Simple and Practical Semi-wet Protein/Peptide Array Utilizing a Micelle-mixed Agarose Hydrogel

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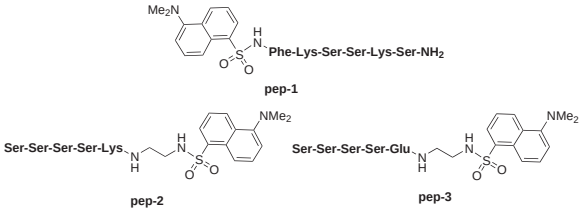
In an agarose hydrogel, triton X-100 formed a micelle with the original critical micelle concentration. The micelle-mixed agarose gel was successfully applied to fabricate a semi-wet protein/peptide array for monitoring enzyme activities and the enzyme inhibitor potency in a high throughput manner.

Protein/peptide microarray is one of the promising tools for a rapid and high throughput assay for many biological events.[1] However, the protein's instability under conventional dry conditions on a plate surface frequently makes it difficult to collect the confident and reproductive data using protein microarray. We have recently reported a "semi-wet protein/peptide array" using a supramolecular hydrogel in which natural proteins and peptides were arranged on a glass plate without denaturation.[2] In the supramolecular hydrogel chip, a well-defined hydrophobic micro-domain consisting of a supramolecular hydrogelator was elaborately employed as a signal transduction site for enzymatic reactions. Here, we describe that the amphiphilic supramolecular hydrogel can be successfully replaced with a micelle-mixed polymer gel for the semi-wet protein array. The hydrophobic domain of the micelle acts as a sensing site for enzyme reaction in the mixed polymer gel.

Agarose, a polymeric hydrogelator composed of polysaccharide, which is widely used in many biological researches was employed as a semi-wet matrix for protein/peptide in this study. In order to introduce a definite hydrophobic domain into agarose gel, a conventional micelle was mixed to produce a micelle-mixed agarose (MmA) hydrogel.[3] As a micelle forming surfactant, nonionic triton X-100[4] and octyl-β-D-glucopyranoside (OG)[5] were



tested. Initially, the micelle formation in an agarose gel (0.25 wt %) was confirmed by the fluorescence change of hydrogel 1-anilinonaphthalene-8-sulfonic acid (ANS) embedded in the mixed hydrogel. The fluorescence intensity of ANS biphasically increased with the raise of the surfactants concentration in the gel. For triton X-100 (Figure 1a), fluorescence of ANS discontinuously increased at a critical concentration ([triton X-100] = 0.2 mM), similar to the system without the agarose gel. The critical micelle concentration (cmc) value of triton X-100 in the agarose gel, thus determined as the discontinuous concentration of the fluorescence increase, was 0.2–0.3 mM (the reported cmc value in aqueous solution is 0.24–0.27 mM).[4] On the other hand, the cmc of OG in the agarose was determined to be 20–25 mM (Figure 1b), the value of which is also same as that in an aqueous solution (25 mM)[5] and 100 times

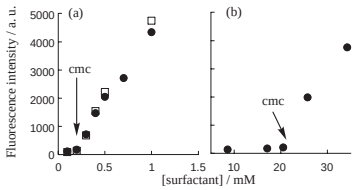


Figure 1. Surfactant concentration dependence for the fluorescence intensity of ANS. a) ● for triton X-100 in an agarose hydrogel; □ for triton X-100 in an aqueous solution. b) for OG in an agarose hydrogel. Conditions: [ANS] = 25 μM in 50 mM tris buffer (pH 8.0) containing 100 mM $CaCl_2$ at room temperature.

higher than the value for triton X-100. Since agarose is nonionic and it does not form a hydrophobic domain in a hydrogel state, triton X-100 and OG should not interact meaningfully with the agarose gel fibers under the studied concentration range. That maybe a reason why the cmc values of the both surfactants in the agarose gel are almost the same as those in a solution.

An enzyme-embedded MmA hydrogel chip was prepared as follows (Scheme 1); a suspended agarose (0.25 wt %) in tris buffer solution containing a surfactant was heated until a homogeneous solution was formed. 10 μL of the hot solution was dropped on a glass surface and the plate was placed under the humid conditions at room temperature for 30 min to afford a hydrogel array (step 1). An enzyme solution (1 μL) was dropped onto each MmA gel spot and the enzyme array was produced after 30 min incubation (step 2). The enzyme assay was carried out by the dropping of a substrate solution onto the enzyme enbedded MmA hydrogel chip (step 3).

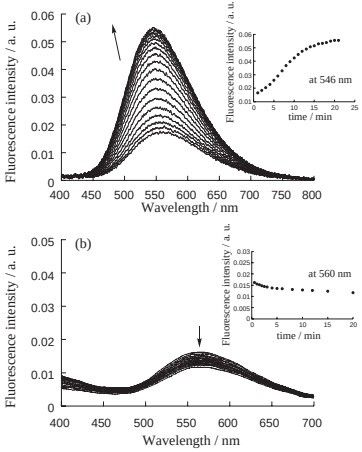
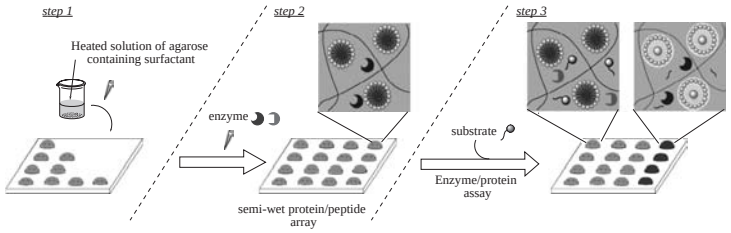


Figure 2. Fluorescent spectral change and its time course (imposed) for the chymotrypsin catalyzed hydrolysis of **pep-1** in an agarose hydrogel-based peptide chip in the presence (a) and absence (b) of a micelle. Conditions: [chymotrypsin] = 10 μM, [**pep-1**] = 100 μM, [agarose] = 0.25 wt %, [triton X-100] = 5.0 mM in 50 mM tris buffer (pH 8.0) containing 100 mM $CaCl_2$ at room temperature.



Scheme 1. Preparation scheme of a semi-wet peptide chip based on a MmA hydrogel. The details are mentioned in the discussion part.

The enzyme activity was monitored by dansyl-labeled peptides as a substrate using fluorophotometer equipped with multichannel photodetector (MCPD). In the case of chymotrypsin[6] in triton X-100 MmA gel ([**pep-1**] = 100 μM, [triton X-100] = 5.0 mM), the fluorescence intensity of the dansyl group increased along with the blue-shift of the fluorescence maximum by 13 nm (from 559 nm to 546 nm) during the enzymatic hydrolysis of **pep-1** (Figure 2a). In contrast, the fluorescence intensity slightly decreased and the maximum did not shifted without triton X-100 (Figure 2b). These indicate that the micelle acts as a sensing domain for the enzymatic reaction. It is conceivable that the cleaved dansyl group lost a hydrophilic peptide moiety to become hydrophobic, and as a result, it migrated and was entrapped in the hydrophobic microdomain of triton X-100 micelle (Scheme 1, imposed as cartoons). On the other hand, we observed the blue-shifted fluorescence (541 nm) before the enzymatic hydrolysis either in an OG MmA gel or in an aqueous solution containing OG micelle ([OG] = 65 mM). This suggests that the peptide substrate was already entrapped in the hydrophobic domain of the OG micelle, probably because the higher concentration is required to form micelle for OG. Consequently, the enzyme-induced fluorescence change did not take place in this OG system. In triton X-100 MmA gel, the activity of trypsin[7] or V8 protease[8] can be similarly monitored by using dansyl-appended peptides bearing appropriate sequence such as **pep-2** or **pep-3**, respectively. The fluorescence intensity increased along with the blue-shift of the emission (λ_{max} due to dansyl group from 560 to 554 nm) as a consequence of the enzymatic cleavage of the peptides (see supporting information). These results are consistent with that for chymotrypsin.

Such fluorescence changes were also detectable with the naked eye. The **pep-1** solution was dropped onto the each MmA hydrogel spot including chymotrypsin, trypsin, V8 protease, or bovine serum albumin (BSA; a carrier protein). As shown in Figure 3a, the stronger green emission appeared at the hydrogel spots containing chymotrypsin. The weak color change was observed at the spots containing trypsin, which is due to that **pep-1** has lysine as the cleavage site for trypsin as well as phenylalanine for chymotrypsin. Interestingly, the DANS-Phe-Lys, a fragment of **pep-1** cleaved by trypsin, is not hydrophobic enough to yield the stronger color change of emission than DANS-Phe, a chymotrypsin-cleavable fragment. Such a color change was not observed at spots containing V8 protease or BSA because of no occurrence of the enzymatic hydrolysis.

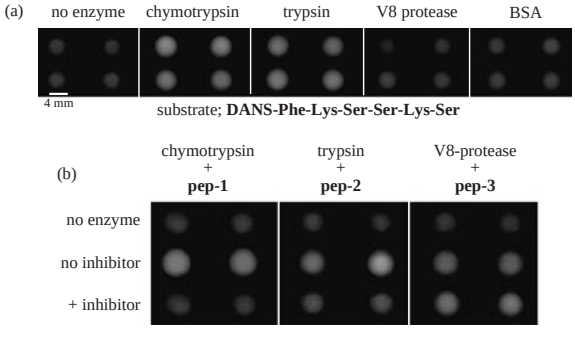


Figure 3. a) Fluorescent enzyme activity assay using a MmA hydrogel-based peptide chip. Conditions: [enzyme or protein] = 10 μM, [**pep-1**] = 100 μM, [agarose] = 0.25 wt %, [triton X-100] = 5.0 mM in 50 mM tris buffer (pH 8.0) containing 100 mM $CaCl_2$ at room temperature. b) Assay of enzyme inhibitors using a MmA hydrogel-based protein chip. Conditions: [enzyme] = 10 μM, [BBI] = 10 μM, [substrate] = 100 μM, [agarose] = 0.25 wt %, [triton X-100] = 5.0 mM in 50 mM tris buffer (pH 8.0) containing 100 mM $CaCl_2$ at room temperature.

The present semi-wet protein array was also applicable to the enzyme inhibitor assay. Bowman-Birk inhibitor (BBI)[9] is a well-known double-headed trypsin/chymotrypsin inhibitor that forms a 1:1 complex with ether trypsin ($K_i = 5.6 \times 10^7\ M^{-1}$) or chymotrypsin ($K_i = 5.0 \times 10^7\ M^{-1}$). BBI (1 μL) was added MmA gel spots containing chymotrypsin, trypsin, or V8 protease, and subsequently, the corresponding substrate was dropped on the hydrogel spots. As shown in Figure 3b, the addition of BBI hampered the color change of the spots containing chymotrypsin and trypsin, but not for the spot of V8 protease which is consistent with the selectivity of BBI. These results indicate that the potency and selectivity of inhibitors can be conveniently estimated using MmA-based protein array.

In conclusion, we succeeded to produce a simple semi-wet protein/peptide array that is constructed with a micelle-mixed agarose hydrogel. In the agarose hydrogel, triton X-100 forms the micelle which functioned as a sensing site for the enzyme activity. Since these two components, agarose, and triton X-100, are commercially available, the present simple system may facilitate a practical application of the semi-wet protein/peptide array.

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