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Enzyme separation and isozyme heterogeneity analysis using non-denaturing two-dimensional electrophoresis

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Carboxylesterase and sorbitol dehydrogenase are separated by non-denaturing two-dimensional electrophoresis (2-DE) of isoelectric focusing separation using 5% carrier ampholyte (pH 6–8) and 1.25% carrier ampholyte (pH 3–10) and size separation. Furthermore, activities of sorbitol, malate and lactate dehydrogenases are sequentially examined when the enzymes are separated by 2-DE and are sequentially reacted to sorbitol, malic and lactic acid, respectively, in the presence of nicotinamide adenine dinucleotide, nitro blue tetrazolium and phenazine methosulphate. Several kinds of enzymes including lactate dehydrogenize isozymes can be simultaneously separated using 2-DE. Furthermore, the binding differences between lactate dehydrogenase isozymes and concanavalin A (con A) can be examined using a combination of 2-DE and non-denaturing stacking gel electrophoresis. The results of this study indicate that non-denaturing 2-DE can be applied to both enzyme separation and isozyme heterogeneity analysis.

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1. Introduction

Enzymes from mouse liver can be separated with retained activity by non-denaturing two-dimensional electrophoresis (2-DE) [1–3]. Because some mouse liver enzymes are located at p*I* 6–8 in isoelectric focusing (IEF) separation, carboxylesterase and sorbitol dehydrogenase are not separated by non-denaturing 2-DE [2,3]. Activities of both the esterase and sorbitol dehydrogenase are thought to be detected on the same gel because the substrate and colour of the chromophores are different. There are many kinds of carrier ampholytes for IEF separation [4], and it is thought that two enzyme may be isolated when enzymes are separated by non-denaturing IEF using different types of carrier ampholyte. For IEF separation of denaturing proteins, immobilized pH gradient strips have been used. However, the use of these strips has problems such as precipitation, low solubility, and aggregation of proteins [5]. During re-swelling of the immobilized pH strips, proteins may be precipitated or aggregated under non-denaturing conditions. Hence, in the present study, we used IEF rods containing various kinds of carrier ampholytes to separate the non-denaturing enzymes. Furthermore, in cases when the enzymes separated by non-denaturing 2-DE (IEF rod and size separation) possess specificity of substrate, sequential activity detection of the enzymes is thought to be performed after enzymes are separated by 2-DE and reacted with different kinds of substrates. Since it has been reported that sol–gel encapsulation of an enzyme is used for optical sensing of the substrate [6], enzymes separated by non-denaturing 2-DE can be used in the analysis of the specific substrate. In addition, it has been reported that glycoproteins can bind to lectins such as concanavalin A (con A) [7], and that serum proteins separated by 2-DE and blotted onto membranes are retained by binding to con A [8]. However, since con A binds to proteins in different ways, isozymes separated using non-denaturing IEF are also thought to bind differently to con A. Therefore, the binding differences between lactate dehydrogenase isozymes and con A can be examined when these isozymes are electrophoresed in the presence of con A after separation using non-denaturing IEF.

The present study indicates that carboxylesterase and sorbitol dehydrogenase are separated by non-denaturing 2-DE of IEF containing 5% carrier ampholyte (pH 6–8) and 1.25% carrier ampholyte (pH 3–10) and size separation. Furthermore, activities of sorbitol, malate and lactate dehydrogenase are sequentially obtained when the enzymes are separated by 2-DE and are sequentially reacted to each substrate in the presence of nicotinamide adenine dinucleotide (NAD), nitro blue tetrazolium (NBT) and phenazine methosulphate (PMS). Several kinds of enzymes including lactate dehydrogenize isozymes can be simultaneously separated by 2-DE. Furthermore, the binding differences between lactate dehydroge-

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nase isozymes and con A can be analyzed using a combination of 2-DE and non-denaturing stacking gel electrophoresis. The present results indicate that non-denaturing 2-DE can be applied to both enzyme separation and isozyme heterogeneity analysis.

2. Materials and methods

2.1. Sample preparation, non-denaturing 2-DE and enzyme activity staining

Mouse livers (Swiss Webster) were purchased from Rockland Inc. (MA). 1.4 g of mouse liver was homogenized in 5.0 ml of 100 mM Tris–HCl buffer (pH 7.2). The homogenate was centrifuged for 5 min at 10,000 × g to obtain the cytosolic fraction. Sucrose was added to the liver cytosolic fraction at a concentration of 40% (w/v). Proteins in the cytosolic fraction (100–300 μg) were subjected to microscale non-denaturing 2-DE. One-dimensional isoelectric focusing (IEF) was performed on rod gels (35 mm × 1.3 mm ID) containing 4% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.05% ammonium persulphate and 0.029% (v/v) N,N,N′,N′-tetramethylenediamine, 1.25% (v/v) pharmalyte (pH 3–10) and 5% (v/v) ampholine (pH 6–8), or containing 4% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.05% ammonium persulphate and 0.029% (v/v) N,N,N′,N′-tetramethylenediamine, 5% (v/v) pharmalyte (pH 3–10). The electrode solutions in the one-dimensional electrophoresis comprised 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). After one-dimensional IEF, the gel was placed on top of the two-dimensional slab gel, which was then run on a 4–17% (w/v) acrylamide linear gradient (0.2–0.85% (w/v) bisacrylamide gradient). The electrode buffer in the two-dimensional electrophoresis comprised 0.05 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) and 0.38 M glycine (pH 8.3). For determination of p*I* in the one-dimensional IEF separation, the IEF gel was cut into 2 mm length pieces after IEF separation. The gel pieces were put into a 0.2 ml vial. An aliquot (50 μl) of boiled water was then added to each vial, which was sealed and left for 2 h. The pH of the solution was measured by using a microelectrode (Beckman Instruments, USA). To find the molecular mass in the second dimension, extracted proteins were mixed with human plasma proteins, and the mixture was separated using non-denaturing 2-DE. The human α_2-macroglobulin, immunoglobulin G (IgG), transferrin, albumin and transthyretin with molecular masses of apparent 500, 170, 90, 70 and 60 kDa, respectively, were used for calibrating separation by the non-denaturing 2-DE. We obtained a calibration curve from these proteins that was used to estimate the molecular mass of proteins on non-denaturing 2-DE as shown in Fig. 1.

In order to analyze the binding interactions of lactate dehydrogenase isozymes and con A, a 4% acrylamide stacking gel containing 0.001–5%(w/v) con A (5 mm thick) was placed onto two-dimensional slab gel. After IEF, the IEF gel was placed on top of the slab gel and the activities of lactate dehydrogenase isozymes were examined using the following staining methods.

For the detection of sorbitol dehydrogenase activity, the proteins in the gel were incubated in 10 ml 0.04 M Tris–HCl buffer at pH 8.0 containing 0.150 g sorbitol, 5 mg NAD, 3 mg NBT and 0.3 mg PMS after washing with H_2O. For analysis of esterase activity on the 2-DE gel, the gel was incubated in 10 ml of 0.2 M phosphate buffer (pH 7.1) containing 0.2 ml of 1% α-naphtyl acetate and 4 mg Fast red TR salt for detection of esterase activity. For the sequential analysis of sorbitol, malate and lactate dehydrogenase activities after the proteins in the 2-DE gel were incubated in the presence of 10 ml of 0.1 M Tris–HCl buffer at pH 7.2 sequential addition of 0.150 g sorbitol, the mixture of 0.125 g Na_2CO_3 and 0.134 g L-malic acid and 10 μl of lactic acid. For examination of only lactate dehydrogenase activity on the 2-DE gel, after cytosolic proteins from mouse liver were separated by non-denaturing 2-DE, the proteins in the 2-DE gel were incubated in the presence of 10 ml of 0.1 M Tris–HCl buffer at pH 7.2 containing 5 mg NAD, 3 mg NBT, 0.3 mg PMS and 0.4 ppb—2% (v/v) lactic acid. In order to detect lactic acid in foods such as yogurt and rice bran, activity of lactate dehydrogenase from mouse liver was used. After cytosolic proteins from mouse liver were separated by non-denaturing 2-DE, the proteins in the 2-DE gel were incubated in the presence of 10 ml of 0.1 M Tris–HCl buffer at pH 7.2 containing 5 mg NAD, 3 mg NBT, 0.3 mg PMS and 0.006% yogurt (or 50 mg rice bran containing lactobacilli). For Coomassie Brilliant Blue R-250 (CBB) staining, the gels were stained with 0.1% CBB, 7% (v/v) acetic acid, and 50% (v/v) methanol for 15 min; and destained in 20% (v/v) methanol and 7% (v/v) acetic acid for 2 h.

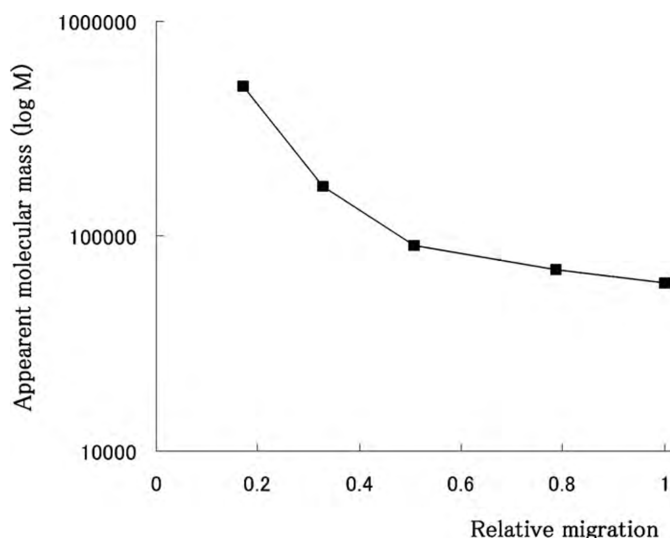

Fig. 1. Apparent molecular mass calibration curve of proteins by non-denaturing 2-DE. Abscissa indicates relative migration of proteins on non-denaturing 2-DE gels, compared with that of transthyretin. Ordinate indicates apparent molecular mass of human plasma proteins (transthyretin: 60,000, albumin: 70,000, transferrin: 90,000, IgG: 170,000, and α_2-macroglobulin: 500,000).

2.2. Peptide mass fingerprinting for protein identification

The peptide mass fingerprinting essentially followed a previously published protocol [9–11]. The protein spots were excised and transferred to a 0.5 ml polypropylene microcentrifuge tube. The excised gel was washed with 200 μl water and was shrunk with acetonitrile. After removing all liquid, the gel was dried in a vacuum evaporator centrifuge (VEC-50, Asahi Techno Glass). This gel was incubated in the presence of 0.1% sodium dodecylsulphate and 10 mM dithiothreitol (DTT) in 0.1 M ammonium hydrogen carbonate at 56 °C for 2 h, and it was then washed and shrunk with acetonitrile. After removing all liquid, the gel was incubated with 55 mM iodeacetamide in 0.1 M ammonium hydrogen carbonate for 20 min at room temperature in the dark. After the incubation, it was washed three times in 50 mM ammonium hydrogen carbonate. The gel was dehydrated with acetonitrile and dried in a vacuum evaporator centrifuge for 30 min, and was incubated in 15 μl of bovine trypsin (12.5 ng/μl) in 50 mM ammonium hydrogen carbonate at 4 °C for 30 min. The remaining supernatant was removed, and 5–25 μl of 50 mM ammonium hydrogen carbonate solution without trypsin was added to the gel, which was incubated at 37 °C for 8 h. The digested polypeptides were recovered with 10 μl solution containing 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile. The extracts were concentrated in the vacuum

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