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

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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.

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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

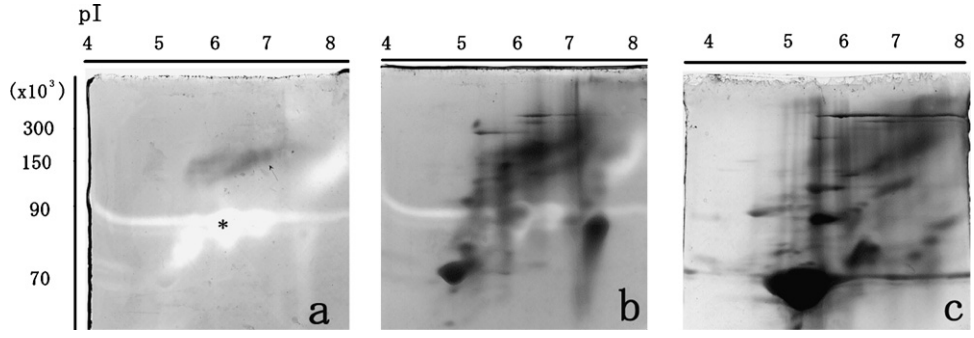

Fig. 1. Staining of retinal dehydrogenase activity using all-*trans* retinal, NAD, NBT, and PMS after separation by non-denaturing 2-DE (a), CBB staining of the cytosol proteins from mouse liver (b) and CBB staining of the mixture of the cytosol proteins and human plasma proteins (c) after separation 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 *.