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Protein Dot Stamp Using Hydrophobin as Immobilization Carrier

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Protein dot spot technology is presented using unique proteins such as hydrophobin HFB (from *Trichoderma reesei*). HFB is a protein of low molecular weight (7.8 kD) and is an outstanding hydrophobic molecule. The authors employ HFB as a carrier for protein dot spots. In this study, fluorescent-labeled HFB was spotted on a hydrophobic glass substrate using a solution drop contact spotter. HFB adsorbed on the glass surface uniformly. The results show that HFB provides prominent adsorption characteristics as a carrier protein. The small HFB molecule can be tagged onto other protein molecules through a conventional genetic procedure. By using HFB as a carrier protein, practical protein chip technology can be realized.

1. Introduction

The Human Genome Project was completed in 2002. At present, we can make full use of a substantial amount of genetic sequencing information. Nowadays, we are more concerned with proteins than with genes, because biological functions are determined by proteins, not by genes. Proteome research has been promoted on the basis of genome information.[1] Proteins play a crucial role in living systems, *e.g.*, signal transduction, catalytic function, immunity and maintenance of a transformed phenotype. These functions can be broadly divided into two categories: molecular selectability (affinity) and catalytic function (enzymatic activity). These functions have been studied for a considerable length of time.[2,3] However, the high-throughput evaluation of protein has not been

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carried out in great detail. This technology is essential for conducting functional and structural studies of proteins derived from a number of genetics information. In proteome research, the specific binding affinity of proteins is very fundamental and indispensable information. Until now, the affinity of the bound function of proteins has been evaluated through assay methods on solid substrates.[1,2,4,5] To perform high-throughput assay on a solid substrate, a protein array is required. There are some methods of protein immobilization for the protein array, *e.g.*, cross linking, entrapment, and carrier binding. Carrier binding is classified into three major methods: ion binding, covalent binding and physisorption. Covalent binding is useful for the protein array because it rigidly binds the target protein to the array. However, this method requires pretreatment prior to protein binding, and sample proteins are affected by steric hindrance because the proteins are modified with a spacer. The ion binding method is not applicable to the protein array since the binding is easily affected by extrinsic factors. The physisorption method can easily but weakly bind proteins. Moreover, bound proteins are affected by extrinsic factors. However, this method has merit for binding proteins to the array chip without a spacer, and can be used for prolonged reactions. Therefore, the physisorption method is useful for the adhesion of proteins to the array chip while maintaining the structure and activity of the proteins.

Hydrophobins are proteins specific to filamentous fungi. Hydrophobins play various roles in fungal physiology, *e.g.*, adhesion to a hydrophobic substrate for growth, formation of protective surface coating, and reduction of surface tension of water.[6] This protein is remarkably stable and withstands temperatures as high as approximately 100°C because of its structure.[7] Hydrophobins have been divided into class I and class II on the basis of hydropathy plots.[8] Class I hydrophobins are from assemblages that are highly insoluble, while class II assemblages and absorbed surface layers dissociate more easily.[9] HFBI and HFBII from *Trichoderma reesei* are class II hydrophobins, and have a size of approximately 75 amino acids.[10] HFB has a unique structure as rigidly amphiphile, with the N- and C-terminal ends are located at the hydrophilic side.[7] Therefore, HFB can be used as a protein array carrier because of its characteristics, and the genetic fusing of protein to HFB can be performed with high-throughput spotting on array chip.

In the present study, HFBI and HFBII are evaluated as carrier proteins for protein immobilization on a solid substrate. A uniform dot of proteins is strongly required for performing solid-phase assay on protein array.

2. Materials and Methods

2.1 *Plasmids*

Plasmids pGZ1/pET81F1+ and pGZ2/pET81F1+ encode HFBI and HFBII, respectively. These plasmids are shown in Fig. 1. FLAG and His-tag were fused to the N-terminus of the HFB gene and cloned into a pET81F1+ vector[11] as an *Nco*I/*Bam*HI insert.

2.2 *Expression and purification of HFB I and HFB II*

Escherichia coli BL21 (DE3) transformed with pGZ1/pET81F1+ and pGZ2/pET81F1+ was grown in Luria Bertani (LB) medium containing ampicillin (100 μg/ml) at 37°C for 12

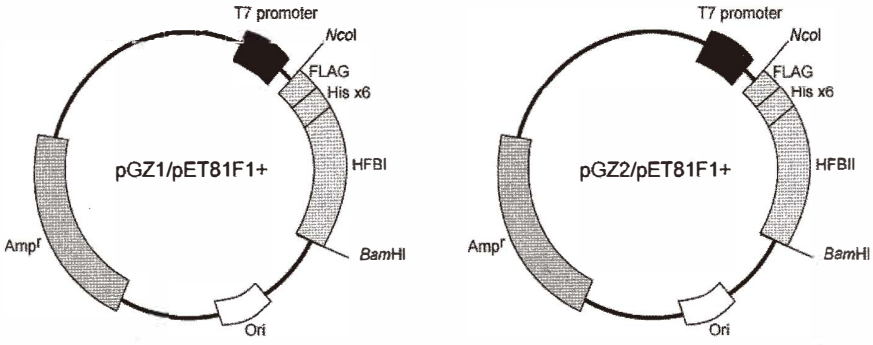

Fig. 1. Expression plasmids pGZ1/pET81F1+ and pGZ2/pET81F1.

h. The precultured medium was then seeded into the incubated LB medium, and the culture was continued at 37°C. When the absorbance at 600 nm reached 0.2, Isopropyl-Thio–β-D-Galactopyranoside as an inducer was added into the culture medium at a final concentration of 0.1 mM. After induction, the cultivation was continued for 4 h at 37°C. The microorganisms were harvested by centrifugation at 8000 rpm for 10 min, and suspended in phosphate buffer (0.1 M, pH 7.4) containing 2% Tween 20. After sonication, each protein sample was purified from the soluble fraction using a His-trap column (Amersham Biosciences, USA). Then, imidazole in the protein-eluted solution was removed by Slide-A-Lyzer (Pierce, USA), and the HFBI and HFBII were obtained. The protein concentration was determined using the method by Lowry.

Both HFBI and HFBII have relatively similar structures, particularly at their N- and C-termini.[10,12] Most differences in the sequence can be found in the middle parts of these proteins. The difference implies that HFBII is more hydrophobic than HFBI due to its residue character.[10,13]

2.3 *SDS-PAGE analysis*

The sample solution was mixed with an equal volume of sample buffer (containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.1 mg/ml bromophenol blue, and 10% 2-mercaptoethanol). The mixture was heated at 95°C for 3 min, and cooled on ice for 5 min. The prepared samples were loaded into wells of a 12.5% SDS-acrylamide gel and separated by electrophoresis. After electrophoresis, the separated proteins were visualized by Coomassie brilliant blue staining. Figure 2 shows SDS-PAGE analyses of HFBI and HFBII. The concentrations of HFBI and HFBII were 1.05 mg/ml and 0.62 mg/ml, respectively.

2.4 *Rhodamine staining*

The sample proteins (HFBI, HFBII, and BSA) were labeled with Rhodamine Red using the FluoReporter Rhodamine Red-X protein labeling kit (Molecular Probes, USA). Labeled proteins were purified and desalted using a PD-10 column (Amersham Biosciences, USA). The degree of rhodamine labeling was determined using the following equation.

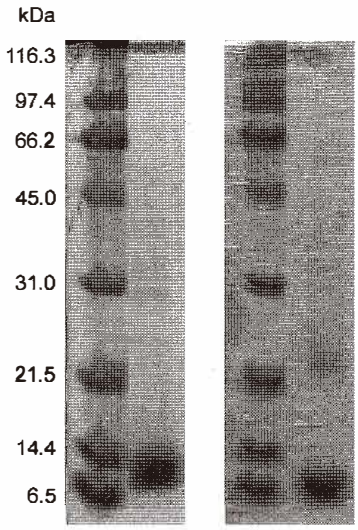

Fig. 2. SDS-PAGE analysis of (A) HFBI and (B) HFBII. Lanes 1 and 3: Molecular weight markers; lane 2: HFBI; lane 4: HFBII. The sample was separated by 12.5% polyacrylamide gel.

Dye per protein weight = Absorbance 570 nm × dilution factor / (120,000 × protein concentration (g/l)); 120,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the dye at 570 nm. The degrees of rhodamine labeling of proteins (HFBI, HFBII, and BSA) were 0.304, 0.150, and 0.153 μmol/mg, respectively.

2.5 *Hydrophobic modification of glass plate*

A glass plate (0.8–1.0 mm thickness) was obtained from Matsunami Glass (Japan). The glass plate was cleaned by ultrasonic irrigation with a 30% diluted solution of clean Ace detergent (As ONE, Japan) for 30 min, and washed with ultrapure water 3 times. The glass plate was treated with 10% sodium ethoxide in ethanol for 1 min, and washed with ethanol and ultrapure water. Then, to silanize the glass plate surface, the glass plate was immersed in 2.5 mM n-octadecyl trichlorosilane / chloroform solution for 15 h at room temperature. The silanized glass plate was washed, sonicated for 10 min with chloroform, washed with ultrapure water twice, and then dried using N_2 gas. Only glass plates with water contact angle of 90° were used for the protein dot spot array.

2.6 *Dot spot and imaging of HFB*

Rhodamine-labeled proteins were spotted at 0.5 μl onto the hydrophobic modified glass plate at room temperature (Fig. 3). After spotting, the protein-spotted glass was washed with ultrapure water containing 2% Tween 20. Spotted proteins were observed by fluorescence microscopy (Nikon, Japan), and fluorescence image analysis was performed using the AQUACOSMOS system (Hamamatsu Photonics, Japan). The extinction and emission filters used were in the range of 510–560 nm, and 590 nm, respectively. All procedures were performed at room temperature.

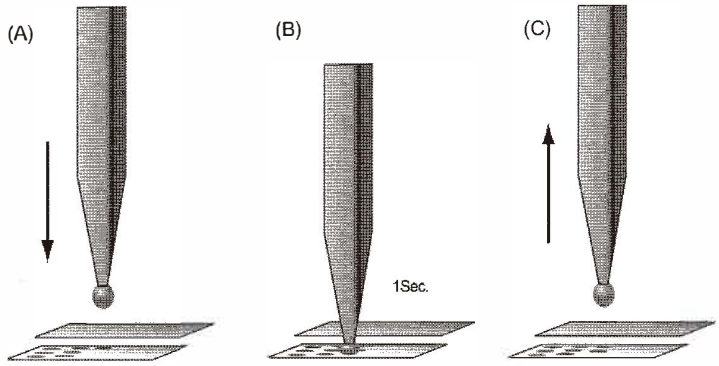

Fig. 3. Protein spotting process. (A) A drop of sample protein solution is made to approach the glass plate using a manipulation system. (B) A drop of sample protein solution is made to contact the glass substrate surface for 1 sec. (C) The drop is then removed from the glass substrate surface.

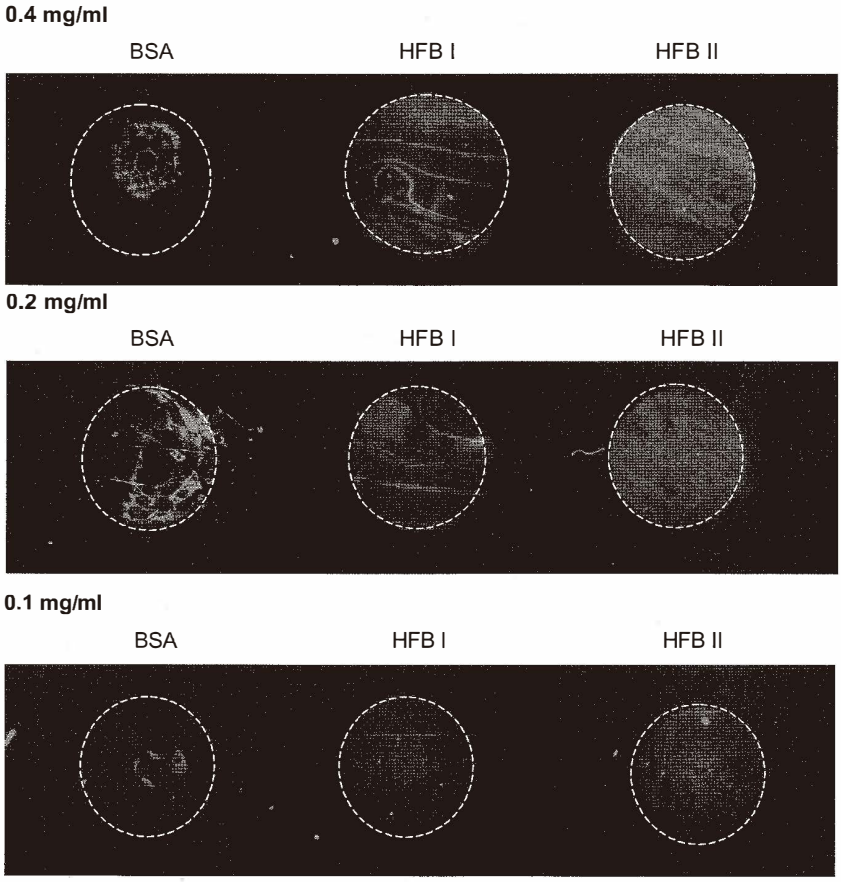

Fig. 4. Microscopic fluorescence images of spotted proteins labeled with rhodamine; BSA, HFBI, and HFBII. The proteins were spotted at concentrations of 0.4, 0.2, and 0.1 mg/ml, respectively.

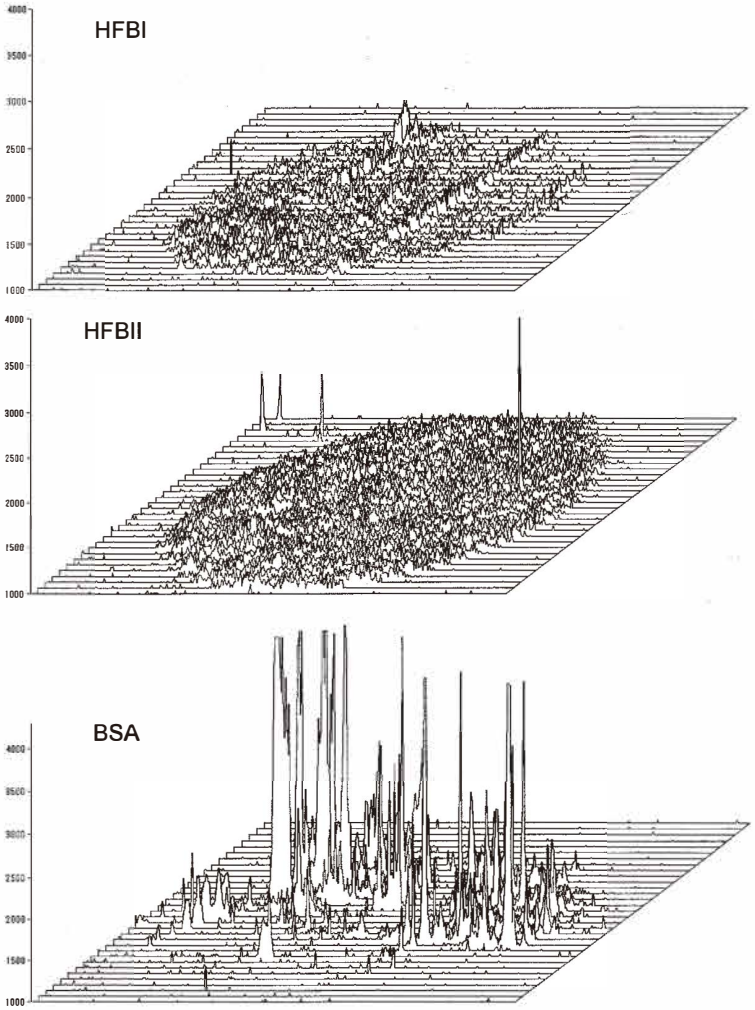

Fig. 5. Profile of fluorescence intensity of spotted protein dot (0.2 mg/ml).

3. Results and Discussion

3.1 *Dot spotting of protein on array chip*

Protein spotting to hydrophobic array chip was systematically performed as illustrated in Fig. 3. A sample dispenser is made to approach a glass substrate with the sample drop contacting the glass substrate surface for a set period of time. Then, the sample dispenser and sample drop were moved away from the substance. Rhodamine-labeled HFB were spotted on a hydrophobic glass plate, and evaluated using a fluorescence microscope with a fluorescence image analyzer (Fig. 4). The protein was spotted at concentrations of 0.4, 0.2, and 0.1 mg/ml. Because BSA is a useful protein for stabilizing an enzyme or

antibody, and can also be used as a carrier protein for a conventional protein array, it was employed as the control protein for dot spotting onto the array chip. As shown in Fig. 4, HFBI and HFBII formed dots uniformly and exhibited fluorescence intensity corresponding to the protein concentration. The uniformity of immobilized protein within the dot was evaluated by profiling of fluorescence strength, as shown in Fig. 5. In the case of HFBI and HFBII, fluorescence was spread throughout the dot. However, BSA aggregated and formed islands within the dotted area. In the case of HFBI/II, the dot size was precisely equal to the size of the sample solution drop (contact area of sample drop). The uniform HFBI/II dot was formed by contact with a drop of HFBI/II solution for only 1 sec. In the present system, HFB solution drop may act as a STAMP protein (Fig. 6). As shown in Fig. 6(A), molecular HFB forms a monolayer at air-water interfaces of the solution drop [6,12,14] before the dots are spotted. Then, the HFB solution drop is made to contact the glass substrate for 1 sec, and then removed. At that moment, the hydrophobic side of the HFB molecule adheres to the hydrophobic glass surface similar to ink transfer using a stamp (Fig. 6(B)–6(C)).

In conclusion, HFBI and HFBII showed good adsorption ability on a hydrophobic surface. HFBI and HFBII have a size of approximately 75 amino acids, and have a similar structure. It is sufficiently low for use as a carrier protein to tag to proteins without functional disturbance. In the present study, we have investigated the adhesion ability of

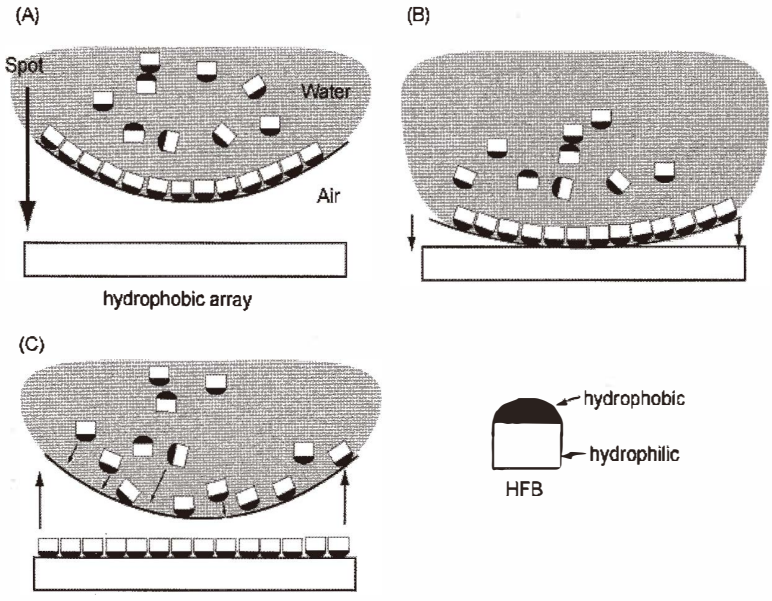

Fig. 6. Schematic diagram of dot plotting of HFB. (A) Monolayer state at air-water interface on sample drop before being spotted. (B) Adsorption of HFB onto hydrophobic array. (C) HFB monolayer on hydrophobic substrate surface.

HFBs on a hydrophobic substrate. The results show a prominent ability of HFB as a carrier for protein immobilization. The drop stamp system, based on the HFB carrier, is quite simple and is practical for producing a protein array chip.

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