Exploring enzymatic catalysis at a solid surface: a case study with transglutaminase-mediated protein immobilization

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The factors affecting enzymatic protein immobilization with microbial transglutaminase (MTG) were explored. As model proteins, enhanced green fluorescent protein (EGFP) and glutathione *S*-transferase (GST) were chosen and tagged with a neutral Gln-donor substrate peptide for MTG (Leu-Leu-Gln-Gly, LLQG-tag) at their C-terminus. To create a specific surface, displaying reactive Lys residues, to be cross-linked with the Gln residue in the LLQG-tag of target proteins by MTG catalysis, a polystyrene surface was physically coated with β-casein. Both recombinant proteins were immobilized onto the β-casein-coated surface only in the presence of active MTG, indicating that those proteins were enzymatically immobilized to the surface. MTG-mediated protein immobilization markedly depends on the pH and ionic strength of the reaction media. The optimal pH range of MTG-mediated immobilization of both recombinant proteins was around 5, at which point the MTG-catalyzed reaction in aqueous solution is not normally preferred. By utilizing a pH-dependent change in EGFP fluorescence, we found that the apparent pH at the surface is likely to be lower than bulk pH, this difference is not attributed to an optimal pH shift in MTG-mediated immobilization. On the other hand, lower yields of protein immobilization at higher ionic strength suggest that electrostatic interaction is a key factor governing MTG catalysis at a solid surface. The results of this study indicate that, in enzymatic catalysis at a solid surface, the concentration of substrates at the surface can enhance the catalytic efficiency, and this could alter the pH dependence of enzymatic catalysis.

Introduction

The immobilization of functional proteins is a key issue in a wide range of protein applications. In particular, protein microarrays have recently become a focus of proteomics, drug discovery and diagnostics due to their potential utility in the rapid screening of thousands of molecular events in a single experiment, which facilitates the study of protein–protein, antigen–antibody, protein–DNA, and protein–small molecule interactions. However, how to maintain the conformation and biological activity of immobilized proteins is a major concern of protein immobilization technology.[1]

Most available immobilization methods for proteins involve the physical adsorption of proteins through electrostatic and hydrophobic interactions or chemical coupling of groups within proteins (carboxyl or amino groups) to surfaces containing reactive groups.[2] In both cases, the orientation of protein molecules immobilized to solid surfaces should be random, and proteins are likely to be partly denatured. To solve these problems, a variety of alternative strategies have been developed. One approach is noncovalent and site-specific immobilization, which can be achieved by the conjugation of an affinity peptide tag, such as the hexahistidine tag (His-tag), to target proteins in order to fuse proteins onto a Ni–NTA-functionalized surface by metal–chelate interactions,[3] biotinylated proteins onto an avidin-coated surface by avidin–biotin interactions,[4] epitope-tagged fusion proteins onto antibody-immobilized surface by antigen–antibody interactions,[5] and a unique peptide tag for the parallel heterodimeric leucine zipper system.[6] However, the specific interactions in these cases are reversible in nature and, thus, are not stable enough over the course of subsequent assays.[1] Other methods introduce reactive peptide tags to effect covalent and site-specific immobilization. For example, azidolated proteins immobilized by Staudinger ligation,[7] hAGT (O^6-alkylguanine DNA alkyltransferase) fusion proteins immobilized onto benzylguanine-displaying surfaces[8] and intein-tagged proteins immobilized using protein splicing[9] have been reported. Although these methods have been shown to be capable of protein immobilization, a relatively large peptide/protein tag and the preparation of specifically modified solid surfaces are required. These issues led us to the development of a simple and efficient method for covalent and site-specific protein immobilization.

To this end, we have focused on the capability of microbial transglutaminase (MTG) in protein cross-linking reactions. Transglutaminase is an enzyme that catalyzes an acyl transfer reaction between the γ-carboxyamide group of peptide-bound Gln residue (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine, with the loss of ammonia.[10] We have recently demonstrated the potential of MTG in the covalent and site-specific immobilization of proteins onto casein adsorbed on a polystyrene surface.[11] However, a quantitative evaluation of enzymatic protein immobilization was not satisfactorily conducted. Indirect estimation of protein

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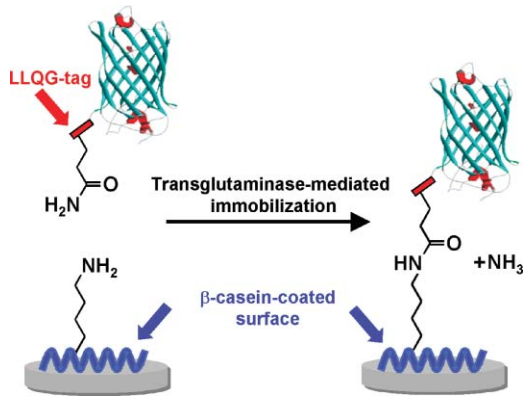
loading onto the surface, on the basis of the catalytic activity of immobilized enzymes, suggested a maximum loading of approx. 35 ng per well.

In this study, we investigated the factors affecting enzymatic protein immobilization in detail. Our goal was thus to understand better the catalytic properties of MTG when applied to protein immobilization, which leads us to a potent alternative route for the design and preparation of new functional biomaterials. For this reason, we prepared two recombinant proteins with similar isoelectric points bearing a neutral Gln-donor substrate peptide at their C-termini. One of them was enhanced green fluorescent protein (EGFP), which facilitated the quantitative evaluation of the present immobilization method. For a solid surface with reactive sites for MTG catalysis, we chose a polystyrene plate physically coated with β-casein possessing reactive Lys residues for MTG.[12] Using β-casein-coated plates that effectively block the non-specific adsorption of proteins while allowing the MTG-mediated immobilization of target proteins, the unique aspects of enzymatic catalysis at a solid surface were investigated (Scheme 1).



Scheme 1 Covalent and site-specific immobilization of proteins onto a β-casein-coated surface through MTG-mediated crosslinking reaction.

Results and discussion

Design of recombinant proteins

We selected EGFP and glutathione *S*-transferase (GST) as model proteins, both of which have been employed frequently in basic studies on protein immobilization.[7–9] These proteins were expressed in *Escherichia coli* as recombinant proteins tagged with a Gln donor substrate peptide (Leu-Leu-Gln-Gly = LLQG-tag) at the C-termini. The peptide sequence of Leu-Leu-Gln-Gly is known to be a good substrate sequence for MTG.[13]

In the EGFP construct, a flexible linker (Gly-Gly-Gly-Ser) was introduced between the protein and the LLQG-tag for the relaxation of steric hindrance upon the binding of MTG to the tag.[11b] It was found that the C-terminal Lys residue (K238) in the wild-type sequence of EGFP turned out to be recognized by MTG upon the extension of the amino acid sequence at the C-terminus (data not shown). Therefore, K238 was substituted to Arg by site-directed mutagenesis (Table 1).

With respect to GST, we found that one out of five Gln residues in the wild-type sequence of GST was recognized by MTG. From an inspection of the tertiary structure of GST,[14] Q207 (the accessible surface area, which scores the highest value of all Gln residues) is likely to be the target site of MTG. Indeed, we confirmed that substitution of Q207 to Ala makes GST an inert substrate for MTG (data not shown). Since the labelling of the extended peptide tag at the C-terminus of GST by small organic molecules could affect substrate recognition,[15] we decided to attach the LLQG-tag directly to the C-terminus of GST to minimize structural perturbation.

Table 1 Amino acid sequences of the recombinant proteins prepared in this study[a]

Protein	Amino acid sequences of N- and C-terminal regions
Wild–type EGFP	MHHHHHHMVSKG–DELY**K**
CQ-EGFP	MHHHHHHMVSKG–DELY**R**GGGS*LLQG*
Wild-type GST	MSPIL–GW**Q**AT–DHPPK
Q207A GST	MHHHHHHGSMSPIL–GWAAT–DHPPK
CQ-GST	MHHHHHHGSMSPIL–GWAAT–DHPPK*LLQG*

[a] Amino acid sequences of N- and C-terminal regions are shown for each protein, with one-letter codes. The substrate peptide sequence for MTG is in italics. Amino acids substituted by site-directed mutagenesis are shown in bold letters. The omitted regions have the same sequence as wild-type for both EGFP and GST.

Finally, we confirmed that neither recombinant protein suffered from a significant loss of native function upon the incorporation of peptide tags at the C-terminus. The resultant recombinant proteins were abbreviated as CQ-EGFP and CQ-GST, respectively (Table 1).

Immobilization of CQ-EGFP and CQ-GST onto a β-casein-coated polystyrene surface

To construct a reactive surface displaying Lys residues for the enzymatic immobilization of recombinant proteins bearing a Gln-donor substrate peptide for MTG, we employed β-casein, because it is a good MTG substrate and substantially prevents non-specific protein adsorption when adsorbed on a plate surface, while providing reactive sites for MTG catalysis.[11a] In fact, casein has been widely employed as a surface-blocking reagent due to its amphiphilic nature, which provides stable protein matrices on a solid surface.

Fig. 1a shows the fluorescence intensity of CQ-EGFP detected on a β-casein-coated plate after immobilization procedures. One

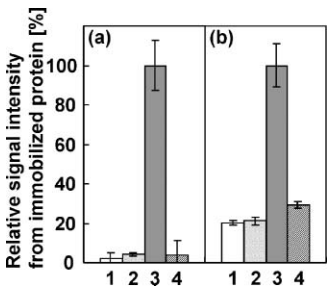


Fig. 1 Immobilization of CQ-EGFP and CQ-GST onto a β-casein-coated surface. The *y*-axis shows the relative signal intensity derived from the proteins immobilized in 50 mM sodium acetate buffer, pH 5, (a) CQ-EGFP and (b) CQ-GST. The maximum signal for each condition was taken as 100%. (Lane 1: LLQG-tagged protein only, lane 2: LLQG-tagged protein + inactivated MTG, lane 3: LLQG-tagged protein + MTG, lane 4: wild-type EGFP (a) or Q207A GST (b) + MTG.)

can see that non-specific adsorption of CQ-EGFP was negligible (lane 1 in Fig. 1a). Incubation with inactivated MTG also provided no significant fluorescence on the surface (lane 2 in Fig. 1a). By contrast, incubation of CQ-EGFP with MTG resulted in an over 20-fold increase in fluorescence compared with control experiments (lane 3 in Fig. 1a), suggesting that the immobilization of CQ-EGFP had, in effect, proceeded enzymatically. Moreover, incubation of wild-type EGFP with MTG resulted in a comparable signal with non-specific adsorption (lane 4 in Fig. 1a). These results indicate that the immobilization of CQ-EGFP is directed through the LLQG-tag. On the other hand, CQ-GST seems to be physically absorbed onto the surface to some extent (lanes 1 and 2 in Fig. 1b). However, the level of CQ-GST activity detected on the plate in the presence of MTG was about 5-fold that of the control experiments, suggesting LLQG-tag-specific immobilization of CQ-GST (lane 3 in Fig. 1b). Since the isoelectric points (pIs) of CQ-EGFP (5.67,[16]) and CQ-GST (6.09,[16]) are comparable, the difference in the non-specific adsorption of these recombinant proteins might not be attributable to electrostatic interaction.

To test the stability of β-casein matrices for protein immobilization, changes in the fluorescence of immobilized CQ-EGFP were followed during repeated washing of the wells. Fig. 2 depicts the number of washing cycles in which the plate was washed three times each with PBST and 1 M NaCl per cycle. As shown in the figure, the fluorescence of immobilized CQ-EGFP was retained perfectly within the experimental errors, even after repeated washing (30 times in total). The results show that β-casein adsorbed on the polystyrene surface can work as a stable matrix for MTG-mediated covalent protein immobilization.

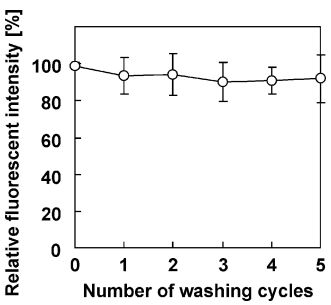


Fig. 2 Stability of immobilized CQ-EGFP during repeated washing. The original fluorescent intensity of immobilized CQ-EGFP was taken as 100%. Each washing cycle involves PBST × 3 and 1 M NaCl × 3.

Effect of pH on CQ-EGFP immobilization

As MTG is an enzyme, solution pH should affect the protein immobilization process. To attain the highest loading on the surface, we checked the pH dependence of MTG-mediated immobilization using CQ-EGFP as a model. Fig. 3a shows a fluorescent image of immobilized CQ-EGFP on the plate. In the absence of MTG, little fluorescence was detected, indicating that non-specific absorption was negligible at all pH values examined. On the other hand, in the presence of MTG, EGFP fluorescence was evident and, interestingly, the efficiency of the immobilization was seen to be markedly dependent on pH. Quantitative evaluation of fluorescent intensity on the plate (Fig. 3b) shows that the optimal pH for CQ-EGFP immobilization is around 5. These results confused us

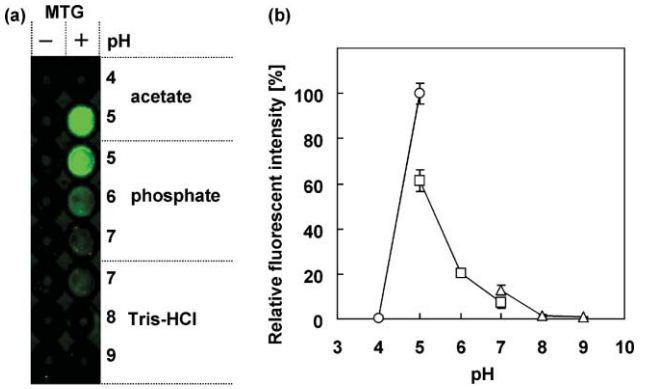


Fig. 3 Effect of pH on MTG-mediated immobilization of CQ-EGFP. (a) Fluorescent image of immobilized CQ-EGFP in the presence (+) and the absence (–) of MTG. (b) Effect of pH on the relative fluorescence of immobilized CQ-EGFP: (○) sodium acetate buffer, (□) potassium phosphate buffer, (△) Tris-HCl buffer. The maximum signal was normalized to 100%. The error bars represent the standard deviation ($n = 3$).

because the optimal pH range of MTG catalysis in solution was reported to be around neutral pH (6 to 7) when hydroxylamine and Z-Gln-Gly were used as substrates.[17] To gain further insight into this phenomenon, we conducted a direct comparison of MTG catalysis in solution with that at the interface, towards the same proteinaceous substrate, CQ-GST.

Comparison of pH dependence of MTG catalysis at a solid surface with that in aqueous solution

We first examined the pH dependence of MTG catalysis in solution by the labelling of CQ-GST with monodansyl cadaverine (MDC). We observed a time-dependent increase in fluorescence due to the labelling of the Gln residue of the LLQG-tag with MDC, by which the local hydrophobicity around the chromophore (dansyl group) is increased by the adjacent Leu residue. Fig. 4a shows the pH–activity profile of MTG-mediated incorporation of MDC into CQ-GST. As a result, the optimal pH was found to be around 8. On the other hand, we found that the optimal pH for the immobilization of CQ-GST is around pH 5 (Fig. 4b), showing good agreement with the pH dependence of CQ-EGFP immobilization (Fig. 3b). The relatively high immobilization efficiency of CQ-GST at pH 6 in comparison with that of CQ-EGFP can be attributed to the difference in pI values, at which point the surface charge of CQ-GST should be close to neutral. The observed discrepancy in optimal pH of MTG catalysis was likely to be derived from the intrinsic characteristics of enzymatic catalysis at the surface. We first assumed that this unique catalytic behavior of MTG is due to a change in the apparent pH at the surface away from that of the bulk solution. To test this idea, we designed the following experiments.

Evaluation of pH at β-casein-coated polystyrene surface by the pH-dependent change in EGFP fluorescence

It is known that the fluorescence intensity of EGFP is pH-dependent.[18] Therefore, immobilized EGFP itself can serve as a pH indicator for estimating local pH at the surface. We immobilized CQ-EGFP onto a β-casein-coated surface under the optimal conditions in Fig. 3 (50 mM sodium acetate buffer, pH 5),

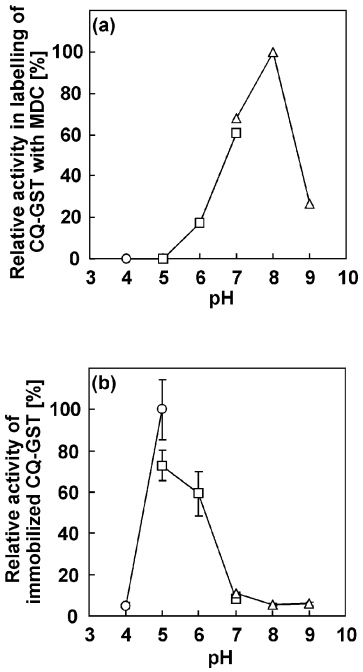


Fig. 4 Effect of pH on the MTG-mediated reactions of CQ-GST. (a) Effect of pH on the relative activity of incorporation of MDC into CQ-GST. (b) Effect of pH on the relative activity of immobilized CQ-GST: (○) sodium acetate buffer, (□) potassium phosphate buffer, (△) Tris–HCl buffer. The maximum activity for each condition was taken as 100%.

then washed out the buffer and measured the fluorescence of immobilized CQ-EGFP by changing the pH of the medium. The results were compared with the pH-dependent fluorescent change of free CQ-EGFP using the same buffered solutions.

Fig. 5 shows the relative fluorescence of free and immobilized CQ-EGFP at different bulk pH. The fluorescence of free CQ-EGFP is comparable to that of wild-type EGFP over the corresponding pH range.[18] On the other hand, the pH–fluorescence profile was shifted toward a higher pH in the case of immobilized EGFP. For instance, at pH 6, the fluorescence of free CQ-EGFP reaches maximum whereas that of immobilized CQ-EGFP is only 40% of the maximum intensity. These results indicate that the apparent pH at the solid surface is lower than that of the bulk solution, resulting in a shift of the pH–fluorescence signal profile toward a higher pH. A similar apparent shift of pH to

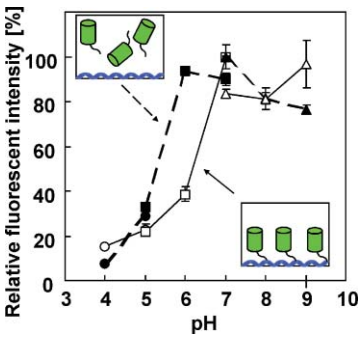


Fig. 5 Effect of pH on the fluorescence of free CQ-EGFP (filled keys) and immobilized CQ-EGFP (open keys) in (●, ○) sodium acetate buffer, (■, □) potassium phosphate buffer; (▲, △) Tris–HCl buffer. The maximum fluorescence for each condition was normalized to 100%.

higher values was observed when EGFP was placed in reverse micelles comprised of an anionic surfactant. This apparent shift was explained by the potential field of the negatively charged surfactant, which attracts protons.[19] In the present case, the surface would also be negatively charged at pH values above 5, due to the isoelectric point (pI) of β-casein (*ca.* 5), possibly accounting for the upward shift in optimal pH. However, this observation cannot be correlated with the downward shift in the optimal pH for enzymatic immobilization because, in this context, the apparent pH at the solid surface in a pH 5 buffered solution is likely to be lower than pH 5, at which point MTG completely loses its catalytic activity.

Effect of ionic strength on MTG-mediated protein immobilization

Next, we focused on the local concentration of substrates at the surface. In the final rate-limiting step of MTG-catalyzed protein immobilization, the acyl–enzyme intermediate (*i.e.* MTG–CQ-EGFP/GST complex) should contact reactive sites (*i.e.* Lys residues in adsorbed β-casein) on the surface. Given that the macromolecular interaction was affected by electrostatic interaction, CQ-EGFP and CQ-GST were immobilized at different ionic strengths—the relative signal intensities from these are shown in Fig. 6. It was found that the higher the salt concentration, the lower the immobilization yields are in both cases. The fact that high ionic strength hinders the immobilization reaction suggests that electrostatic interactions among protein components participating in the reaction are crucial for MTG-mediated protein immobilization. With respect to CQ-GST, the effect of ionic strength was not significant, implying that electrostatic interaction is not the only factor that controls its immobilization. In fact, non-specific physical adsorption of CQ-GST was evident in Fig. 1b, which could result in a lower sensitivity of CQ-GST immobilization to the ionic strength of the bulk solution.

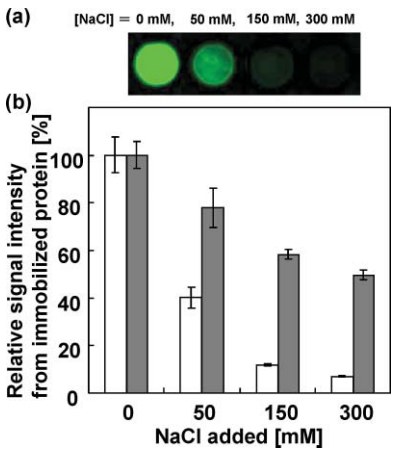


Fig. 6 Effect of salt concentration on the MTG-mediated immobilization of CQ-EGFP. (a) Fluorescent image of CQ-EGFP immobilized with MTG at different ionic strengths. (b) Relative signal intensity obtained from immobilized CQ-EGFP (open bars) and CQ-GST (filled bars). The maximum signal was normalized to 100%.

Taking electrostatic interactions into consideration, we reconsidered the results of Fig. 3b and 4b. The quite-similar pH dependence of immobilization of CQ-EGFP and CQ-GST (the amino acid sequences of which are significantly different) could

be attributed to their similar charge properties. As MTG (pI of 8.9[17]) is positively charged below pH 7, at which point enzymatic protein immobilization was observed, here we focused solely on substrate proteins. As the LLQG-tag is neutral, it is reasonable to assume that the pIs of both recombinant proteins are consistent with the wild-type proteins. At pH 7, EGFP, GST and β-casein (pI of 4.6–5.1[20]) are all negatively charged. Hence, the electrostatic interaction between EGFP (or GST) and β-casein is repulsive. Lowering the bulk pH to 5, the repulsive interaction should be significantly diminished. This should increase the local concentration of substrates at the surface, resulting in higher immobilization yields. At pH 4, enzymatic immobilization did not occur because MTG is considered to be inactive. As a result, the pH dependence of MTG catalysis in protein immobilization differed from that observed in aqueous solution, implying some unique aspects of enzymatic catalysis at a solid surface.[21]

Finally, we attempted to estimate the protein loading on the casein-coated surface by virtue of the fluorescence of EGFP under the optimized conditions in this study. On the basis of the calibration curve generated with free CQ-EGFP, the highest protein loading onto the β-casein-adsorbed polystyrene surface attained in 5 mM sodium acetate buffer (pH 5) (the fluorescent image at the far left in Fig. 6a) was estimated to be 240 ± 20 ng per well (Fig. 7). Given that the solution structure of EGFP is like a cylinder with a diameter of 3 nm and a height of 5 nm,[22] we calculated the surface coverage of immobilized CQ-EGFP as it stands perpendicular to the surface. The maximum surface area that can be adsorbed by β-casein under the present experimental conditions was calculated to be 0.96 cm^2 per well (100 μl of a β-casein solution per 0.7 cm diameter well). To this surface, *ca.* 8.9×10^{-12} mol per well of CQ-EGFP molecules was immobilized, which corresponds to *ca.* 0.38 cm^2 per well. Therefore, the surface coverage by CQ-EGFP was calculated to be 40%, although a better method for accurate estimation of the protein loading should be designed. According to technical information from the manufacturer, the maximum protein loading onto the polystyrene plate is *ca.* 650 ng cm^{-2}, when immunoglobulin G was employed by physical adsorption.[23] In our case, *ca.* 250 ng cm^{-2} of a recombinant protein was site-specifically immobilized by MTG with the above estimation.

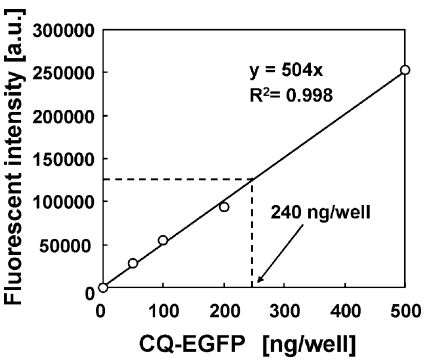


Fig. 7 Estimation of protein loading onto a casein-coated polystyrene surface based on the calibration curve constructed with free CQ-EGFP. The horizontal broken line indicates the fluorescence intensity derived from immobilized CQ-EGFP.

Conclusions

The new enzymatic protein immobilization method presented here has several advantages over conventional immobilization techniques. Firstly, the experimental procedure is quite simple because this method only requires inexpensive proteinaceous adsorbents, such as casein, and the attachment of a substrate peptide for MTG to target proteins by standard genetic manipulation. Secondly, the size of the peptide tag required for protein immobilization is quite small. In comparison with other peptide and protein tags for protein immobilization [such as the intein tag (*ca.* 450 amino acids), the hAGT tag (207 amino acids) and the leucine zipper tag (43 amino acids)], the LLQG-tag, which comprises 4 amino acids, works well for protein immobilization. Thirdly, as an enzyme mediates the immobilization, the immobilization process can be made highly specific under mild conditions. These features are attractive for the fabrication of solid protein formulations such as protein microarrays and immobilized enzymes; however, further improvements must be achieved, especially in terms of immobilization yields. Inspired by the marked contribution of the electrostatic interactions of protein components at a solid surface, we are currently designing a new surface for MTG-mediated protein immobilization.

Experimental

Materials

MTG was provided by Ajinomoto Co., Inc. (Japan). The EGFP and GST genes were cloned from pET32-b562-EGFP[24] and pGEX6P-3 (GE Healthcare Bio-Science Co., USA), respectively, as template plasmids. A 96-well polystyrene plate (Nunc Maxi Sorp) was purchased from Nalge Nunc (Denmark). Bovine β-casein sodium salt was purchased from Sigma-Aldrich (USA). All other chemicals were of commercially available analytical grade.

Genetic manipulation

Recombinant proteins were constructed with a C-terminal extension of a Gln-donor substrate peptide tag (Leu-Leu-Gln-Gly = LLQG-tag). The resultant recombinant proteins were abbreviated as CQ-EGFP and CQ-GST. To construct CQ-EGFP and CQ-GST, EGFP and GST, gene fragments were amplified by the polymerase chain reaction (PCR) from pET32-b562-EGFP and pGEX6P-3, respectively, and cloned into the expression vector pET22b(+) (Novagen, USA). PCR amplification of CQ-EGFP gene fragments utilized the upstream primer (5′-G GAA TTC CAT ATG CAC CAC CAC CAC CAC CAC ATG GTG AGC AAG GGC GAG G-3′) containing a *Nde* I site with a translation initiation codon (ATG) and hexahistidine tag for purification, and the downstream primer (5′-CGG GGT ACC GCG GCC GCT TTA TCA ACC CTG CAG CAG GGA ACC ACC ACC CTT GTA CAG CTC G-3′) containing a *Not* I site. The PCR product was double digested with *Nde* I and *Not* I, and cloned into the pET22b(+) vector, *via Nde* I and *Not* I sites, to yield pET22b-CQ-EGFP. PCR amplification of CQ-GST gene fragments utilized the upstream primer (5′-CGG GAA TTC CAT ATG CAC CAC CAC CAC CAC CAC GGA TC-3′) containing a *Nde* I site with a translation initiation codon (ATG), and the downstream primer (5′-G CCC AAG CTT TCA ACC CTG CAG TTT TGG AGG

ATG GTC GCC AC-3′) containing a *Hind* III site. The PCR product was double digested with *Nde* I and *Hind* III, and cloned into the pET22b(+) vector, *via Nde* I and *Hind* III sites, to yield pET22b-CQ-GST.

Expression and purification of recombinant proteins

Recombinant plasmids were transformed into *E. coli* BL21 (Novagen, USA), and transformants were grown in Luria–Bertani (LB) medium supplemented with 100 μg ml^{-1} ampicillin at 37 °C in a shaker at 200 rpm. After reaching an OD600 of ~0.6, protein expression was induced with 0.5 mM isopropyl thiogalactosidase (IPTG) and the culture was incubated overnight at room temperature (*ca.* 25 °C). Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl and 1 mM EDTA) and lysed by sonication on ice. The cell debris was pelleted by centrifugation and the supernatants were prepared for protein purification. The supernatant obtained for CQ-EGFP with an N-terminal hexahistidine tag was purified by Ni–NTA spin columns (GE Healthcare Bio-Science Co., USA). The supernatant obtained for CQ-GST was purified by glutathione columns (GE Healthcare Bio-Science Co., USA). The purified proteins were employed as CQ-EGFP and CQ-GST.

Physical adsorption of β-casein onto a polystyrene plate

An aqueous solution of β-casein (5 mg mL^{-1}) was prepared in 50 mM Tris-HCl buffer (pH 7). One hundred microliters of the β-casein solution was added to each well of a 96-well polystyrene plate, which was then incubated at 4 °C for 16 h. Wells were washed with PBST [phosphate buffer saline plus 0.05% (v/v) Tween20] three times, to wash out the residual soluble β-casein fraction. The surface-treated plate was employed as the β-casein-coated surface.

Immobilization of CQ-EGFP and CQ-GST onto β-casein-coated surface

A typical procedure for the immobilization of CQ-EGFP and CQ-GST onto β-casein-coated plates was carried out as follows. Reaction mixture solutions of which total reaction volume 100 μl per well were prepared for CQ-EGFP (10 μg per well) or CQ-GST (10 μg per well) and MTG (0.042 U per well) in 50 mM sodium acetate buffer (pH 5), and added in each well of the β-casein-coated plate. Enzymatic immobilization was conducted at *ca.* 25 °C for 2 h. As controls, physical adsorption of CQ-EGFP and CQ-GST was investigated in the absence of MTG and in the presence of thermally-inactivated MTG under the same conditions. Inactivated MTG was prepared by heating the aqueous solution of MTG at 95 °C for 1 h. After these enzymatic and physical immobilization procedures, the wells were washed with PBST three times before the fluorescence or enzymatic activity measurements.

Effect of pH on MTG-mediated protein immobilization

The immobilization of CQ-EGFP and CQ-GST onto the β-casein-coated plates was conducted in different pH conditions. Reaction mixture solutions were prepared for CQ-EGFP (10 μg per well) or CQ-GST (10 μg per well) and MTG (0.042 U per well) in 50 mM buffered solutions: sodium acetate buffer (pH 4, 5), potassium phosphate buffer (pH 5, 6, 7), and Tris-HCl buffer (pH 7, 8, 9). Enzymatic immobilization was conducted in each well at room temperature (*ca.* 25 °C) for 2 h. After incubation, the wells were washed with PBST three times before the measurement of protein function.

Effect of ionic strength on MTG-mediated protein immobilization

The immobilization of CQ-EGFP and CQ-GST onto the β-casein-coated plates was carried out under different salt concentrations. Reaction mixture solutions were prepared for CQ-EGFP (10 μg per well) or CQ-GST (10 μg per well) and MTG (0.042 U per well) in 5 mM acetate buffer (pH 5.0) plus 50, 150 and 300 mM NaCl. After enzymatic immobilization at room temperature (*ca.* 25 °C) for 2 h, the wells were washed with PBST three times, then the fluorescence or enzymatic activity in each well was measured.

Measurement of fluorescent image and intensity of immobilized EGFP

Fifty mM Tris-HCl buffer (pH 7.0, 100 μL) was added to each well of the EGFP-immobilized plate. The fluorescence intensity was measured at 25 °C, at the excitation wavelength of 488 nm with a 530 nm band pass filter using a Molecular Imager FX Pro (Bio-Rad Inc., USA). The background signal from non-modified β-casein-coated plates was subtracted from the raw data, to evaluate the fluorescence signal solely due to the immobilized CQ-EGFP.

Measurement of enzymatic activity of immobilized GST

The activity of immobilized GST was measured at 25 °C using 1 mM GSH and 1 mM 1-chloro-2,4,-dinitrobenzene (CDNB) dissolved in 100 mM potassium phosphate buffer (pH 6.5) as the substrate. The substrate solution (100 μL) was added to each well of the GST-immobilized plate to initiate the enzymatic reaction. Product formation was followed at 340 nm and ΔA_{340} was measured for 5 min using a Power Wave X (Bio-Tek Instrument Inc., USA).

Measurement of the catalytic activity of MTG in aqueous solution at different pH values

Reaction mixture solutions were prepared for CQ-GST (50 μg mL^{-1}), monodansylcadaverine (MDC) (20 μM) and MTG (0.84 U mL^{-1}) in various 50 mM buffers: sodium acetate buffer (pH 4, 5), potassium phosphate buffer (pH 5, 6, 7), Tris–HCl buffer (pH 7, 8, 9). The incorporation of MDC into CQ-GST was followed by the change in fluorescence intensity with excitation and emission wavelengths set at 340 and 550 nm, respectively, for 30 min at 25 °C.

Fluorescence measurements of immobilized CQ-EGFP and soluble CQ-EGFP

CQ-EGFP was immobilized onto β-casein-coated plates in 50 mM sodium acetate buffer (pH 5) at *ca.* 25 °C overnight. After washing wells with PBST three times, 50 mM buffered aqueous solutions with different pH (see effect of pH on MTG-mediated protein immobilization) were placed in each well and the fluorescence of EGFP was measured. In the case of soluble CQ-EGFP, CQ-EGFP aqueous solutions (1 μg mL^{-1}) were prepared using the same-buffered solution. Solutions (100 μL) were placed in each well of a

β-casein coated plate, and the fluorescence of free CQ-EGFP was measured. The amount of immobilized EGFP was estimated by the calibration curve constructed from 100 μL of pH 7 phosphate buffered solution of CQ-EGFP with different concentrations. The linearity range was 50–500 ng per well.

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