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A microfluidic device containing membrane-immobilized antibodies for successively capturing cytosolic enzymes

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ABSTRACT

A microfluidic device containing membrane-immobilized anti-esterase (ES) antibodies and anti-lactate dehydrogenase (LDH) antibodies was prepared. The membrane was prepared by transferring antibodies that had been separated by non-denaturing two-dimensional electrophoresis to a polyvinylidene difluoride membrane, which was then stained and cut into small pieces (16 mm^2). In this microfluidic device, >0.014 Unit mL^{-1} of the purified porcine carboxylesterase was specifically captured by membrane-immobilized anti- ES antibodies and >147 Unit mL^{-1} of purified porcine LDH was specifically captured by membrane-immobilized anti-LDH antibodies. Furthermore, ES and LDH in micro-scale aliquots of porcine liver cytosol were successively captured by membrane-immobilized antibodies in the device, and the enzyme activities were quantitatively analyzed by spectrofluorometry. The results indicate that the microfluidic device containing membrane-immobilized antibodies can be used to investigate the activities of several types of intact enzymes.

1. Introduction

Accumulating evidence indicates that immunoaffinity can be incorporated in devices and methods that can then be used to isolate specific target analytes [1–4]. Such devices can be used to isolate enzymes [5]. To investigate the activities of intact enzymes, it is necessary to produce a device that can capture intact enzymes without impairing their activities. We have previously shown that membrane-immobilized antibodies (i.e. immunoaffinity membranes) produced by antibodies isolated by non-denaturing two-dimensional electrophoresis (2-DE) can be transferred to a polyvinylidene difluoride (PVDF) membrane, which we then stained [6–8]. The immunoaffinity membranes were small: therefore, they could be placed on a microdevice. Antigen–antibody interactions can occur on the surface of such membrane within a microdevice, and the antigens captured by the membrane are expected to retain their functions such as enzyme activities. In addition, because the immunoaffinity membranes can be simultaneously produced [7], several types of the immunoaffinity membranes are placed on the microdevice for capturing several types of antigens.

It has been shown that the activities of several enzymes, such as carboxylesterase and lactate dehydrogenase (LDH), can be quantitatively analyzed by fluorometric methods [9,10]. Furthermore, because enzyme activities are altered due to some diseases [11], quantitative analysis of enzyme activity is considered useful for detecting these diseases. The activities of enzymes can be analyzed by using fluorometric methods even when the intact enzymes in biological samples have been captured by an immunoaffinity membrane within a microdevice.

Here, we present a microfluidic device containing membrane-immobilized anti-esterase (ES) antibodies and anti-LDH antibodies. Both ES and LDH were captured by the membrane-immobilized antibodies within the device from porcine liver cytosol, and ES and LDH activities were quantitatively analyzed by fluorometric methods. The results indicate that the cytosolic enzymes were successively captured by the microfluidic device that contained several types of membrane-immobilized antibodies and that the enzymes retained their activities.

2. Materials and methods

2.1. Chemicals and sample preparation

Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10) were purchased from Daiichi Pure Chemicals Co. Ltd. (Osaka, Japan)

Abbreviations: PVDF, polyvinylidene difluoride; TEMED, *N,N,N′,N′*-tetramethylenediamine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NAD, nicotinamide adenine; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate

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and GE healthcare (Uppsala, Sweden), respectively. Polyclonal anti-porcine liver esterase antibody and anti-rabbit muscle lactate dehydrogenase antibody were purchased from Rockland Inc. (Gilbertsville, PA). PVDF was purchased from Merck-Millipore (Bedford, MA). Nicotinamide adenine (NAD) and purified porcine heart LDH were purchased from Oriental yeast co. LTD (Tokyo, Japan). Cyto Tox-ONE ™ was purchased from Promega Corp. (Madison, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Osaka, Japan). Porcine livers were homogenized in 0.1 M Tris–HCl buffer (pH 7.2). The homogenate was centrifuged for 10 min at 10,000 × *g* to obtain the cytosol fraction. Sucrose was added to commercial rabbit anti-porcine liver ES antibody and anti-rabbit muscle LDH antibody anti-rabbit muscle lactate dehydrogenase antibody at a final concentration of 40% (w/v).

2.2. Production of immunoaffinity membranes

Five to ten micro-liter of antibodies (10–20 μg) were subjected to micro-scale non-denaturing 2-DE using a previously reported method [6,12]. To produce two kinds of immunoaffinity membranes such as anti-ES antibodies immobilized membrane (ES antibody membrane) and anti-LDH antibodies immobilized membrane (LDH antibody membrane), the antibodies were transferred to a PVDF membrane using a semi-dry transblotting apparatus for immobilization after separation [13].

2.3. Antigen–antibody interactions in the microfluidic device

To trap specific antigens using the constructed immunoaffinity membranes, the anti-ES antibody membrane was first blocked with 1% bovine serum albumin (BSA) in 0.1 M Tris–HCl buffer (pH 7.0) for 1 h, after which the cytosolic fraction from a porcine liver sample was added to this membrane and incubated for more than 2 h. The porcine liver fraction was collected and then, added to the anti-LDH antibody membrane. The membranes were washed three times with 5 mL of 0.1 M Tris–HCl buffer (pH 7.0). The anti-ES membrane was then incubated in 10 mL of 0.2 M phosphate buffer (pH 7.1) containing 0.2 mL of 1% α-naphthyl acetate and 4 mg of Fast red TR salt to allow the ES activity on the membrane to be analyzed. The anti-LDH membrane was incubated in 10 mL of Tris–HCl buffer (pH 7.2) containing 10 μL of lactic acid, 5 mg β-nicotinamide adenine dinucleotide (NAD), 3 mg nitro blue tetrazolium (NBT) and 0.3 mg phenazine methosulfate (PMS) to allow the LDH activity on the membrane to be analyzed.

The specific antigens were successively captured in the microfluidic device following the procedure described next (Fig. 1). Immunoaffinity membranes (4 × 4 mm) were prepared and incubated with 1% BSA in 0.1 M Tris–HCl buffer (pH 7.0) for 1 h, then placed in the well (4 mm i.d.) of the microfluidic device, as is shown in Figs. 1 and 2a. The ES antibody and LDH antibody membranes were placed in a microfluidic device (Fig. 2b), and the microfluidic device was connected to a peristaltic pump and filled with 50 mM Tris–HCl at pH 7.0 (Fig. 1). To check for the amounts of antibodies on these membranes, different quantities of anti-ES and LDH antibodies (0.1–10 μg) were immobilized on PVDF (4 × 4 mm) membranes. To examine the trap capability and specificity of the antigens by the membrane-immobilized antibodies, different amounts of purified porcine carboxylesterase (0.0014–0.14 Unit mL^{-1}) and LDH (54–300 Unit mL^{-1}) were applied to the microfluidic device, and a liquid contained a sample was circulated through the microfluidic device system at a flow rate of 2.2 mL min^{-1}. In addition, a 10 μL aliquot of the cytosolic fraction of porcine liver was applied to the microfluidic device, and the liquid containing the sample was circulated. The ES and LDH antigens in the cytosolic fraction were captured by the ES antibody and LDH antibody membranes, respectively, as shown in Fig. 2b. The captured ES and LDH enzyme activities were analyzed by spectrofluorometry.

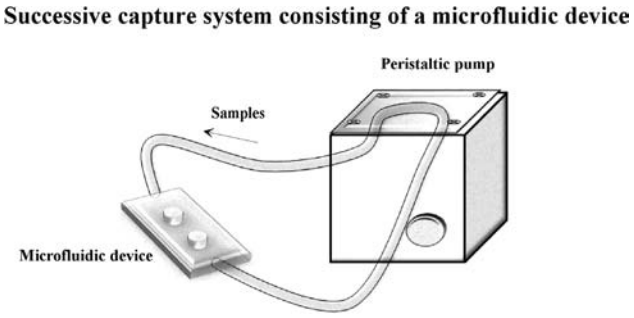

Fig. 1. Schematic of the successive capture system consisting of a microfluidic device connected to a peristaltic pump. A 10 μL aliquot of porcine liver cytosol was used in the system in our tests.

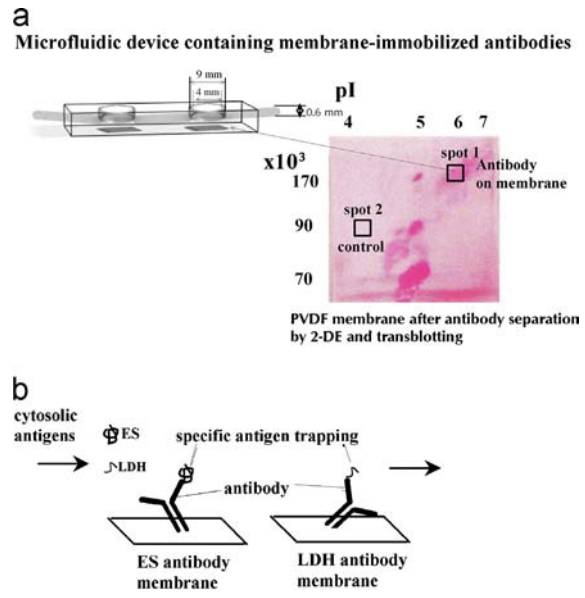

Fig. 2. (a) Schematic of the microfluidic device containing membrane-immobilized antibodies. The membranes were prepared by using antibodies separated by non-denaturing two-dimensional electrophoresis, which were transferred to a polyvinylidene difluoride membrane, and they were stained and cut into small pieces (16 mm^2). (b) Schematic of the device containing membranes with immobilized anti-ES and anti- LDH antibodies. ES and LDH were successively captured from porcine liver cytosol by the membrane-immobilized anti-ES and anti-LDH antibodies, respectively.

2.4. Quantitative analysis of ES and LDH activity using spectrofluorometry

Once the ES and LDH in the cytosolic fraction of porcine liver had been captured by using the ES antibody and LDH antibody membranes, respectively, their fluorescences were analyzed by using a spectrofluorometer (FP-8200; JASCO, Tokyo, Japan). Compound 4-methylumbelliferyl acetate (1 mg) was dissolved in 0.5 mL of *N*,*N*-dimethylformamide for quantitatively analyzing the ES activity. The solution was diluted by a factor of 1000 with 0.1 M phosphate buffer at pH 6.9 (4-MB solution). The 4-MB solution (100 μL) was mixed with the ES captured by the ES antibody membrane (spot 1 in Fig. 2a) and by the control membrane, without the antibodies (spot 2 in Fig. 2a), and this mixture was reacted for 30 min at 25 °C. The reaction solution was then diluted by a factor of 100 with ultra-pure water. The ES activity in the solution was determined by using an excitation

wavelength of 360 nm and an emission wavelengths of 460 nm. The fluorescence intensity of the 4-MB substrate solution was set to 1.0 when it was not reacted (i.e. without the membrane) to allow the relative fluorescence intensity of the ES activity to be calculated. The relationship between the specific activity and the fluorescent signal from purified porcine liver carboxylesterase (>15 U mg^{-1}) was used to analyze the specific activity of the ES captured by the ES antibody membrane.

CytoTox-ONE™ was used for the quantitative analysis of the LDH activity. The LDH captured by the LDH antibody membrane (spot 1 in Fig. 2a) and by the control membrane, without the antibodies (spot 2 in Fig. 2a) was soaked in 100 μL of CytoTox-ONE reagent containing the substrate and the assay buffer, and the solution was mixed for 30 s. The mixture was allowed to react for 30 min at 25 °C in the dark, and a stop solution was added. A 10 μL aliquot of the reaction solution was diluted by a factor of 10 with ultra-pure water. The LDH activity in the solution was measured by using an excitation wavelength of 560 nm and an emission wavelength of 582 nm. The fluorescence intensity of the CytoTox-ONE reagent solution was set to 1.0 when it was not reacted with the membrane to allow the relative fluorescence intensity of the LDH activity to be calculated. The relationship between the specific activity and the fluorescent signal for purified porcine heart LDH (300 U mg^{-1}) was used to analyze the specific activity of the LDH captured by the LDH antibody membrane. The data were analyzed with the Student's *t*-test.

3. Results and discussion

3.1. Specific capture of ES and LDH from a porcine liver cytosol fraction by the immunoaffinity membranes

Fig. 3 shows the (a) ES and (b) LDH activity staining after the cytosolic fraction of porcine liver was added to the membranes with immobilized anti-ES and anti-LDH antibodies, respectively. The ES in the porcine liver extract was captured by the membrane-immobilized anti-ES antibodies, and the LDH in the porcine liver extract was captured by the membrane-immobilized anti-LDH antibodies. The ES was not trapped by the membrane-immobilized anti-LDH antibodies, and the LDH was not trapped by the membrane-immobilized anti-ES antibodies (data not shown). The enzymes captured by the immunoaffinity membranes were, therefore, specific to each membrane, and the captured enzymes retained their activities.

3.2. Successive capture of enzymes in a microfluidic device containing the immunoaffinity membranes

When 0.1–10 μg of anti-ES antibody and 0.2–10 μg of anti-LDH antibody were immobilized on a PVDF membrane (4×4 mm), purified porcine carboxylesterase and LDH were specifically captured by the membrane-immobilized anti-ES antibody and LDH antibody, respectively (data not shown). A membrane with immobilized anti-ES antibodies and a membrane with immobilized anti-LDH antibodies were prepared (spot 1 in Fig. 2a) and incubated with 1% BSA, and they were placed in the well of a microfluidic device, as shown in Figs. 1 and 2a, and the enzymatic reactions of the captured enzymes were analyzed. Enzymes in the cytosolic fraction were captured by using membranes not containing antibodies (spot 2 in Fig. 2a) in the microfluidic device, as controls, and the enzymatic reactions in these were examined. When purified porcine carboxylesterase (0.0014–0.14 Unit mL^{-1}) was applied to the microfluidic device contained a membrane with immobilized anti-ES antibodies and a membrane with immobilized anti-LDH antibodies, >0.014 Unit mL^{-1} of carboxylesterase was specifically trapped on the membrane with immobilized anti-ES antibodies compared to the membrane with immobilized anti-LDH antibodies (Fig. 4a). In addition, when the purified porcine LDH (54–300 Unit mL^{-1}) was applied to the microfluidic device contained a membrane with immobilized anti-ES antibodies and a membrane with immobilized anti-LDH antibodies, >147 Unit mL^{-1} LDH was specifically trapped to the membrane with immobilized anti-LDH antibodies compared to the membrane with immobilized anti-ES antibodies (Fig. 4b). Thus, specific immunoreactivity on the membrane immobilized antibodies was retained within the microfluidic device. However, the amount of ES trapped by the membrane-immobilized anti-ES antibody was 1050 times greater than that for LDH trapped by the membrane-immobilized anti-LDH antibody. Because the detection sensitivity for esterase activity was 11 times higher than that for LDH activity, the difference in these enzymes' activity on the immunoaffinity membranes was thought to result from the differences in the immunoreactivity on these membranes. There were two possibilities for the suppressed enzyme activity. (1) Because the amounts of LDH on the membrane-immobilized anti-LDH antibody were limited, LDH activity could be suppressed. (2) Because enzyme active sites overlap with interaction sites for antigens with antibodies, LDH activity could be suppressed. Fig. 5 shows changes in (a) the 4-MB fluorescence spectra of ES captured on the membrane with immobilized anti-ES antibodies, and the control underwent hydrolysis reactions in the microfluidic device and (b) the relative fluorescence intensity at $\lambda_{em}=460$ nm ($\lambda_{ex}=360$ nm) when the ES

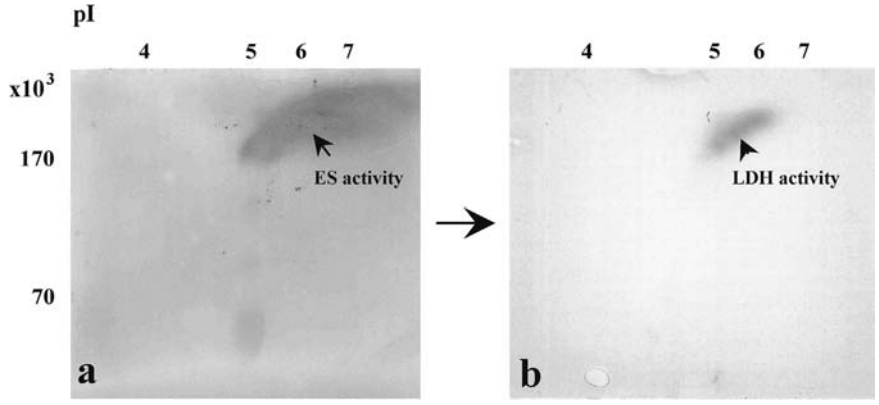

Fig. 3. Activity staining of (a) ES bound to the membrane-immobilized anti-ES antibody and (b) LDH bound to the membrane-immobilized anti-LDH antibody after the cytosolic fraction of a porcine liver extract was applied to the membrane with immobilized anti-ES antibodies and then to the membrane with immobilized anti-LDH antibodies.

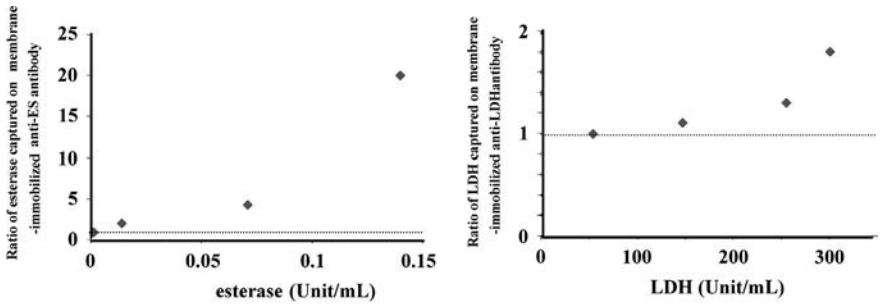

Fig. 4. (a) Ratio of carboxylesterase captured on membrane-immobilized anti-ES antibody as compared to a membrane-immobilized anti-LDH antibody (b) ratio of LDH captured on a membrane-immobilized anti-LDH antibody as compared to membrane-immobilized anti-ES antibody. Ratio of captured enzyme >1 (dashed line) indicates specific enzymes binding to a membrane-immobilized antibody.

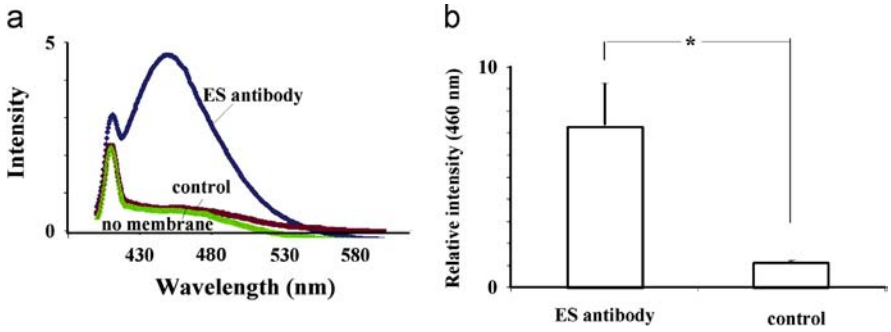

Fig. 5. (a) Fluorescence emission spectra of 4-methylumbelliferyl acetate after hydrolysis by ES bound to membrane-immobilized anti-porcine ES antibodies (spot 1 in Fig. 2a; ES antibody) and non-antibodies (spot 2 in Fig. 2a; control), and after no enzymatic reaction (no membrane) after trapping ES from porcine liver cytosol in the microfluidic device. (b) Relative fluorescence intensity at $\lambda_{em}=460$ nm ($\lambda_{ex}=360$ nm) of 4-methylumbelliferyl acetate after hydrolysis by ES bound to membrane-immobilized antibodies (ES antibody) and non-antibodies (control) after trapping ES from porcine liver cytosol in the microfluidic device.

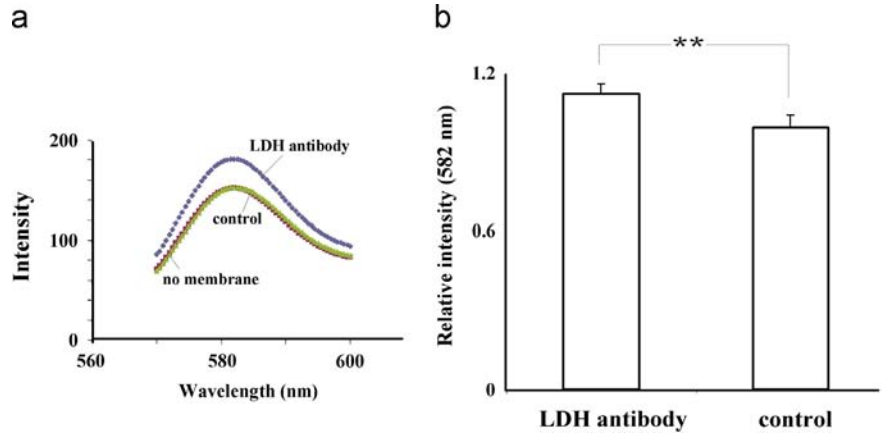

Fig. 6. (a) Changes in the fluorescence spectra of resorufin synthesized from reductive β-nicotinamide adenine dinucleotide (NADH) and resazurin by diaphorase after NADH had been synthesized by captured LDH bound to membrane-immobilized anti-LDH antibodies (spot 1 in Fig. 2a; LDH antibody) and non-antibodies (spot 2 in Fig. 2a; control), and after no NADH synthesis by LDH (no membrane) after trapping LDH from porcine liver cytosol in the microfluidic device. (b) Relative fluorescence intensities at $\lambda_{em}=582$ nm ($\lambda_{ex}=560$ nm) of the resorufin synthesized from reductive NADH and resazurin by diaphorase after the NADH had been synthesized by the LDH captured by membrane-immobilized anti-LDH antibodies (LDH antibody) and membrane-immobilized non-antibodies (control) after trapping LDH from porcine liver cytosol in the microfluidic device.

bound to the membrane-immobilized anti-ES antibodies and the control underwent hydrolysis reactions after trapping ES from porcine liver cytosol in the microfluidic device (Figs. 1 and 2). The specific fluorescent peak at $\lambda_{em}=460$ nm was measured to determine the ES bound to the membrane-immobilized anti-ES antibodies, because 4-MB could be hydrolyzed by the captured ES (Fig. 5a). The enzymatic reaction did not occur on the control membrane (Fig. 5a). The fluorescent peak at $\lambda_{em}=460$ nm was 6.54 times higher for the membrane with immobilized anti-ES antibodies than for the control membrane (Fig. 5b). The cytosolic ES in the porcine liver extract was specifically trapped by the membrane-immobilized anti-ES antibodies in the microfluidic device, and the amount of ES captured by the membrane-immobilized anti-ES antibodies was estimated to be 0.02 units. Fig. 6 shows changes in the fluorescence spectra of the resorufin synthesized by the reductive reaction between NADH and resazurin caused by diaphorase after the NADH was synthesized by LDH captured by the membrane with immobilized anti-LDH antibodies and control. The fluorescent peak at $\lambda_{em}=582$ nm was used to measure the amount of LDH captured by the membrane with immobilized anti-LDH antibodies, because the NADH synthesized by the LDH could have been oxidized by diaphorase (Fig. 6a). The fluorescent peak at $\lambda_{em}=582$ nm was significantly higher for the membrane with anti-LDH antibodies

than for the control (Fig. 6b). However, among the cytosolic enzymes of porcine liver, the enzymatic activity of the LDH captured by the membrane-immobilized LDH antibodies was only a factor of 0.18 times that of the ES captured by the membrane-immobilized ES antibodies. This was because 0.0065 units of LDH were captured by the LDH antibody membrane, whereas 0.02 units of ES were captured by the ES antibody membrane. Quantitative analyses of cytosolic enzyme activities have been developed and sensitive analyses of enzyme activities have been reported [14,15]. In the present study, a high sensitivity for detecting ES activity but not LDH activity was achieved. Thus, the sensitivities for detecting enzyme activities probably depend on the amounts of enzymes trapped on the imunoaffinity membrane in this microfluidic device. Different forms of a protein in tissue samples and biological fluids are analyzed by immunosorbent assays [16]. When antibodies that recognize the minute structural differences of an antigen are immobilized on a membrane, various forms of enzymes, such as their active and inactive forms, are considered to be trapped in the present device. Furthermore, antigens can be captured by using a combination of the immunoaffinity technique and a microfluidic device [17,18]. Other types of techniques and devices could be used to capture other antigens. The immunoaffinity membranes used in the method presented here can be easily exchanged so that other antigens can be captured because different immunoaffinity membranes can be produced by other reported methods [7]. Many different types of enzymes could, therefore, be captured and analyzed by this method. Immunoaffinity methods can also be used to capture intact proteins, protein complexes on intact organelles, intact bacteria, and prostate cancer cells [19–22]. Therefore, the immunoaffinity method presented here could be used to capture such macromolecules and cells.

4. Conclusions

Carboxylesterase and LDH in porcine liver cytosol can be successively captured by using a microfluidic device containing membrane-immobilized antibodies. The activities of the enzymes captured can be quantified by fluorometric methods. The microfluidic device containing membrane-immobilized antibodies can, therefore, be used to investigate the activities of several types of intact enzymes.

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