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Journal of Chromatography B, 860 (2007) 180–184
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Direct analysis of retinal dehydrogenase activity on an electroblotting membrane following separation by non-denaturing two-dimensional electrophoresis

Youji Shimazaki *, Takahiro Kuroda

Graduate school of Science and Engineering (Science section) and Venture Business Laboratory, Ehime University, Matsuyama, Japan

Received 22 May 2007; accepted 18 October 2007
Available online 9 November 2007

Abstract

The reaction from retinal to retinoic acid catalyzed by retinal dehydrogenase on a polyvinylidene difluoride (PVDF) membrane was examined using laser desorption ionization time of flight mass spectrometry (LDI-TOF MS) when the enzyme was separated by non-denaturing two-dimensional electrophoresis (2-DE), transferred onto the membrane, and stained without impairing the enzyme activity. Furthermore, the enzyme was analyzed by *de novo* sequencing using electrospray ionization tandem mass spectrometry (ESI-MS/MS) after proteins from mouse liver were separated by non-denaturing 2-DE, blotted onto the membrane, and stained. The results indicated that the reported methods could be applied for the direct examination of changes in retinoid catalyzed by enzymes on such membranes.

Keywords: Retinal dehydrogenase; Retinal; Retinoic acid; Identification; *De novo* sequencing

1. Introduction

Retinoids such as retinylester, retinal, retinol and retinoic acid are metabolized in animal liver and retina; and a number of enzymes and proteins such as serum retinal binding protein, cellular retinal-binding proteins, retinol dehydrogenases, retinal dehydrogenases and cellular retinoic acid-binding proteins are involved in the cascade of retinoid metabolic reactions [1]. In order to examine the cascade of retinoid metabolic reactions *in vitro*, it is necessary to analyze the enzymes and proteins related to retinoid metabolism, and measure the changes in the amounts of retinoids. It has been reported that human plasma proteins can be separated by non-denaturing two-dimensional gel electrophoresis (2-DE), and the isoelectric point (pI) and molecular masses of human plasma proteins can be examined in detail [2,3]. Further, we previously reported the analysis of enzyme function and *de novo* sequence analyses after the separation of native proteins from the animal's liver and retina by non-denaturing 2-DE [4–7]. For the detection of enzyme activity, the substrates and chromophores are added to the enzymes in the gel. However, since the products and chromophores combine with the enzymes in the gel following the enzymatic reaction, it might be difficult to continuously examine the changes of substrates catalyzed by the enzymes. So, in order to examine the continuous changes of substrates catalyzed by enzymes, it is preferable that enzymatic reactions are performed on the surface of a support without using chromophores. Since phosphatidylcholine and lipids of high density lipoprotein are hydrolyzed by an esterase on a polyvinylidene difluoride (PVDF) membrane after separation by non-denaturing 2-DE and electroblotting onto the membrane [8], retinoids such as retinylester, retinal, retinol and retinoic acid can be metabolized by enzymes on the membrane. It has been reported that abundant radical molecular ions [$M^{+\bullet}$] of retinoids such as retinylester, retinal, retinol, and retinoic acid can be examined using laser desorption ionization time of flight mass spectrometry (LDI-TOF MS) [9,10]. Therefore, changes

Abbreviations: PVDF, polyvinylidene difluoride; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; 2-DE, two-dimensional gel electrophoresis; LDI, laser desorption/ionization; TOF, time of flight; CBB, coomassie brilliant blue; NAD, β-nicotinamide adenine dinucleotide; PMS, phenazine methosulfate; NBT, nitroblue tetrazolium.

* Corresponding author. Tel.: +81 89 927 9617; fax: +81 89 927 9590.
E-mail address: yoji@dpc.ehime-u.ac.jp (Y. Shimazaki).

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doi:10.1016/j.jchromb.2007.10.028

in retinoid catalyzed by enzymes on a PVDF membrane can be exmined using LDI-TOF MS. The enzymes processing retinoids on such a membrane can be identified, since it has been reported that *de novo* sequence analysis of enzymes on membranes can be performed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) [11].

The present study reports the enzymatic reaction from retinal to retinoic acid using LDI-TOF MS after the enzyme was separated using non-denaturing 2-DE, transferred onto a membrane, and stained without impairing the enzyme activity. The enzyme on the membrane was identified as retinal dehydrogenase by ESI-MS/MS. The methods are believed to be applicable for the direct examination of changes in retinoids catalyzed by enzymes on such membranes.

2. Materials and methods

2.1. Reagents, sample preparation, non-denaturing 2-DE and electroblotting

Acrylamide, ampholine (pH 3.5–10 and pH 3.5–5, respectively), and bovine trypsin (sequence grade) were purchased from Daiichi pure Chemical Co. Ltd. (Tokyo), Amersham Pharmacia Biotech (Piscataway, NJ) and Roche (Mannheim), respectively. PVDF membranes (0.45 μm, Immobilon^{TM}) were purchased from Millipore (Bedford, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Wako Pure Chemicals (Osaka). Mouse liver (swiss webster) was purchased from Rockland Inc. (Gilbertsville, PA). Liver was taken out mouse as follows (age: 2–3 months, weight: 25–30 g, gender: male or female).

Mouse liver (1.4 g) was homogenized using a homogenizer (As one, Osaka) in 5.0 ml of 100 mM Tris–HCl buffer (pH 7.2), and the homogenate was centrifuged for 5 min at 10,000 × *g* to obtain the cytosol. Sucrose was added to the cytosol fraction until a concentration of 40% (w/v) sucrose was attained. The protein concentration in the fraction was estimated from their ultraviolet light absorption with the assumption that the absorbance at 280 nm of a 1 mg/ml albumin solution was 1.0 [12]. Proteins in the fraction (100–500 μg) were subjected to microscale non-denaturing 2-DE using a previously reported method [2,3,4–7]. IEF was done on rod gels (35 mm long × 1.3 mm i.d.). A mixed solution of 4% acrylamide (0.2% Bis) containing 2% ampholine (pH 3.5–10), 1% ampholine (pH 3.5–5) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.05% ammonium persulfate, 0.029% TEMED. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). Following IEF, the IEF gel was placed on top of the second-dimension slab gel, which was then run on a 4%–17% acrylamide linear gradient (0.2%–0.85% Bis gradient). The IEF gel was equilibrated in a buffer containing 0.01 M Tris and 0.076 M glycine, pH 8.3. The electrode buffer contained 0.05 M Tris and 0.38 M glycine (pH 8.3). The gels were stained with 0.1% CBB, 7% (v/v) acetic acid, and 50% (v/v) methanol for 15 min; and were destained in 20% (v/v) methanol and 7% (v/v) acetic acid for 2 h. For determination of pI and molecular masses of the cytosol proteins from mouse liver, the cytosol fraction and human plasma were mixed, and the mixture was applied to the non-denaturing 2-DE. Since pI and molecular masses of human plasma proteins on the non-denaturing 2-DE were examined in detail [2,3], these are used for the determination of pI and molecular masses of cytosol proteins from mouse liver.

Once proteins were separated using non-denaturing 2-DE, they were transferred onto a polyvinylidene fluoride (PVDF) membrane using a wet-type transblotting apparatus in order to immobilize the separated proteins onto the membrane [13]. For the detection of the proteins on the PVDF membrane, the membrane was soaked in 0.01% direct blue 71 in 10 ml 20% (v/v) methanol and 7% (v/v) acetic acid for 15 min, and then destained in 20% (v/v) methanol and 7% (v/v) acetic acid for 2 h [14].

2.2. Detection of enzyme activity

After cytosol proteins were separated by non-denaturing 2-DE or were blotted onto the PVDF membrane after the separation, retinal dehydrogenase activity was analyzed by a modified method, as described previously [7]. Proteins in the 2-DE gel or on the PVDF membrane were incubated in 10 ml 0.04 M Tris–HCl buffer (pH 8.0) containing 2.5 mg retinal (solubilized in ethanol), 5 mg β-nicotinamide adenine dinucleotide (NAD), 3 mg nitroblue tetrazolium (NBT), and 0.3 mg phenazine methosulfate (PMS) for 10 min at 37 °C. The position of the enzymatic

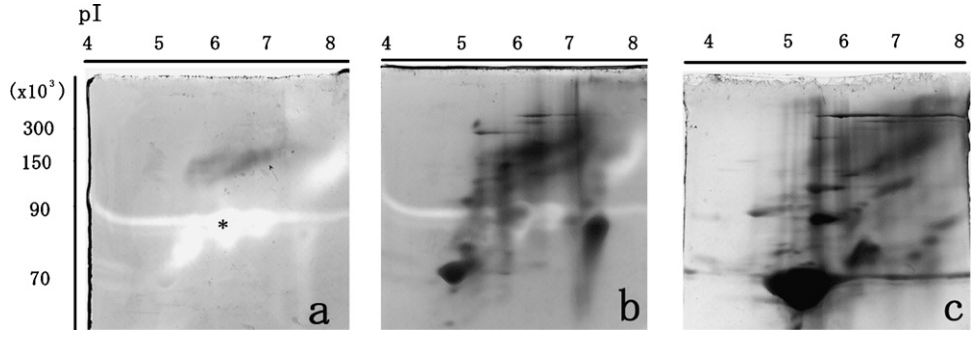

Fig. 1. Staining of retinal dehydrogenase activity using all-*trans* retinal, NAD, NBT, and PMS after separation by non-denaturing 2-DE (a), CBB staining of the cytosol proteins from mouse liver (b) and CBB staining of the mixture of the cytosol proteins and human plasma proteins (c) after separation by non-denaturing 2-DE. Retinal dehydrogenase activity was obtained at pI 5.8–7.2/150,000 (arrow in a). Human plasma proteins were used for determination of pI and molecular masses. SOD-like activity is indicated by *.

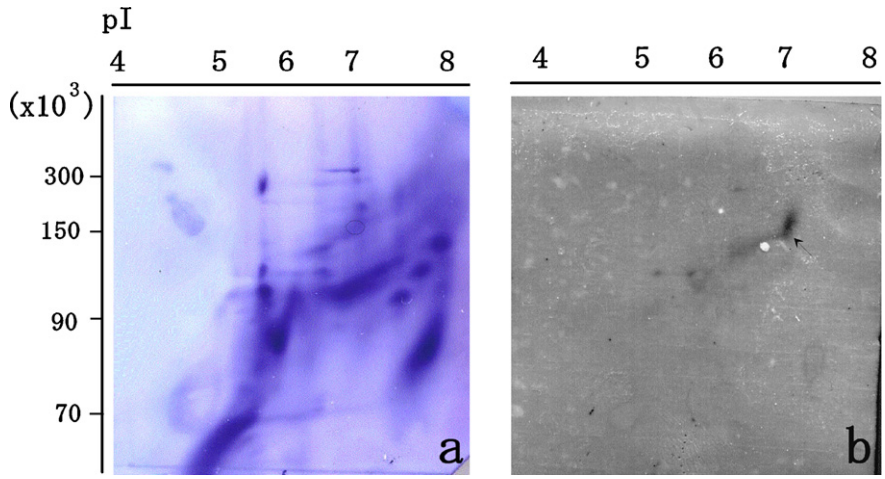

Fig. 2. Staining of direct blue 71 (a) and of retinal dehydrogenase activity using all-*trans* retinal, NAD, NBT, and PMS (b) after separation by non-denaturing 2-DE and blotting onto the membrane. The protein spot at pI 7.1/150,000 (circle in a) was excised from the PVDF membrane for analysis by ESI-MS/MS. Retinal dehydrogenase activity was obtained at pI 7.1/150,000 (arrow in b).

reaction on the 2-DE gel or the PVDF membrane was stained by formazane dye. After enzymatic reaction, the gel or the PVDF membrane was washed with waster.

2.3. Sequence structure analysis by ESI-MS/MS

Protein spots were excised from the PVDF membrane, and digested on the membrane by trypsin using a previously described method [15–18]. After excision of protein spots, the spots were washed three times with 50 mM ammonium hydrogen carbonate. The spots were incubated in 10 μl of bovine trypsin (12.5 ng/μl) in 50 mM ammonium hydrogen carbonate at 37 °C for 4 h. The digested polypeptides were recovered using 10 μl of a solution containing 1% formic acid and 80% acetonitrile. Extracts were concentrated using a vacuum evaporator centrifuge, and were suspended in 20 μl 1% formic acid. The obtained polypeptides were concentrated and desalted using C_{18} Zip Tip, and placed into a spraying capillary (Protana, Odense, Denmark) for nano ESI mass spectrometry. The capillary was attached to a nanospray ion source on the electrospray quadrupole time-of-flight instrument (QSTAR) from Applied Biosystems (Framingham, MA, USA). From the TOF-MS spectra, doubly charged peptide ions were selected, and MS/MS spectra were analyzed by increasing the collision energy. From the MS/MS spectra, peptide sequences were identified by manual *de novo* peptide sequencing using Bioanalyst™ (Applied Biosystems, Foster city, CA, USA). Peptide sequences were then searched for PepSea™ server (Toronto, Canada) against the NCBInr. database (2002-07-01) to identify the proteins.

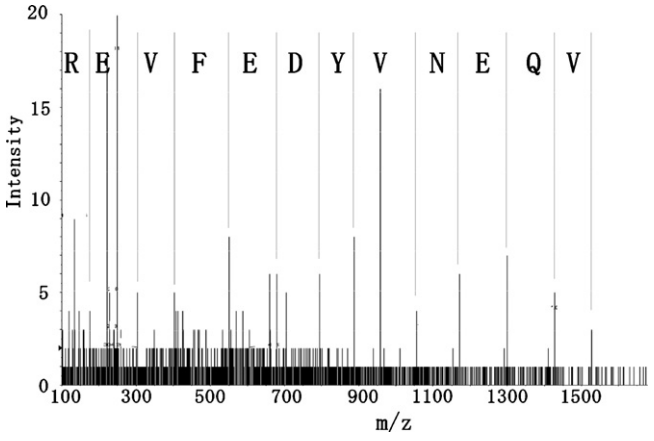

Fig. 3. The MS/MS spectrum of the doubly charged peptide ion at *m/z* 887.67 with fragment ions and deduced peptide sequences.

2.4. Examination of retinal dehydrogenase activity on the membrane using laser desorption ionization TOF MS

Once proteins were separated using non-denaturing 2-DE, and transferred onto the membrane, proteins were stained with 0.01% direct blue 71 in 10 ml 0.1 M Tris–HCl buffer solution (pH 7.0). For the analysis of retinal dehydrogenase activity by laser desorption ionization TOF MS, several regions were excised from the membrane after staining with direct blue 71. Proteins in each excised piece were incubated with 70 μl 20 mM Tris–HCl buffer solution containing 30% (v/v) ethanol, 12.5 mg/ml all-*trans* retinal, and 5 mg/ml NAD for 60 min at 30 °C. After incubation, the entire liquid portion was collected. Then, 1 μl of the liquid was put on a stainless steel sample plate (sample plate for Voyager DE PRO; Applied Biosystems, Framingham), and dried. For the desorption and ionization of retinoid derivatives, a pulsed nitrogen laser with a wavelength of 337 nm was used. Mass detection was done using (non-matrix-assisted) LDI-TOF MS (Voyager DE PRO; Applied Biosystems, Framingham) in a positive ion linear mode.

3. Results and discussion

Fig. 1 shows detection of retinal dehydrogenase activity (a) and CBB staining following the enzyme activity staining (b) after cytosol proteins of mouse liver are separated by non-

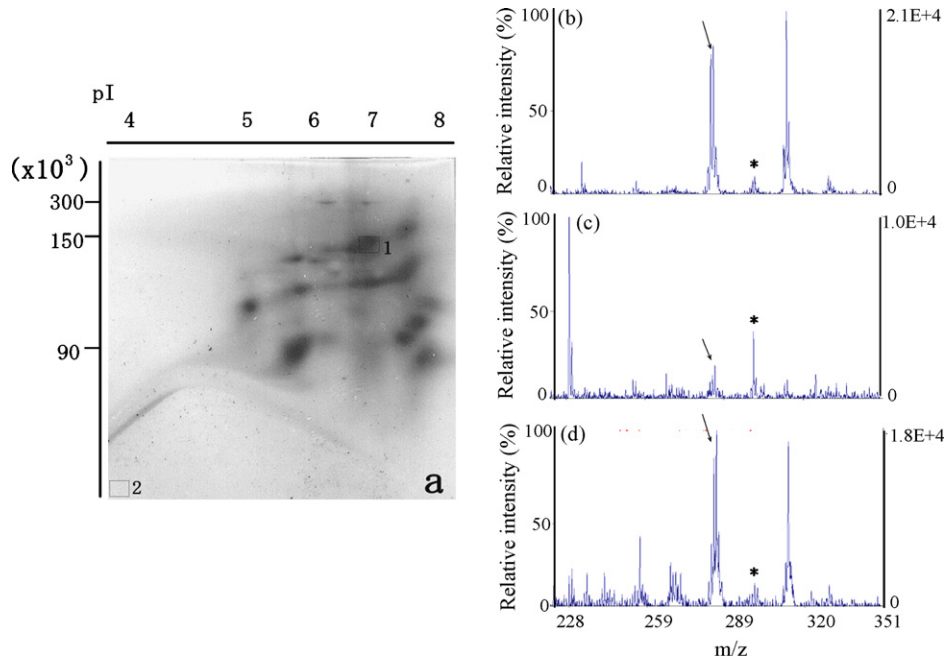

Fig. 4. Protein staining on the PVDF membrane using direct blue 71 in a neutral solution after separation of cytosol proteins by non-denaturing 2-DE and blotting onto the membrane (a). LDI-TOF MS spectrum before the incubation of retinal (b), after the incubation of retinal and spot 1 in a (c), and after the incubation of retinal and spot 2 in a (d). Peaks of retinal and retinoic acid are indicated by the arrow and *, respectively.

denaturing 2-DE. Retinal dehydrogenase activity was observed when retinal, NAD, NBT, and PMS were added to the gel after the separation of cytosol proteins (Fig. 1a), and the activity spot was located at pI 5.8–7.2 /150,000 after CBB staining. Human plasma proteins were used for determination of pI and molecular masses on the non-denaturing 2-DE (Fig. 1c), since pI and molecular masses of human plasma proteins are examined in details [2,3].

Fig. 2 shows direct blue staining (a) and retinal dehydrogenase staining (b) on the PVDF membrane after separation by non-denaturing 2-DE and blotting onto the membrane. Retinal dehydrogenase activity was observed at pI 7.1/150,000 as shown in Fig. 2b. For the analysis of the sequence structure of the protein possessing retinal dehydrogenase activity, the spot at pI 7.1/150,000 was excised from the PVDF membrane, and digested on the membrane by trypsin after the proteins separated by non-denaturing 2-DE were transferred onto the membrane, and were stained with direct blue. Fig. 3 shows the MS/MS spectrum of the doubly charged peptide ion at 887.67, and the fragment ions and the deduced peptide sequences. From the deduced sequence, the mass and the deduced sequence tag of the peptide fragment ions gave an exact matching to mouse aldehyde dehydrogenase, corresponding to the internal tryptic fragment VQENVYDEFVER. And from the deduced peptide sequence, the protein was identified as mouse aldehyde dehydrogenase (AHD-M1). Further, a subunit molecular mass 52, 700 was obtained when protein spot at pI 7.1/150,000 was excised, treated with 0.1% SDS and 10 mM dithiothreitol and analyzed by MALDI-TOF MS (data not shown).

The spectra of retinoid derivatives using LDI-TOF MS are shown in Fig. 4 before the incubation of retinal (b) and after the incubation of retinal and spot 1 in Fig. 4a (c), and after the incubation of retinal and spot 2 in Fig. 4a (d). The peak of the abundant radical molecular ions ($M^{+\bullet}$) of retinal ($m/z = 284$ u) was higher than that of retinoic acid ($m/z = 300$ u) before the incubation of retinal (Fig. 4b), after the incubation of retinal and spot 2 in Fig. 4a (Fig. 4d). Since there was no enzyme present at spot 2 in Fig. 4a, retinal was not converted to retinoic acid. On the other hand, the peak of the abundant radical molecular ions ($M^{+\bullet}$) of retinoic acid ($m/z = 300$ u) was higher than that of retinal ($m/z = 284$ u) after the incubation of retinal and spot 1 in Fig. 4a (Fig. 4c). Since the protein in spot 1 was identified as aldehyde dehydrogenase (Fig. 3), retinal was converted to retinoic acid by this enzyme on the membrane. Since it has been reported that the conversion from retinal to retinoic acid is catalyzed by some cytosolic aldehyde dehydrogenases [19–21], the same reaction can be obtained on such membranes. Wingerath et al. reported that purified retinoids were analyzed by LDI- and MALDI-TOF MS [9,10]. The present study indicated that the conversion from retinal to retinoic acid catalyzed by retinal dehydrogenase on the membrane was directly observed using LDI-TOF MS after separation and blotting onto the membrane under non-denaturing conditions.

4. Conclusions

The present study reports the enzymatic reaction from retinal to retinoic acid using LDI-TOF MS after the enzyme was separated using non-denaturing 2-DE, transferred onto a membrane, and stained without impairing the enzyme activity. The methods are believed to be applicable for the direct examination of changes in retinoids catalyzed by enzymes on such membranes.

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