°C for 1 h. After washing in TBST for 5 min three times, the TBST containing horseradish peroxidase conjugated anti-rabbit IgG (1/10000 dilution) was added to the membrane, and the membrane was incubated at 25°C for 1 h. Thereafter, the membrane was washed with TBST for 5 min at 3 times, and was then soaked in Clarity Western ECL substrate solution for 5 min in darkness. Immuno-detection was performed using LAS-4000 mini Luminescent Image Analyzer (Fujifilm, Tokyo, Japan).

Detection of hydrolytic activity with phosphatidylcholine

To analyze the hydrolysis of phosphatidylcholine (PC 16:0/18:2) by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS), several regions associated with hydrolytic activities were excised from the 2DE gel using the combined methods of nondenaturing IEF and blue native electrophoresis. Proteins in each piece were incubated with 100 μ L of 0.5 mg/mL PC for 2 h at 37°C. After incubation, all the liquid was collected. Then, 1 μ L of the liquid was mixed with 1 μ L of a solution containing saturated α -cyano-4-hydroxycinnamic acid, 0.1% v/v trifluoroacetic acid, and 60% v/v acetonitrile. The mixture of sample and matrix was put on a stainless steel sample plate (sample plate for AXIMA-TOF²; Shimadzu, Kyoto, Japan) and dried. Mass analysis was carried out using a MALDI-TOF MS system (AXIMA-TOF²; Shimadzu) in positive ion reflector mode.

Elution and immobilization of dye-binding esterase

After a region involved in hydrolytic activities was excised from the 2DE gel using native IEF and blue native electrophoresis, the excised piece was placed on a gel column (50 mm id), as shown in Fig. 1b. Then, the dye-binding protein was mobilized by native electrophoresis. During

mobilization, a current of 1 mA per nondenaturing gel was applied. Dye-binding enzyme was collected at the cathode end of gel. An equivalent volume of 60% polyethylene glycol (PEG) #4000 was added to the collected enzyme. The mixture was then kept at 4°C for more than 2 h and centrifuged for 30 min at 10,000 $\times$ g for protein precipitation. The precipitate was dissolved in 10 μ L of 0.1 M Tris-HCl (pH 7.2). After 2 μ L of the obtained solution was divided to 4 tubes, 8 μ L of 0.1 M Tris-HCl (pH 7.2) was added to each tube. The solution in each tube was then aspirated and dispensed using a pipette tip containing chromatography media ZipTip C4 for 10 cycles to ensure binding of the complex mixtures after swelling the ZipTip with 0.1 M Tris-HCl (pH 7.2). Then, four disposable ZipTip binding the same amount of enzyme were made.

To measure esterase activity in the ZipTip, 10 μ L of 50 mM Tris-HCl (pH 7.2) containing 0.92 mM 4-methylumbelliferyl acetate (4-MUA) was aspirated in the presence or absence of pigment-binding protein (or pigment-binding 1.5–150 nunits porcine liver esterase), and incubated at 37°C for 30 min. After incubation, samples were diluted 500 times. The fluorescence was analyzed using a spectrofluorometer (FP-8200 from JASCO, Tokyo, Japan) at an excitation wavelength of 360 nm and emission wavelengths of 400–500 nm and 450 nm. The data were analyzed with the Student's *t*-test.

RESULTS AND DISCUSSION

After the cytosolic proteins in the mouse liver sample were separated using the combined methods of native IEF and blue native electrophoresis, several spots were stained during electrophoresis (Fig. 2a), esterase activity was examined on the same gel (Fig. 2b), and the spots were stained by CBB-R250 (Fig. 2c). Stained blots identifying esterase using anti-esterase antibody are shown in (Fig. 2d) following separation by the combinational methods and electroblotting of the membrane. The spots at *pI* 6.7/180,000 Da, *pI* 6.5/100,000 Da and *pI* 6.4/70,000 Da (* in Fig. 2a) were stained using blue native electrophoresis. These spots retained esterase activity (Fig. 2b), as determined by using an anti-esterase antibody (Fig. 2d).

Fig. 2 Detection of spots stained by using blue native electrophoresis (a), esterase activity staining (b), and CBB-R250 staining (c) after combined native IEF and blue native electrophoresis. Stained blot for identification of esterase using anti-esterase antibody (d) after separation using combined native IEF and blue native electrophoresis followed by blotting onto a PVDF membrane.

The spot at pI 6.7/180,000 Da contained two molecules, one of 120,000 Da and another of 70,000 Da, whereas the protein at pI 6.4/70,000 Da contained molecules of less than 70,000 Da (data not shown). The spots were thought to consist of protein complexes, since it has been reported that protein complexes may contain esterase^{14, 15}. Several spots at pI 7–8 were stained by CBB-R250 after separation by native IEF and blue native electrophoresis (Fig. 2c). The proteins separated at pI 6–7 by native IEF were thought to be stained during blue native electrophoresis, whereas the proteins separated at pI 7–8 were not stained during electrophoresis. Differences in the staining can be caused by not only electrostatic but also hydrophobic interactions between proteins and CBB-G 250 during blue native electrophoresis. A total of 43% less esterase activity was determined when 0.1% w/v CBB-G 250 was added to the 15 nunits of the purified esterase. Since the stacking gel contained 0.1% w/v CBB-G 250, the proteins were thought to be attached to less than 0.1% w/v CBB-G 250 during blue native electrophoresis. Thus, more than 57% of the esterase activity was thought to be retained even after separation by the combined methods of native IEF and blue native electrophoresis.

Fig. 3 shows the 2DE pattern of combined native IEF and blue native electrophoresis (a), and the MALDI-TOF MS spectra of PC 16:0/18:2 changes in the absence (b) and presence of the spot at pI 6.7/180,000 Da (spot 1) and the spot at pI 6.4/70,000 Da (spot 2) of the 2DE (c and d). The

peaks of PC 16:0/18:2 are shown at m/z 760.4 $[M+H]^+$ (arrow) and 782.4 $[M+Na]^+$ (arrowhead). PC 16:0/18:2 was incubated with the excised gels containing esterase at spots 1 and 2 (Fig. 3a), after separation by nondenaturing IEF and blue native electrophoresis. The peaks at m/z 496.2 $[M+H]^+$ (*) and 522.2 $[M+H]^+$ (**) clearly increased when PC 16:0/18:2 was incubated with the excised gels containing esterase at the positions of both spots 1 and 2 (Figs. 3c and 3d), in comparison with those displayed in Fig. 3b. It is remarkable that, besides the generation of lysophosphatidylcholine 16:0 ($m/z=496.2$), there was also a lysophosphatidylcholine species containing an unsaturated fatty acid residue such as oleic acid ($m/z=522.2$). The results indicated that the enzyme in the gel cleaved the fatty acid residues at both the sn-1 and sn-2 positions of PC 16:0/18:2. The properties of the lytic enzyme could be examined by the hydrolysis of PC using MALDI-TOF MS as it has been reported that PC hydrolysis by lipolytic enzymes can be examined using this method¹⁶. Thus, the characteristics of esterase after separation using native IEF and blue native electrophoresis can be examined using this method. Since CBB-binding esterase separated by 2DE possesses a strong negative charge, these enzymes can be migrated and eluted by native electrophoresis. Following elution of the esterase within the electrophoretic column, it can be recognized because it binds CBB.

Fig. 4a shows the fluorescent emission spectra of esterase activity when 4-MUA was hydrolyzed by the dye-binding

Fig. 3 Detection of spots after combined method of native IEF and blue native electrophoresis (a). MALDI-TOF MS spectra of PC following incubation in the absence (b) and presence of spot 1 (c) and spot 2 (d). Peaks of PC are shown at m/z 760.4 $[M+H]^+$ (arrow) and 782.4 $[M+Na]^+$ (arrowhead). Peaks at m/z 496.2 $[M+H]^+$ (*) and 522.2 $[M+H]^+$ (**) are found in lysophosphatidylcholine.

Fig. 4 Fluorescent emission spectra of esterase activity when 4-methylumbelliferyl acetate was hydrolyzed by the dye-binding esterase eluted from the gel and immobilized on chromatography media ZipTip C4 (a). The relative fluorescence intensity at $\lambda_{em}=450$ nm with the dye-binding esterase eluted from the gel and immobilized on ZipTip C4 (b). Each data point indicates the mean $\pm$ standard deviation of more than four individual measurements. ** $P < 0.005$ (Student's *t*-test)

esterase eluted from spots 1 and 2 of the gel (Fig. 3a) and immobilized on the chromatography media ZipTip C4. The relative fluorescence intensities at $\lambda_{em}=450$ nm were 3.0 and 2.4 times, respectively, greater than that of no enzyme (Cont.), as seen in Fig. 4b. The activity of the esterase was less than 150 nunits after the enzyme from the mouse liver cytosolic fraction was separated, eluted, and immobilized on the ZipTip. It is reported that the elution and extraction of macromolecular proteins in the native condition are difficult using electrophoresis¹⁷⁾. However, when CBB is attached to the proteins, protein-CBB complexes are thought to possess a strong negative charge. The complexes can, therefore, be eluted from the gel and immobilized on the chromatography media. Many kinds of enzymes are utilized after purification and immobilization^{18–20)}. In addition, many can be separated by the combined method of native IEF and blue native electrophoresis, because the method enables high resolution protein separation²¹⁾. Thus, the method presented in this study can be applied in the production of enzyme reactors after the enzymes and their complexes from biological samples have been separated, eluted, and immobilized.

CONCLUSIONS

A combination of native IEF and blue native electrophoresis enabled the separation and detection of enzymes from biological samples. Enzyme reactors can then be produced after enzyme extraction and immobilization on chromatography media.

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The MALDI-TOF MS analysis was carried out on an AXIMA-TOF²; Shimadzu at the division of Analytical Bio-Medicine the Advanced Research Support Center (ADRES), Ehime University.

No potential conflicts of interest were disclosed.

ABBREVIATIONS

BSA, Bovine serum albumin; 4-MUA, 4-methylumbelliferyl acetate; PC, phosphatidylcholine

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