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

Youji Shimazaki [a,b,*], Kenta Shimizu [b], Suzuna Masaoka [b]

[a] Graduate School of Science and Engineering (Science Section) and Venture Business Laboratory, Ehime University, Matsuyama, Japan
[b] Faculty of Science, Ehime University, Matsuyama, Japan

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ABSTRACT

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.

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-

* Corresponding author at: Graduate School of Science and Engineering (Science Section), Ehime University, 2-5 Bunkyo-cho, Matsuyama City 790-8577, Japan. 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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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.

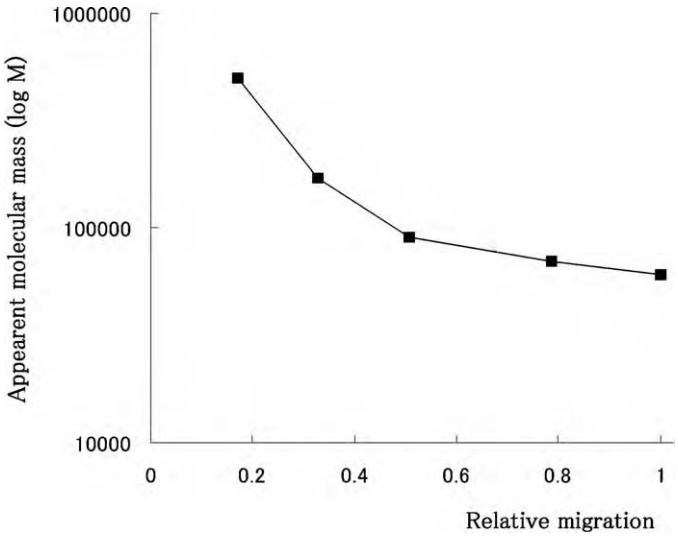

Fig. 1. Apparent molecular mass calibration curve of proteins by non-denaturing 2-DE. Abscissa indicates relative migration of proteins on non-denaturing 2-DE gels, compared with that of transthyretin. Ordinate indicates apparent molecular mass of human plasma proteins (transthyretin: 60,000, albumin: 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

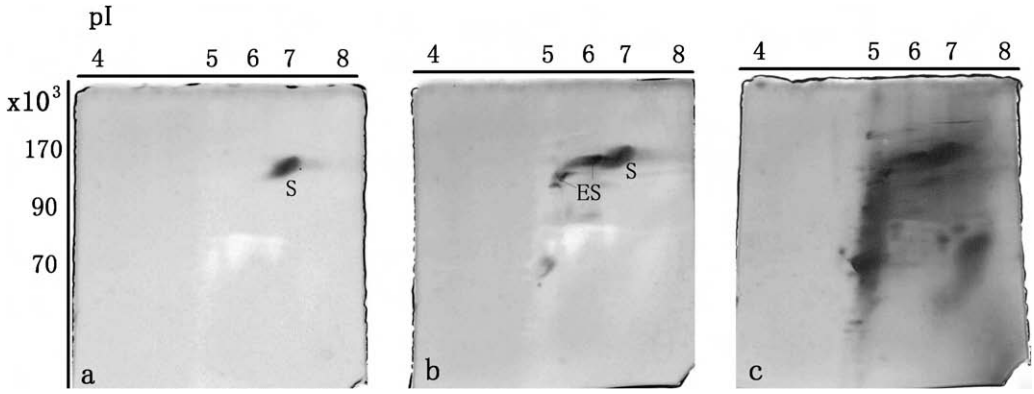

Fig. 2. Activity staining of sorbitol dehydrogenase (a), activity staining of esterase following sorbitol dehydrogenase (b) and CBB staining following both activity staining (c) after cytosol proteins in mouse liver are separated by non-denaturing 2-DE using IEF gel containing 5% pharmalyte (pH 3–10). S: sorbitol dehydrogenase and E: esterase.

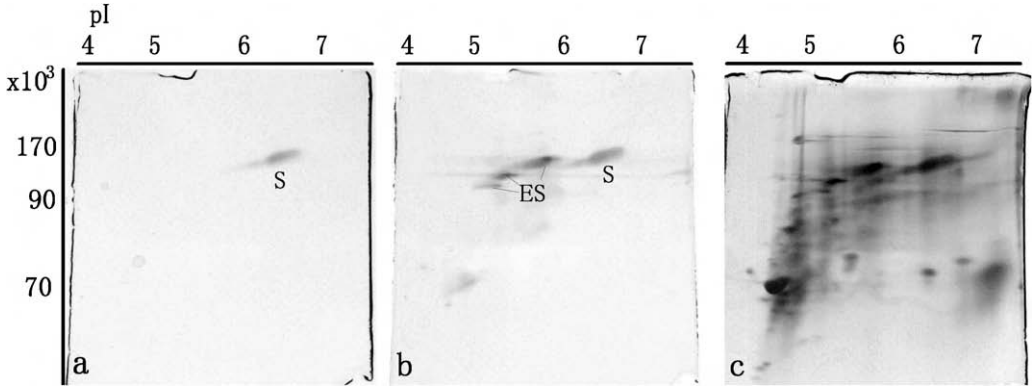

Fig. 3. Activity staining of sorbitol dehydrogenase (a), activity staining of esterase following sorbitol dehydrogenase (b) and CBB staining following both activity staining (c) after cytosol proteins in mouse liver are separated by non-denaturing 2-DE using IEF gel containing 1.25% pharmalyte (pH 3–10) and 5% ampholine (pH 6–8). S: sorbitol dehydrogenase and E: esterase.

evaporator centrifuge and were suspended in 20 μl of 0.1% TFA. The polypeptides obtained were concentrated and desalted using C18 Zip-Tip from Millipore (Bedford), and were mixed with α-cyano-4-hydroxycinnamic acid. Mass analysis was done using MALDI-TOF MS, operating in a positive-ion reflector mode. The spectra were analyzed using the mass values for monoisotopic peaks that were used for searches (Mascot, http://www.matrixscience.com/) against Swiss Prot database. Monoisotopic peaks of trypsin autodigest (*m*/*z* 842.51) and ACTH 18-39 (*m*/*z* 2465.1989) were used for internal calibration. The database was searched using the following terms: taxonomy (*Mus.*), trypsin digest (one missed cleavage allowed), cysteine modified by carbamidomethylation, and mass tolerance of 50 ppm, using internal calibration and oxidation of methionines. The criteria used to accept identification included the extent of sequence coverage, the number of peptides matched and the probabilistic score ($p < 0.05$ for a random match).

3. Results and discussion

Fig. 2 shows that sorbitol dehydrogenase activity (a), and activity of esterase following sorbitol dehydrogenase (b) were obtained after the proteins were separated by a non-denaturing 2-DE

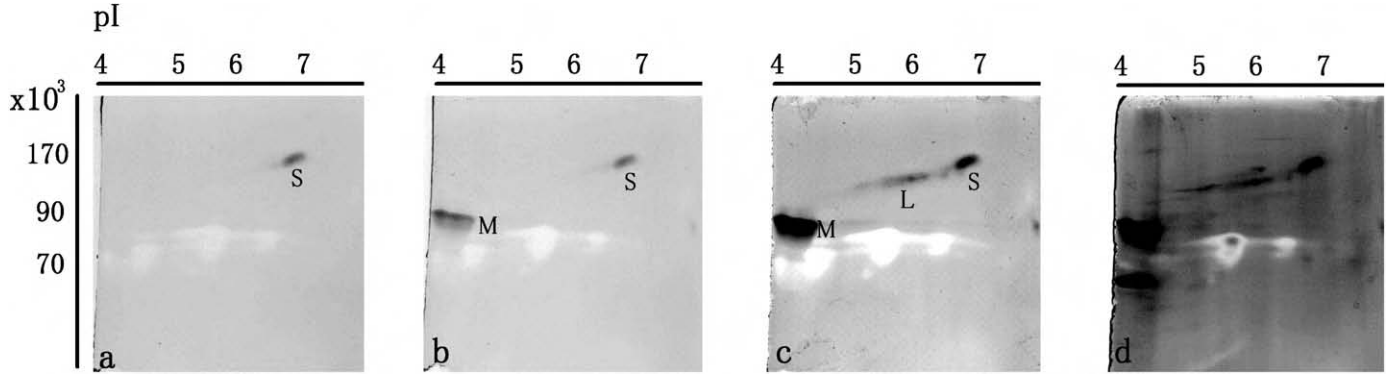

Fig. 4. Activity staining of sorbitol dehydrogenase (a), activity staining of malate dehydrogenase following sorbitol dehydrogenase (b), activity staining of lactate dehydrogenase following both sorbitol and malate dehydrogenases (c) and CBB staining following three kinds of the enzyme activity staining (d) after cytosol proteins in mouse liver are separated by non-denaturing 2-DE using the IEF gel containing 1.25% pharmalyte (pH 3–10) and 5% ampholine (pH 6–8). S: sorbitol dehydrogenase, M: malate dehydrogenase and L: lactate dehydrogenase.

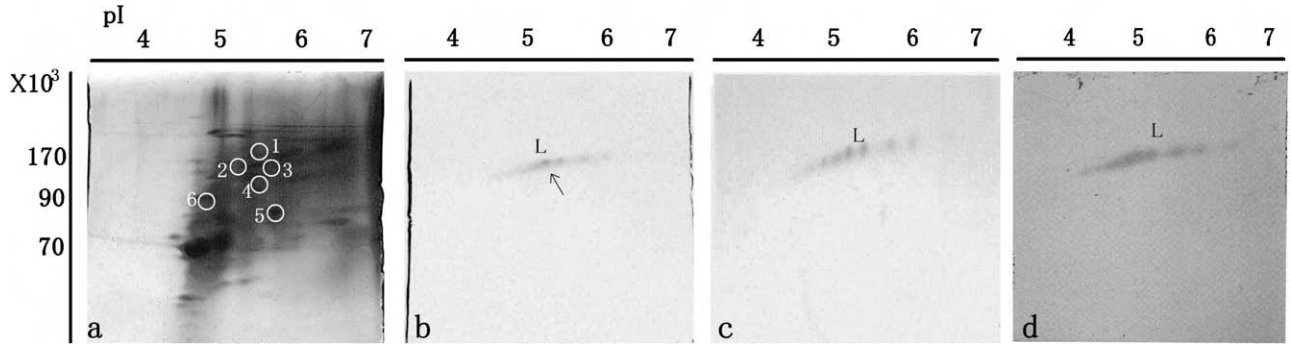

Fig. 5. CBB staining of proteins (a), activity staining of lactate dehydrogenase in the presence of 20 ppm lactic acid (b), activity staining of lactate dehydrogenase in the presence of 0.006% yogurt (c) and 0.5% rice bran containing lactobacilli (d) after cytosol proteins in mouse liver are separated by non-denaturing 2-DE using IEF gel containing 1.25% pharmalyte (pH 3–10) and 5% ampholine (pH 6–8).

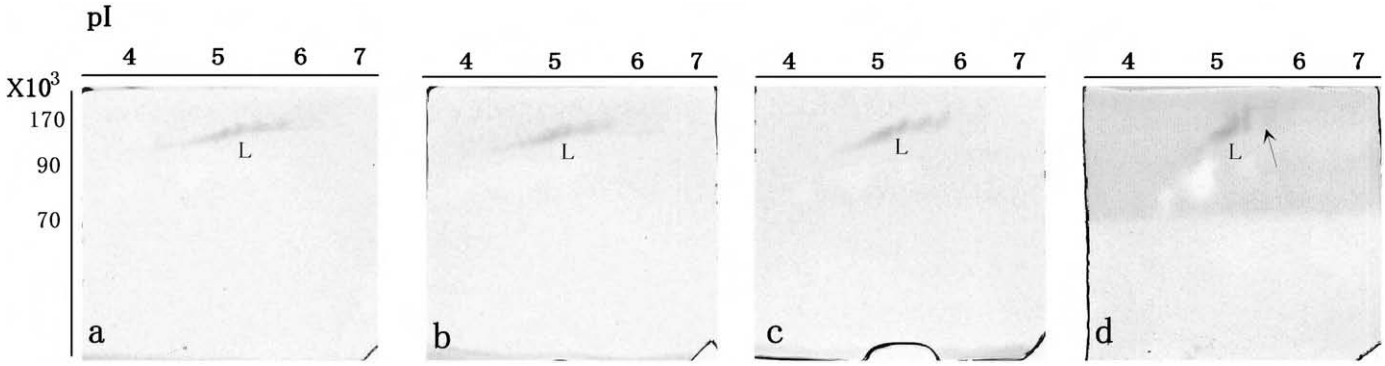

Fig. 6. Activity staining patterns of lactate dehydrogenase isozymes after separation using a combination of 2-DE and non-denaturing stacking gel electrophoresis containing 0.001% (a), 0.1% (b), 1% (c) or 5% (d) con A. L: lactate dehydrogenase. The loss of lactate dehydrogenase isozyme is indicated by the arrow.

method of IEF containing 5% carrier ampholyte (pH 3–10), and size separation. Esterase activity was retained even after sorbitol dehydrogenase activity was analyzed on the 2-DE gel (Fig. 2b). However, these two enzymes were not separated by this method. After activities of esterase and sorbitol dehydrogenase were examined by 2-DE, the separated proteins were stained by CBB (Fig. 2c). Many proteins were separated at p*I* 5–7.5 on the 2-DE gel, whereas few proteins were separated at p*I* 4–5. However, a wide area of 2-DE was obtained at p*I* 4–5 in 2-DE (Fig. 2c). Therefore, the separation of p*I* 5–7.5 in the IEF should be improved using a different p*I* range of carrier ampholyte (i.e. pH 6–8). Fig. 3 shows that sorbitol dehydrogenase activity (a), and activity of esterase following sorbitol dehydrogenase (b) were obtained after proteins were separated by a non-denaturing 2-DE method of IEF separation containing a mixture of 5% carrier ampholyte (pH 6–8) and 1.25% carrier ampholyte (pH 3–10) and size separation. Activity spots of sorbitol dehydrogenase and esterase were clearly separated by this 2-DE (Fig. 3b). Furthermore, the separation of proteins at p*I* 4.5–7 was improved using IEF that mainly contained carrier ampholyte (pH 6–8) after the activities of esterase and sorbitol dehydrogenase were examined. It is considered that after the separation of enzymes by 2-DE, substrate-specific enzymes would be evident on the 2-DE gel. Fig. 4 shows the sequential activity analysis of sorbitol dehydrogenase (a), malate dehydrogenase following sorbitol dehydrogenase (b) and lactate dehydrogenase following both the activity of sorbitol and malate dehydrogenase (c), after proteins were separated by a non-denaturing 2-DE method of IEF separation containing a mixture of 5% carrier ampholyte (pH 6–8) and 1.25% carrier ampholyte (pH 3–10) and size separation. These dehydrogenases were separated by 2-DE, and the enzymes recognised each specific substrate. CBB stain of proteins are shown in Fig. 5a after cytosolic proteins were separated by non-denaturing 2-DE of IEF separation containing a mixture of 5% carrier ampholyte (pH 6–8) and 1.25% carrier ampholyte (pH 3–10) and size separation. The spots of the non-denaturing 2-DE are labeled nos. 1–6 to indicate spots analyzed by peptide mass fingerprinting (PMF) using matrix–assisted laser desorption/ionization-time of flight (MALDI-TOF MS) (Fig. 5a). Table 1 shows that proteins in the spots were identified by PMF after separation by non-denaturing 2-DE. Spot no. 2 in 2-DE possesses lactate dehydrogenase activity and 2-DE detected 20 ppm of lactic acid in the enzyme (Fig. 5b). In addition, more than seven separated activity spots of lactate dehydrogenase were obtained

Table 1
Protein spot analysis of non-denaturing 2-DE and PMF using MALDI-TOF MS.

Spot number	Entry name	Protein name	Accession number	Protein score[a]	Sequence coverage
1	ALDH2	Aldehyde dehydrogenase	P17738	78	15%
2	DEMSLM	L-Lactate dehydrogenase	A25205	80	31%
3	HEM2_MOUSE	Delta-aminolevulinic acid dehydrase	P10518	72	23%
4	ARLY_MOUSE	Arginosuccinate lyase	Q91Y10	78	29%
5	SODC_MOUSE	Superoxide dismutase	P08228	60	31%
6	MDHC_MOUSE	Malate dehydrogenase	P14152	81	23%

[a] Protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 60 are significant ($p < 0.05$).

in 2-DE (Fig. 5b). More than 20 ppb of lactic acid was detected in this enzyme by 2-DE (data not shown). When the concentration of lactate dehydrogenase was decreased, the activity was obtained at one spot of p*I* 5.2/140,000 (arrow in Fig. 5b). It has been reported that five isozymes are found in the electrophoretic pattern because of the random association of the different subunits [12]. The heterogeneity of the isoenzymes in the present study may be due not only to the random association of different subunits, but also due to posttranslational modification such as glucosylation. So, this 2-DE in the present study can be applied to not only several kinds of enzymes separation but also lactate dehydrogenase isozymes separation. Existence of lactic acid in foods, such as 0.006% yogurt (c) and 0.5% rice bran containing lactobacilli (d), was examined by non-denaturing 2-DE (Fig. 5). It has been reported that lactate dehydrogenase after the sol–gel encapsulation may be used for optical sensing of L-lactate [6]. Therefore, lactate dehydrogenase can be applied as a sensor of lactic acid after the enzyme is separated by non-denaturing 2-DE gel and is encapsulated in the gel. Furthermore, since more than 20 ppb of lactic acid was detected by 2-DE, foods containing more than 20 ppb of lactic acid could be examined by this method. In addition, other substances such as sorbitol, malic acid and lipids can be examined because enzymes separated by 2-DE gel react specifically to each substrate. Fig. 6 shows the activity staining patterns of lactate dehydrogenase isozymes after separation using a combination of 2-DE and non-denaturing stacking gel electrophoresis containing 0.001% (a), 0.1% (b), 1% (c) or 5% (d) con A. In the presence of 1% con A, the band shifts of isozymes (p*I* 5.2–6) were reduced (c). Additionally, no isozyme activity (p*I* 5.5–6) was measured in the presence of 5% con A (d). It has been reported that lactate dehydrogenase activity is not activated by the addition of con A [13]. Therefore, these results indicate that some lactate dehydrogenase isozymes (p*I* 5.2–6) interact with con A. A possible explanation for these findings is that the glycosylation of isozymes differs among isozymes, and that some isozymes can be bound to con A.

4. Conclusion

We demonstrate here that not only several kinds of enzymes but also lactate dehydrogenize isozymes are separated by non-denaturing 2-DE. Further, isozyme heterogeneity can be analyzed by a combinational method of 2-DE and non-denaturing stacking gel containing con A. This method can be applied to examine relationship between enzyme activity and posttranslational modification such as glycosylation.

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