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Retaining activity of enzymes after capture and extraction within a single-drop of biological fluid using immunoaffinity membranes

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ABSTRACT

The purpose of this study was the measurement of enzyme activity within a single-drop of biological fluid after micropurification. Esterase and lactate dehydrogenase (LDH) retained their enzymatic activities after being captured by membrane-immobilized antibodies, which were prepared by non-denaturing two-dimensional electrophoresis, transferred to polyvinylidene difluoride and then stained by Ponceau S. The activities of both enzymes were also measured after being captured by antibodies and biotinylated antibodies bound to membrane-immobilized protein A or avidin, respectively. After esterase and LDH were captured from biological samples by membrane-immobilized protein A or avidin, their activities were semi-quantitatively measured on the surface of the membrane using fluorescence determination. More than 51% of enzyme activities were retained even after the enzymes were captured by biotinylated antibody bound to membrane-immobilized avidin and eluted by rinsing with 5 μL of 1% Triton X-100, compared with the activities of the enzyme on the immunoaffinity membrane.

1. Introduction

Technologies have been reported for analyzing molecules within small sample volumes such as microlitres or nanolitres [1,2]. These techniques have progressed to examining molecules in one-drop of biological fluids such as blood [3,4]. The analytical techniques can help to lighten the burden imposed on patients, because it requires a low-volume of a biological sample. It has been reported that the enzyme activity within a droplet of biological fluid can be examined by spectrofluorometry using fluorogenic substrates [5]. For characterizing small amounts of enzymes, the technique of microfluidic separation is required. Microfluidic immunoaffinity separation can be applied for the selective isolation of a target from biological samples [6]. We have reported that proteins can be isolated and extracted using three types of immunoaffinity membrane. One of these is prepared by non-denaturing two-dimensional electrophoresis (2-DE), transferred to polyvinylidene difluoride (PVDF) and then stained [7], and the others comprise antibodies and biotinylated antibodies bound to membrane-immobilized protein A and avidin, respectively [8,9]. Because the adsorption and extraction of proteins are performed on the membrane surface, antigens in a single-drop of biological fluid are directly applied to immunoaffinity membranes. Thus, the method of immunoaffinity membranes can be applied to isolate antigens in biological fluid samples that are as small as a single-drop. Furthermore, because structural damage to an antigen is minimal during adsorption and extraction of the protein on the membrane surface, extracted proteins are thought to retain their activities. In fact, the activities of enzymes are retained even after their adsorption on various materials, such as polydopamine films, and subsequent extraction [10–13]. After extracting enzymes using the detergent, Triton X-100, their activities can be examined [12,13]. To examine changes in enzyme activity during the process of isolation and extraction using an immunoaffinity membrane, a quantitative analytical method of measuring enzyme activity may be necessary. It has previously been reported that the activities of enzymes such as carboxylesterase and lactate dehydrogenase (LDH) can be quantitatively analyzed by fluorometric methods [14,15]. Because fluorometric methods are sensitive in measuring enzyme activity, the activities of enzymes can be measured even after their purification within a single-drop of biological fluid using immunoaffinity membranes.

Abbreviations: PVDF, polyvinylidene difluoride; TEMED, *N,N,N,N*-tetramethylenediamine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NAD, β-nicotinamide adenine dinucleotide; bis, *N,N*-methylenbisacrylamide; BSA, bovine serum albumin; 4-MB, 4-methylumbelliferyl acetate; LDH, lactate dehydrogenase.

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After esterase and LDH were captured from biological samples by either antibodies bound to membrane-immobilized protein A or biotinylated antibodies bound to membrane-immobilized avidin, their activities were semi-quantitatively measured on the surface of the membrane using fluorescence determination with single-drop analysis. The activities of the enzymes were retained even after they were captured by a biotinylated antibody bound to membrane-immobilized avidin and eluted by rinsing with 5 μL of 1% Triton X-100. The methods of immunoaffinity membranes can be applied to capture and extract enzymes in a low-volume of biological fluid samples.

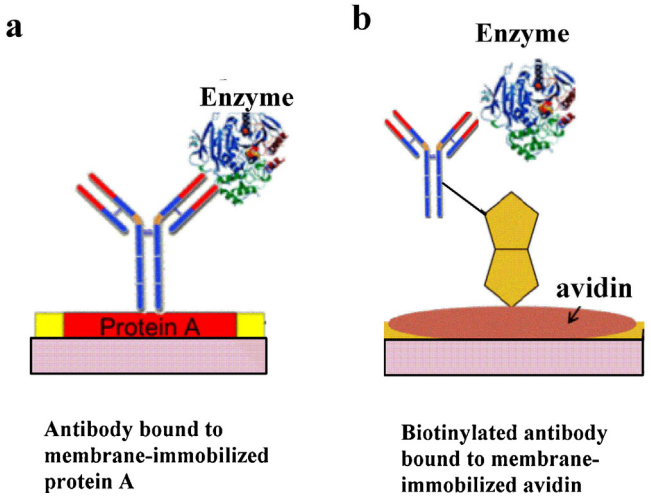
2. Materials and methods

2.1. Materials

Antisera against rabbit lactate dehydrogenase and poricine esterase were purchased from Rockland (Gilbertsville, PA, USA). Antiserum against against human transferrin was purchased from Dako (Glostrup, Denmark). Cyto Tox-ONE TM was purchased from Promega Corp. (Madison, WI, USA). Carrier ampholyte (Pharmalyte, pH 3–10), Polyvinylidene difluoride (PVDF) membrane, biotin Labeling Kit—NH2 and acrylamide were purchased from GE Healthcare (Uppsala, Sweden), Merck-Millipore (Bedford, MA, USA), Dojindo Laboratories (Kumamoto, Japan) and Daiichi Pure Chemical (Osaka, Japan), respectively. Nicotinamide adenine (NAD) and purified porcine heart LDH were purchased from Oriental yeast co., LTD (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Osaka, Japan).

2.2. Preparation of immunoaffinity membranes

For the production of a membrane-immobilized antibody, sucrose was added to antisera, avidin and protein A at a final concentration of 40% (w/v). A sample of 5–10 μL antiserum (10–20 μg protein) was subjected to micro-scale non-denaturing 2-DE using a previously reported method [16,17]. Isoelectric focusing (IEF) was performed on rod gels (35 mm long × 1.3 mm i.d.). The gel solution contained 4% (w/v) acrylamide and 0.2% (w/v) *N*,*N*-methylenbisacrylamide (bis), 2% (v/v) Pharmalyte (pH 3–10), 0.05% (w/v) ammonium persulphate and 0.029% (v/v) *N*,*N*,*N*',*N*'-tetramethylenediamine (TEMED). The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). The resulting IEF gel was placed on top of a two-dimensional slab gel, which was run on a gel containing 5.7% (w/v) acrylamide and 0.28% (w/v) bis. The electrode buffer contained 0.05 M Tris and 0.38 M glycine (pH 8.3). The antibody was transferred to a PVDF membrane using a semi-dry transblotting apparatus for immobilization after separation [18]. For the detection of proteins on the membrane, the membrane was soaked in 0.5% Ponceau S in 10 mL of 0.1 M acetate buffer. The orientation of antibodies on the membrane is not controlled in the case of antibodies coupled to immunoaffinity column [19], whereas antibodies bound to membrane-immobilized protein A and biotinylated antibodies bound to membrane-immobilized avidin can be controlled by altering the interactions between the antigen and antibody on the membrane surfaces. Scheme 1 shows (a) an antibody bound to membrane-immobilized protein A and (b) a biotinylated antibody bound to membrane-immobilized avidin. For the production of membrane-immobilized avidin, avidin (50 μg) was applied to the top of a non-denaturing electrophoresis gel in many separate lanes of 4 mm width. The gel solution contained 5% acrylamide, 0.25% bis and 0.13 M Tris–HCl (pH 6.9). The electrode buffer contained 0.05 M Tris and 0.38 M glycine (pH 8.3). Because avidin is a basic protein, it migrates towards a cathode. When the bottom of the gel was connected to the cathode, avidin migrated to the bottom of the gel. After separation by non-denaturing electrophoresis, the protein was transferred to a PVDF membrane [18]. To detect avidin, the membrane was soaked in 0.001% Ponceau S in 10 mL of 50 mM Tris–HCl buffer (pH 7.0). For the production of membrane-immobilized protein A, protein A (10 μg) was applied to the top of a non-denaturing electrophoresis gel in many separate lanes of 4 mm width. The gel solution contained 7.5% acrylamide, 0.38% bis and 0.13 M Tris–HCl (pH 8.8). After separation by electrophoresis, the protein was transferred to a PVDF membrane [18]. The membrane was soaked in 0.1% Ponceau S in 10 mL of 0.1 M acetate buffer (pH 5.2). The membranes were then destained with water.



Scheme 1. Membrane-immobilized antibody bound to membrane-immobilized protein A (a) and biotinylated antibody bound to membrane-immobilized avidin (b).

2.3. Enzyme activity detection after antigens capture and and extraction using immunoaffinity membranes

The porcine liver fraction was collected and was added to the produced immunoaffinity membranes (membrane immobilized antibody, antibody bound to membrane-immobilized protein A and biotinylated antibody bound to membrane-immobilized avidin) after 20 μL of 1% bovine serum albumin (BSA) was added to the membranes, and was incubated for 30 min for blocking. The membranes were washed three times with 5 mL of 0.1 M Tris–HCl buffer (pH 7.0). For detection of LDH activity, the membrane or gel 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). For detection of esterase activity, the membrane or gel was 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.

For trapping esterase or LDH by membrane-immobilized protein A, 10–15 μL of 1% BSA solution containing 8–10 μg anti-porcine esterase antibody, anti-rabbit LDH antibody or anti-transferrin antibody (control) was added to the membrane-immobilized protein A, and was incubated for 2 h after 10 μL of 1% BSA was added to the membrane, and was incubated for 30 min for blocking. Then, after 10 μL of 1 μg/mL of purified porcine esterase, purified rabbit LDH or porcine liver cytosol was added to the antibody bound to membrane-immobilized protein A, and it was incubated for 2 h. After washing with 0.1 M Tris–HCl buffer (pH 7.0) for 3 times, the esterase or LDH activity on the membrane was examined by the