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Activity control of autodisplayed proteins on the same outer membrane layer of *E. coli* by using Z-domain/streptavidin/ and lipase/foldase systems

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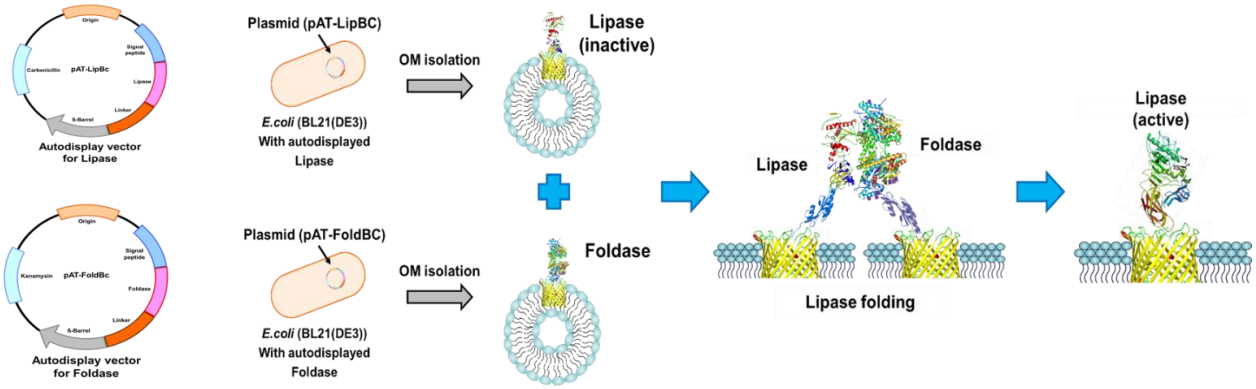
Graphic abstract



Highlights

- ㅇ Outer membrane (OM) fractions were isolated after autodisplay of model proteins on OM of *E. coli*.
- ㅇ OM Layer were made by mixing OM fractions of two kinds of autodisplayed proteins with controlled ratio.
- ㅇ Two model protein-pairs were selected to be Z-domain-streptavidin and lipase-foldase.
- ㅇ Each protein on OM layer were determined to have relative activity according to the mixing ratio.
- ㅇ Activity of lipase could be optimized according to the mixing ratio of foldase in OM layer.

Abstract

The autodisplay technology has been applied for expression of a desired protein on the outer membrane (OM) of *Escherichia coli*. In this work, the OM fractions of *E. coli* with two autodisplayed proteins were separately prepared and mixed to demonstrate the feasibility of control over the ratio of two autodisplayed proteins. As the first model, Z-domain and streptavidin were autodisplayed, and their activities were tested by means of the combined OM layer in a 96-well microplate and a surface plasmon resonance (SPR) biosensor. As the second model, lipase and foldase were autodisplayed which required an interaction between two proteins to obtain the activity of lipase. The OM fractions of *E. coli* with an autodisplayed lipase and foldase were separately prepared and mixed to demonstrate the feasibility of control over the ratio of two autodisplayed proteins when the interaction of two proteins is required within the same OM layer for the activity of the lipase.

Keywords: autodisplay, co-transfection, Z-domain, streptavidin, lipase, foldase

1. Introduction

The autodisplay technology is applied for expression of a target protein on the outer membrane (OM) of *Escherichia coli*, and various desired proteins have been

expressed successfully, e.g., as a fusion protein of a β-barrel of AIDA-1 [1-4]. Owing to the high efficiency of expression (10^5 protein molecules per *E. coli* cell), many target proteins have been successfully expressed by means of the autodisplay technology, such as Z-domain [5-6], streptavidin [7], adrenodoxin [8], casein [9-10], and Ro protein/SSA [11-12]. In particular, the autodisplayed Z-domain and streptavidin have been often used for improvement of immunoassay sensitivity. For the application to immunoassays, whole *E. coli* cells with autodisplayed proteins can be used [13-15], and the OM fraction of *E. coli* with autodisplayed proteins can be isolated and layered again on 96-well microplates, metal surface of sensors, or microbeads [16-18].

Recently, we reported that the simultaneous expression of two proteins is possible by means of the autodisplay technology. When Z-domain and casein were simultaneously autodisplayed by co-transfection of two autodisplay vectors, the two proteins were found to be autodisplayed in the ratio of 1:1 on the OM of *E. coli* [19]. However, the ratio of two autodisplayed proteins on the OM of *E. coli* was found to vary depending on the kind of the autodisplayed proteins. As a method to control the ratio of two autodisplayed proteins in the OM layer, OM fractions of *E. coli* with two autodisplayed proteins were separately prepared and mixed in various ratios of the two autodisplayed proteins, as shown in Fig. 1(a). In the present work, Z-domain and streptavidin were selected as model proteins, and their activities were assayed by means of the OM layer in a 96-well microplate, using a surface plasmon resonance (SPR) biosensor. These experiments were designed to

show that the activities of proteins in the OM layer resulting from mixing of the OM fractions are quantitatively in proportion to the mixing ratio of the OM fractions containing the autodisplayed Z-domain and streptavidin.

The control over the protein ratio in the OM layer was also demonstrated for two autodisplayed proteins that require an interaction for their activities. For example, lipases from *Burkholderia* and *Pseudomonas* species were reported to require an additional enzyme called lipase-specific foldase (Lif), which can fold the lipase into an active conformation [20-21]. Recently, the active lipase from *Burkholderia* was obtained via coautodisplay of the lipase and a Lif on the same *E. coli* cell, as shown in Fig. 1(b) [22]. The activity of the lipase was ensured by the mobility of the β-barrel serving as an anchor for autodisplay of the lipase and foldase within the OM. owing to such phenomena as dimerization or multimerization of subunits, active or functional enzymes were assembled on the surface of *E. coli,* even when they were expressed as monomers [23-26]. In the present study, the OM fractions of *E. coli* with the autodisplayed lipase and foldase were separately prepared and mixed in a controlled ratio of the two autodisplayed proteins to optimize the activity of the lipase, as shown in Fig. 1(c). The aim of these experiments was to show that (1) the activity of lipase in the OM layer could be achieved by mixing two OM fractions with separately autodisplayed lipase and foldase, and (2) the activity of the lipase in the OM layer can be controlled via the mixing ratio of the two OM fractions.

2. Materials and methods

2.1. Materials

Luria-Bertani (LB) broth was purchased from Duchefa (Haarlem, The Netherlands). Aprotinin was acquired from Roche Korea (Seoul, Korea). Triton X-100, phenylmethylsulfonyl fluoride (PMSF), carbenicillin, kanamycin, lysozyme, DNase, bovine serum albumin (BSA), C-reactive protein (CRP), and other reagents were purchased from Sigma-Aldrich Korea (Seoul, Korea). An allophycocyanin (APC)-labeled anti-human antibody (rabbit polyclonal), biotinylated atto-550, a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (rabbit polyclonal), anti-CRP antibody (rabbit polyclonal), biotinylated anti-HBsAg antibody (goat polyclonal), and a FITC-conjugated anti-CRP antibody (goat polyclonal) were purchased from Abcam Co. (Cambridge, UK).

2.2. Autodisplay of proteins

E. coli strains UT5600(DE3) [F−ara-14, leuB6, trpE38, secA6, lacY1, proC14, tsx-67, Δ(ompT-fepC)266, entA403, rfbD1, rpsL109(Str^R), xyl-5, mtl-1, thi-1, and λ(DE3)] and BL21(DE3) [B, F−dcm, lon, hsdS(rB^-mB^-), ompT, gal, λ(DE3)] were used to express autotransporter fusion proteins [22,28]. For co-expression of a lipase and foldase, BL21 (DE3) strain containing one of the autodisplay vectors, pAT-LipBc (encoding

lipase), was cultured to obtain electrocompetent cells. Another plasmid, pAT-FoldBc (encoding foldase), was then transfected into the electrocompetent cells by electroporation, generating strain BL21 (DE3) pAT-LiFoBc, whose name implies co-expression of the lipase and foldase. *E. coli* cells were grown at 37°C in LB broth containing carbenicillin (100 µg/ml), or kanamycin (50 µg/ml), or both antibiotics and supplemented with ethylene diaminetetraacetate (EDTA) and 10 mM β-mercaptoethanol. *E. coli* cells were grown until optical density at the wavelength of 600 nm (OD_{600}) reached 0.5. Protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For the autodisplay of Z-domain and streptavidin, plasmids pET-Z-18–3 and pST001 were transfected into the *E. coli* UT5600 (DE3) strain by electroporation. Bacteria were grown overnight at 37°C with vigorous shaking in the LB medium containing carbenicillin (50 µg/ml). Protein expression was induced by addition of 1 mM IPTG and subsequent incubation for 1 h at 30°C with vigorous shaking [1,5,13-14,19].

2.3. Preparation of the OM layer

After harvesting and washing of *E. coli* cells expressing the autodisplayed proteins by means of PBS, we used lysozyme (0.04 mg/mL) to prepare cell lysates. In addition, 1 M sucrose and 1 µM EDTA were added to the cell lysates, with subsequent incubation for 10 min. Sequentially, extraction buffer (2% Triton X-100, 50 mM Tris-HCl pH 8.0, and 10 mM $MgCl_2$) and 1 mg/ml DNase were also added to the cell lysates, followed by incubation for 25 min. The cell lysates were centrifuged at 5000

g for 5 min to remove large cell debris. After centrifugation, the supernatants were transferred to a new centrifugation tube. The supernatants corresponding to purified cell lysates were centrifuged at 35,000 g for 10 min in order to acquire membrane protein fraction. The resulting OM fraction was re-suspended in phosphate-buffered saline [17, 29].

2.4. Fluorescence imaging of Z-domain and streptavidin activity

To test the activity of mixed OM fractions from Z-domain-expressing *E. coli* and streptavidin-expressing *E. coli*, the OM fraction with autodisplayed streptavidin was mixed with the OM fraction containing autodisplayed Z-domain, in the mixing ratios of 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, or 0% (only the OM fraction with Z-domain). The total protein concentration in the OM fractions was adjusted to 300 μg/ml in PBS. For preparation of the OM layer on a silanized glass slide, each OM fraction was incubated for 2 h and then washed three times with PBS containing 0.1% of Tween 20. An allophycocyanin (APC)-labeled anti-human IgG antibody (20 μg/ml) and biotinylated atto-550 (20 μg/ml) were reacted with the OM layers coating the silanized slide glass. Fluorescent images were obtained and analyzed on a microarray scanner at 635 nm and 534 nm (GenePix 4100A).

2.5. Assay of Z-domain and streptavidin activity in a 96-well microplate

OM fractions (300 μg/ml) were applied to a 96-well microplate (for coating) with incubation for 2 h; then, the plate was washed three times with PBS containing 0.1% of Tween 20. Biotinylated HRP (or an anti-mouse IgG antibody) in the concentration range of 1–1000 ng/ml was added to each well of the microplate coated with the OM layers and was allowed to react for 1 h at room temperature. Finally, 3, 3′, 5, 5′-tetramethylbenzidine solution Pierce, NY, USA) was used in the chromogenic reaction. After quenching with 2 M sulfuric acid as a stop solution, the OD_{450} value was measured on a microplate reader (Molecular Devices, CA, USA) [14,30].

2.6. Assay of Z-domain and streptavidin activity with an SPR biosensor

An SPR biosensor with a lab-made SPR chip was used for assay of the autodisplayed Z-domain and streptavidin activity in the OM layer. The SPR chip was fabricated by sputtering an adhesive layer of titanium (2 nm) and then a layer of gold (48 nm) on BK-7 glass (10 × 10 mm^2). An SPR biosensor from KMac Co. (Yousung, Korea) was used, as previously described [5]. The sample solution and the washing buffer were injected into the sensor chip, using an injection valve and a peristaltic tubing pump. The flow rate was controlled (1 ml/min), and flow of the solution was stopped by regulation of the peristaltic tubing pump during the incubation step. For detection of activity of an OM protein by SPR signal measurement, OM fractions with autodisplayed streptavidin and Z-domain were injected into the SPR chip for preparation of the OM layer. Then, the SPR chip was washed three times with PBS containing 0.1% of Tween 20. Subsequently, the anti-CRP antibody (rabbit

polyclonal) and biotinylated anti-HBsAg antibody (goat polyclonal), at a concentration of 20 μg/ml, were injected for 1 min and incubated for 3 min. After washing, the SPR signal was calculated by comparison of signals before and after injection of the proteins [31-33]

2.7. Assay of lipase activity

Lipolytic activity of the lipase was quantified using whole *E. coli* cells, according to Winkler and Stuckmann's protocol, by means of the chromogenic substrate *p*-nitrophenylpalmitate (*p*-NPP) [22, 34]. Freshly prepared *E. coli* cells (10^9/ml) in potassium phosphate buffer (25 mM, pH 7.4) were washed three times with potassium phosphate buffer and kept in the same buffer at the concentration of OD_{578} = 10. For the lipase activity assay, the *p*-NPP substrate was prepared in ethanol at a final concentration of 7.9 mM and finally diluted with potassium phosphate buffer to a working concentration of 0.29 mM. The *p*-NPP solution was kept at 25°C for 1 h. For analysis of the lipase activity, a suspension of whole cells at OD_{578} = 10 (20 μl) was reacted with a 0.29 mM *p*-NPP solution in a 96-well microplate for 1 h. The lipase activity was determined by measuring the yellow color of the nitrophenylate ion at 405 nm on a microplate reader (Molecular Devices, CA, USA). To measure the lipase activity of mixed OM layers, OM fractions were prepared from *E. coli* BL21 (DE3) carrying pAT-LipBc (lipase) and *E. coli* BL21 (DE3) carrying pAT-FoldBc (foldase). Next, the two OM fractions were mixed physically in

the ratio of 1:3 to prepare the OM mixture. Three types of OM fractions at the concentration of 200 μg/ml (lipase only, foldase only, and a mixture of lipase and foldase) were used at 1 mg/ml to coat polystyrene microbeads (60 μl) for 2 h at room temperature. Subsequently, the microbeads coated with OM fractions were washed three times with a sufficient amount of potassium phosphate buffer. Next, OM-coated microbeads were reacted with the 0.29 mM *p*-NPP solution for 45 min. The lipase activity was measured as described above.

3. Results and discussion

3.1. OM layer of two autodisplayed proteins without interaction

When two proteins were simultaneously autodisplayed on the OM of *E. coli*, their ratio was nearly impossible to control. For control over the ratio of two autodisplayed proteins within the same OM layer, the OM layers of the two proteins were prepared by 1) isolation of the separate OM fractions of the two autodisplayed proteins, and then 2) mixing and layering the two OM fractions. Next, the activities of the two autodisplayed proteins in the OM layer were assayed to determine whether the activities were in the same ratio as the mixing ratio. In this work, two model proteins, Z-domain and streptavidin, were selected, as shown in Fig. 1(a). Since the autodisplayed Z-domain has binding affinity toward the F_c region of immunoglobulin G, and streptavidin binds to biotin, these two proteins have independent activities [5-7].

First, we tested the feasibility of control over the ratio of autodisplayed proteins in an OM fraction as a result of mixing the OM fractions from different *E. coli* strains. The OM fraction of *E. coli* with autodisplayed streptavidin was mixed with the OM fraction of intact *E. coli* in the ratio of 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, or 0% (only the OM fraction from intact *E. coli*). As shown in Fig. 2(a), intact *E. coli* showed no protein band with the molecular weight of streptavidin, and the intensity of protein bands of streptavidin (68.5 kDa) was found to gradually diminish in accordance with the mixing proportion of the OM fraction from intact *E. coli*. The relative amount of streptavidin was estimated by densitometry to be 100%, 63%, 44%, 27%, and 0% for the mixing ratios of 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, and 0% (only the OM fraction from intact *E. coli*), respectively. In the case of Z-domains, SDS-PAGE results showed that the protein band size of Z-domain (67 kDa) also diminished according to the mixing ratio; the OM fraction from intact *E. coli* did not yield any protein band with the molecular weight of Z-domain, as shown in Fig. 2(b). The relative amount of Z-domain was estimated by densitometry to be 100%, 77%, 54%, 42%, and 0% for the mixing ratio of 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, and 0% (only the OM fraction from intact *E. coli*), respectively. Then, the OM fractions from *E. coli* with autodisplayed Z-domain (68.5 kDa) and streptavidin (67 kDa) were mixed in different ratios: 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, or 0% (only the OM fraction with autodisplayed Z-domain). As shown in

Fig. 2(c), SDS-PAGE results revealed that the protein bands near the molecular weights of Z-domain (68.5 kDa) and streptavidin (67 kDa) in the aliquots used for mixing had similar intensity regardless of the aliquot, for each mixing ratio of OM fractions from *E. coli* with the two autodisplayed proteins. The relative amount of proteins (streptavidin and Z-domain) in the mixed ratio of OM fraction was estimated by densitometry to be 100%, 99%, 99%, 98%, and 96% for the aliquots used to achieve the mixing ratios of 100% (only the OM fraction with autodisplayed Z-domain), 75%, 50%, 25%, and 0% (only the OM fraction with autodisplayed streptavidin), respectively. These results implied that the OM fraction of autodisplayed proteins with the desired proportion of autodisplayed proteins could be prepared by mixing OM fractions from different *E. coli* strains.

Next, the activity of two autodisplayed proteins in the same OM layer, which was prepared by mixing two OM fractions in a desired ratio of autodisplayed Z-domain and streptavidin, was assayed in a reaction with fluorescently labeled ligands. As shown in Fig. 3(a), the OM layer was prepared by mixing two OM fractions in ratios of the two autodisplayed proteins, such as 100% (only the OM fraction from *E. coli* with autodisplayed streptavidin), 75%, 50%, 25%, and 0% (only the OM fraction from Z-domain). For the assay of activity of Z-domain, an anti-human IgG antibody labeled with APC (λ_{Ex} = 650 nm, λ_{Em} = 660 nm) was reacted with the OM layers with different proportions of Z-domain, and then fluorescence image analysis was performed to measure fluorescent signals for the labeled antibody. As shown in Fig. 3(b), the fluorescent signal increased in proportion with the amount of the OM

fraction containing autodisplayed Z-domain with the ratio of 5.7 [fluorescence intensity (arbitrary units; AU)/ratio of Z-domain (%)]. For streptavidin activity assay, biotin labeled with Atto-550 (λ_{Ex} = 554 nm, λ_{Em} = 576 nm) was reacted with the OM layers containing various proportions of streptavidin, and then fluorescence image analysis was performed to measure fluorescent signals for the antibody labeled with Atto-550. As shown in Fig. 3(c), the fluorescent signal increased in proportion with the amount of the OM fraction containing autodisplayed streptavidin in the ratio of 2.2 [fluorescence intensity (AU)/ratio of streptavidin (%)]. These results showed that the activity of two autodisplayed proteins in the OM layer was in proportion to the mixing ratio of the two OM fractions.

For quantitative analysis of the relation between the activity of autodisplayed protein and the mixing ratio of OM fractions, the activity of autodisplayed proteins was assayed in the OM layer from OM fractions at different ratios. As shown in Fig. 4(a), the OM fractions from *E. coli* with autodisplayed Z-domain and streptavidin were mixed and layered at different proportions: 100% (only the OM fraction with autodisplayed streptavidin), 75%, 50%, 25%, and 0% (only the OM fraction with autodisplayed Z-domain). For the autodisplayed Z-domain activity assay, the HRP-conjugated anti-mouse antibody was reacted with the OM layers at different ratios of OM fractions, and then the enzymatic activity of HRP was measured in the chromogenic reaction with a substrate called 3,3′,5,5′-tetramethylbenzidine (TMB). As shown in Fig. 4(b), the binding activity of the autodisplayed Z-domain increased in proportion with the amount of the OM fraction containing autodisplayed Z-

domain. For the streptavidin activity assay, biotin-labeled HRP was reacted with the OM layers at different ratios of OM fractions, and then the activity of streptavidin was measured in the reaction of HRP bound to biotin by means of a chromogenic reaction with TMB. As shown in Fig. 4(c), the activity of streptavidin increased in proportion with the amount of the OM fraction containing autodisplayed streptavidin. These results indicated that the activity of proteins in the OM layer prepared by mixing of OM fractions was quantitatively in proportion to the mixing ratio of the OM fractions containing autodisplayed Z-domain and streptavidin.

The activity of the two autodisplayed proteins in the OM layer was also assayed using the SPR biosensor. As shown in Fig. 5(a), the OM fraction containing autodisplayed Z-domain was layered on the SPR biosensor. For the binding assay of the autodisplayed Z-domain, a murine anti-CRP antibody and a biotin-labeled goat anti-HBsAg antibody were reacted in sequence, and then the SPR signal was measured to be 990.16 and 187.98, respectively. Since the autodisplayed Z-domain bound to mouse immunoglobulins with far stronger affinity than to goat immunoglobulins, the anti-CRP mouse antibody made a big difference in SPR signals in comparison with the goat antibody. As shown in Fig. 5(b), the OM fraction with autodisplayed streptavidin was also layered on the SPR biosensor. For the streptavidin binding assay, a murine anti-CRP antibody and biotin-labeled goat anti-HBsAg antibody were reacted in sequence, and then the SPR signal was measured to be less than zero and 137.71, respectively. These results meant that the biotin-labeled goat anti-HBsAg antibody could bind to the OM layer made of

the OM fraction containing the autodisplayed streptavidin. In comparison with the OM layer containing the autodisplayed Z-domain, the activity of binding to biotin was less than that of Z-domain to murine immunoglobulins. As shown in Fig. 5(c), the OM fractions with autodisplayed Z-domain and streptavidin were mixed in the ratio of 1:1, and the OM layers with various ratios of OM fractions were prepared on the SPR biosensor. For the assay of binding to the OM layer containing autodisplayed Z-domain and streptavidin, the murine anti-CRP antibody and the biotin-labeled goat anti-HBsAg antibody were reacted in sequence, and then the SPR signal was measured to be 122.34 and 27.36, respectively. These results indicated that both the murine anti-CRP antibody and biotin-labeled goat anti-HBsAg antibody bound to the autodisplayed Z-domains and streptavidin. The difference in signals for the binding activity of Z-domain and streptavidin resulted from the binding activity of the autodisplayed Z-domain and streptavidin as in the case of Fig. 3(b) and Fig. 3(c). These results showed that the OM fractions from different *E. coli* cells were mixed successfully and that the OM fraction with various desired proportions of autodisplayed proteins could be obtained. These results suggested that the activity of proteins in the OM layer on the SPR biosensor could be quantitatively varied in proportion to the mixing ratio of OM fractions containing autodisplayed Z-domain and streptavidin.

3.2. OM layer of two autodisplayed proteins with interaction

The autodisplayed Z-domain and streptavidin had independent protein activities, which required no interaction between the two proteins. As shown in the previous work, the activity of each protein was determined from the mixing ratio because the expressed proteins of Z-domain/streptavidin system made no direct interaction. In this work, two co-autodisplayed proteins in the OM layer of the same *E. coli* cell were tested to determine whether these proteins made interactions by using lipase/foldase system. In this case, the activity of lipase were changed according to the relative amount of foldase. Additionally, the feasibility of control over the activity of a target protein was verified by preparing an OM layer with different ratios of OM fractions with two autodisplayed proteins. As previously mentioned, lipases from *Burkholderia* and *Pseudomonas* species were reported to require an additional enzyme called lipase-specific foldase (Lif) which can fold the lipase into an active conformation [20-21]. Accordingly, lipases from *Burkholderia* and lipase-specific foldase (Lif) were selected as model proteins.

First, two kinds of proteins on OM of the same *E. coli*, the lipase and the lipase-specific foldase were co-autodisplayed on the same *E. coli* by co-transfection of autodisplay vectors of lipase (pAT-LipBc) and foldase (pAT-FoldBc), as shown in Fig. 1(b) [22]. Next, two OM fractions with the autodisplayed lipase and foldase were separately prepared by separate transfection of autodisplay vectors of lipase (pAT-LipBc) and foldase (pAT-FoldBc), as shown in Fig. 1(c). As shown in Fig. 6(a), SDS-PAGE results revealed that the protein bands of the separately autodisplayed lipase (82 kDa) and foldase (80 kDa) had different intensities even when OM fractions had

the same amount of total protein (5 μg) in each lane. When lipase and foldase were autodisplayed by co-transfection of two autodisplay vectors, the protein bands of the separately autodisplayed lipase (82 kDa) and foldase (80 kDa) also showed different intensity values (lane 3). The ratio of protein expression for the co-autodisplayed lipase and foldase was estimated by densitometry to be 1.0:1.8 (lipase: foldase). These results showed that (1) the ratio of autodisplayed lipase and foldase could vary even when two proteins were co-autodisplayed on the same OM of *E. coli*, and (2) the optimal ratio of lipase and foldase should be controlled to obtain the maximal lipase activity. The feasibility of creation of an OM layer with a controlled ratio of the lipase and foldase was tested by mixing different amounts of OM fractions containing the autodisplayed lipase and foldase, in the following ratios: 100% (only the OM fraction with autodisplayed lipase), 75%, 50%, 25%, or 0% (only the OM fraction with autodisplayed foldase). The relative amount of lipase was estimated by densitometry to be 100%, 71%, 48%, 28%, and 0% for the mixing ratio of 100% (only the OM fraction with autodisplayed lipase), 75%, 50%, 25%, and 0% (only the OM fraction with autodisplayed foldase). As shown in Fig. 6(b), SDS-PAGE results revealed that the intensity of protein bands near the molecular weight of lipase (82 kDa) and foldase (80 kDa) changed according to the mixing ratio of the OM fractions. These results indicated that the OM layer with the autodisplayed lipase and foldase in the desired ratio could be prepared by mixing OM fractions.

The activity of the autodisplayed lipase was assayed in whole *E. coli* cells when the foldase was also autodisplayed by co-transfection of autodisplay vectors of the

lipase (pAT-LipBc) and foldase (pAT-FoldBc). As shown in Fig. 7(a), whole *E. coli* cells were immobilized on the surface of modified microplates [27], and then the lipase activity was measured by means of the hydrolysis reaction of ester bonding with a chromogenic substrate called *p*-nitrophenyl palmitate (pNPP, transparent). When the substrate was hydrolyzed, p-nitrophenylate ion (yellow, λ_{max} = 405 nm) was produced. For the comparison of lipase activity, one unit of lipase was defined to be the production of 1 μmol p-nitrophenylate ion (extinction coefficient, ε_{405nm}=18,000 $M^{-1}cm^{-1}$) per 1 min. As shown in Fig. 7(b) and (c), no significant lipase activity at the different concentrations of substrate was observed when *E. coli* autodisplayed only lipase or only foldase on the OM layer. When lipase and foldase were simultaneously autodisplayed by co-transfection of two autodisplay vectors encoding lipase and foldase, the lipase activity was detected, as shown in Fig. 7(d). These results revealed that the autodisplayed foldase engaged in an interaction with the co-autodisplayed lipase on the OM layer of the same *E. coli*. As previously mentioned, the activity of lipase resulted from the mobility of the β-barrel serving as an anchor for autodisplay of the lipase and foldase within the OM [22-26].

The feasibility of control over the lipase activity was tested after formation of the OM layer by mixing separately prepared OM fractions containing autodisplayed lipase and foldase. First, the lipase activity was assayed in the OM fraction of *E. coli* containing the autodisplayed lipase and foldase by co-transfection of autodisplay vectors encoding the lipase and foldase. As shown in Fig. 8(a), a significant lipase activity (approximately 269.13 mU/mg) was detected in the OM fraction prepared

from *E. coli* with co-autodisplayed lipase and foldase in comparison with the OM fraction of *E. coli* with the autodisplayed lipase only (no lipase activity). Next, the OM fractions of *E. coli* with the autodisplayed lipase only and those with only the autodisplayed foldase were prepared and mixed in different ratios: 100% (only the OM fraction with autodisplayed lipase), 75%, 50%, 25%, and 0% (only the OM fraction with autodisplayed foldase). As shown in Fig. 8(b), the OM fractions containing only the autodisplayed lipase and only the autodisplayed foldase showed a significantly lower lipase activity than did the OM fractions that contained the mixture of the autodisplayed lipase and foldase. In particular, the OM fraction at the mixing ratio of 1:3 for the autodisplayed lipase and foldase showed a significantly higher lipase activity (approximately 149.48 mU/mg) than did the OM fractions containing only one kind of protein and the OM fraction at the mixing ratio of 1:1 (approximately 43.16 mU/mg). These results showed that (1) the activity of lipase could be obtained by mixing two OM fractions containing the separately autodisplayed lipase and foldase, and (2) the activity of the lipase can be controlled by means of the mixing ratio of the two OM fractions. When an OM layer was prepared by mixing OM fractions of the autodisplayed lipase and foldase, the lipase activity was also assayed. In this work, polystyrene (PS) microbeads with the diameter of 1 μm were used for preparation of the OM layer by mixing the OM fractions containing autodisplayed lipase and foldase. The most significant benefit of autodisplayed proteins in comparison with soluble enzyme fractions will be no requirement of purification steps because the autodisplayed proteins could be

expressed on the outer membrane of *E. coli*. Additionally, the optimal condition for maximum activity could be obtained by mixing the outer membrane fractions. As shown in Fig. 8(c), the OM layer containing only the autodisplayed lipase or the autodisplayed foldase in the ratio of lipase to foldase of 1:3 showed far higher sensitivity in comparison with the OM layer containing only the autodisplayed lipase or the autodisplayed foldase. Such an activity of lipase from the optimal mixing ratio was observed to be even higher than the case of co-autodisplay of lipase and foldase on the same *E. coli* cell. These results suggested that (1) the activity of the lipase in the OM layer could be obtained by mixing two OM fractions with a separately autodisplayed lipase and foldase, and (2) the optimal activity of the lipase in the OM layer can be achieved by controlling the mixing ratio of the two OM fractions.

4. Conclusions

Two proteins can be expressed on the same *E. coli* OM by co-transfection of two autodisplay vectors. However, the ratio of the two autodisplayed proteins cannot be controlled. In this study, the OM fractions of *E. coli* with two autodisplayed proteins were separately prepared and mixed to demonstrate the feasibility of control over the ratio of the two autodisplayed proteins. Z-Domain and streptavidin were selected as model proteins, and the activities of Z-domain and streptavidin

were tested in the combined OM layer in a 96-well microplate, using an SPR biosensor. The activity of the two autodisplayed proteins in the same OM layer (which was prepared by mixing two OM fractions at the desired ratio of autodisplayed Z-domain and streptavidin) was assayed in a reaction of fluorescently labeled ligands and an enzyme called HRP. According to fluorescence image analysis and enzymatic activity assay, the activity of proteins in the OM layer resulting from the mixing of OM fractions was quantitatively in proportion to the mixing ratio of the OM fractions containing autodisplayed Z-domain and streptavidin. The SPR biosensor assay also showed that the activity of proteins in the OM layer on the SPR biosensor could be quantitatively varied in proportion to the mixing ratio of OM fractions containing autodisplayed Z-domain and streptavidin. As the second step, the control over a protein ratio in the OM layer was also demonstrated for two autodisplayed proteins that require an interaction for their activities. In this work, the OM fractions of *E. coli* with an autodisplayed lipase and foldase were separately prepared and mixed to demonstrate the feasibility of control over the proportion of two autodisplayed proteins when interaction of two proteins is required in the same OM layer for the activity of the lipase. The ratio of protein expression for the co-autodisplayed lipase and foldase was estimated by densitometry to be 1.0:1.8 (lipase: foldase). According to the lipase assay, the OM fraction at the mixing ratio of 3:1 and 1:3 for the autodisplayed lipase and foldase showed a significantly higher lipase activity than the OM fractions containing only one kind of protein. These results showed that (1) activity

of the lipase could be obtained by mixing two OM fractions with a separately autodisplayed lipase and foldase, and (2) the activity of lipase could be controlled by varying the mixing ratio of the two OM fractions.

Acknowledgements

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Figure legends

Fig. 1. A schematic diagram of OM layer formation by mixing of isolated OM fractions with autodisplayed proteins. (a) OM layer formation by means of OM fractions isolated from *E. coli* with autodisplayed Z-domain and streptavidin. (b) OM layer formation by means of the OM fraction isolated from *E. coli* with co-autodisplayed lipase and foldase. (c) OM layer formation by means of OM fractions isolated from *E. coli* with an autodisplayed lipase and foldase.

Fig. 2. Control over the protein amount in different mixing ratios of OM fractions containing autodisplayed Z-domain and streptavidin. (a) SDS-PAGE analysis of mixed OM fractions from *E. coli* with autodisplayed streptavidin and intact *E. coli*. (b) SDS-PAGE analysis of mixed OM fractions from *E. coli* with autodisplayed Z-domain and intact *E. coli*. (c) SDS-PAGE analysis of mixed OM fractions from *E. coli* with autodisplayed streptavidin and Z-domain.

Fig. 3. An assay of activity of the autodisplayed Z-domain and streptavidin by fluorescence image analysis. (a) A schematic view of the assays of activity of autodisplayed Z-domain and streptavidin. (b) An assay of activity of the autodisplayed Z-domain with APC-labeled immunoglobulin (λ_{Ex} = 650 nm, λ_{Em} = 660 nm). (c) An assay of activity of the autodisplayed Z-domain with biotinylated atto-550 (λ_{Ex} = 554 nm, λ_{Em} = 576 nm).

Fig. 4. An assay of activity of the autodisplayed Z-domain and streptavidin in a 96-well microplate. (a) A schematic view of assays of activity of autodisplayed Z-domain and streptavidin. (b) An assay of activity of the autodisplayed Z-domain with HRP-labeled immunoglobulin. (c) An assay of activity of the autodisplayed Z-domain with biotin-labeled HRP.

Fig. 5. An assay of activity of the autodisplayed Z-domain and streptavidin using an SPR biosensor. (a) An assay of activity of the OM layer containing the autodisplayed Z-domain by means of an anti-CRP antibody and biotin-labeled anti-HBsAg antibody. (b) An assay of activity of the OM layer containing the autodisplayed streptavidin by means of an anti-CRP antibody and biotin-labeled anti-HBsAg antibody. (c) An assay of activity of the OM layer containing the autodisplayed Z-domain and streptavidin by means of an anti-CRP antibody and biotin-labeled anti-HBsAg antibody.

Fig. 6. Control over the protein amount at different mixing ratios of the OM fractions containing an autodisplayed lipase and foldase. (a) SDS-PAGE analysis of OM fractions from *E. coli* with the autodisplayed lipase and foldase. (b) SDS-PAGE analysis of mixed OM fractions at a controlled ratio of the autodisplayed lipase and foldase.

Fig. 7. An assay of activity of the autodisplayed lipase in whole *E. coli* cells in a 96-

well microplate. An assay of activity of the lipase was carried out for *E. coli* with autodisplayed lipase ("Lipase"), *E. coli* with autodisplayed foldase ("foldase"), and *E. coli* with co-autodisplayed lipase and foldase ("co-autodisplayed lipase and foldase") by means of a chromogenic substrate called pNPP.

Fig. 8. An assay of activity of the autodisplayed lipase in the OM fraction and OM layer. (a) An assay of activity of the lipase in the OM fraction from *E. coli* with the co-autodisplayed lipase and foldase. (b) An assay of activity of the lipase in mixed OM fractions from *E. coli* with a separately autodisplayed lipase and foldase. (c) A microbead assay of activity of the lipase in the OM layer formed by mixing the OM fractions from *E. coli* with a separately autodisplayed lipase and foldase.

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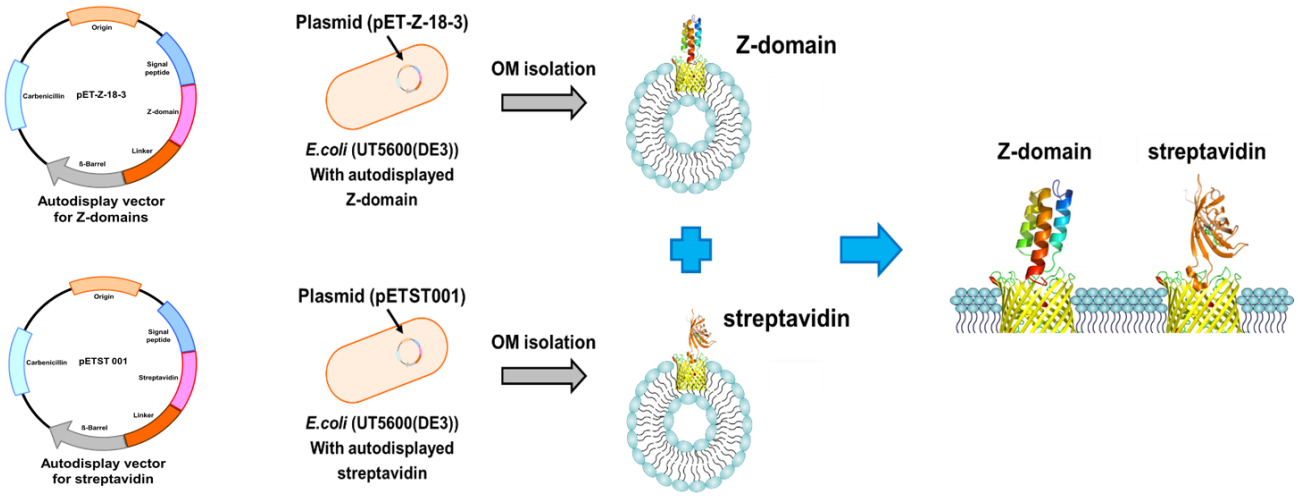

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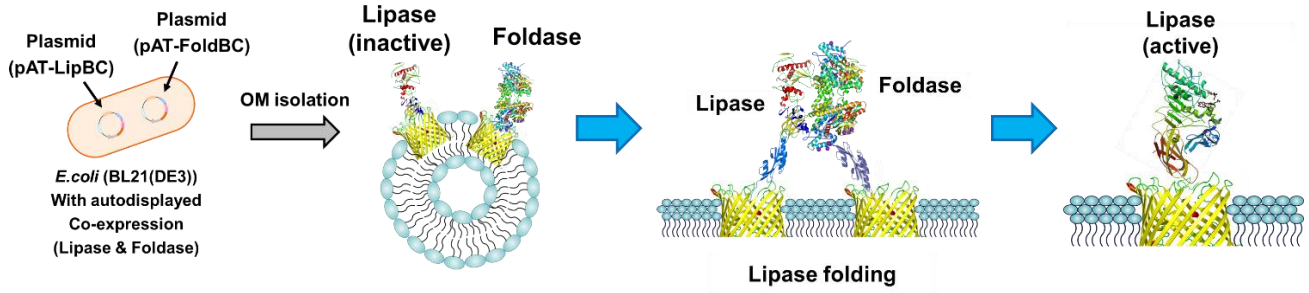

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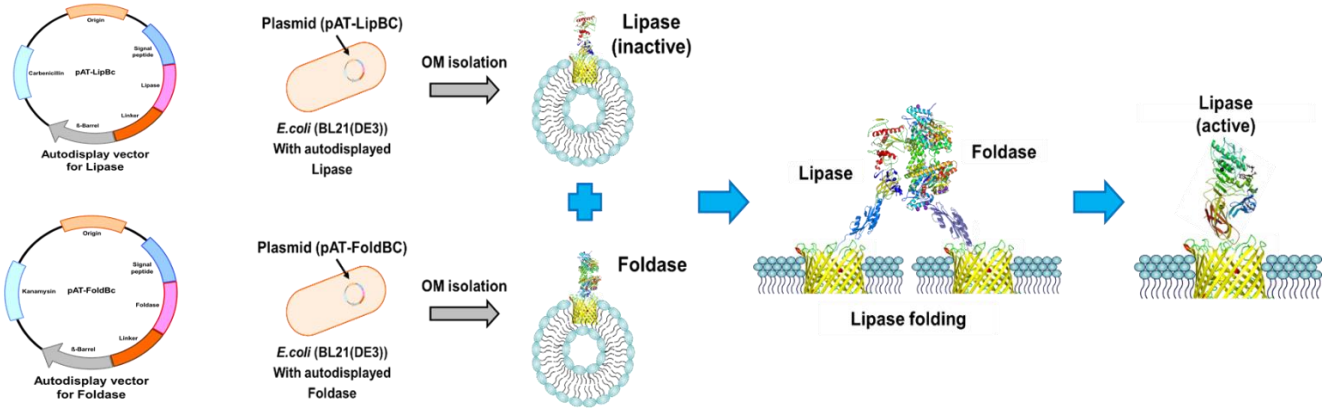

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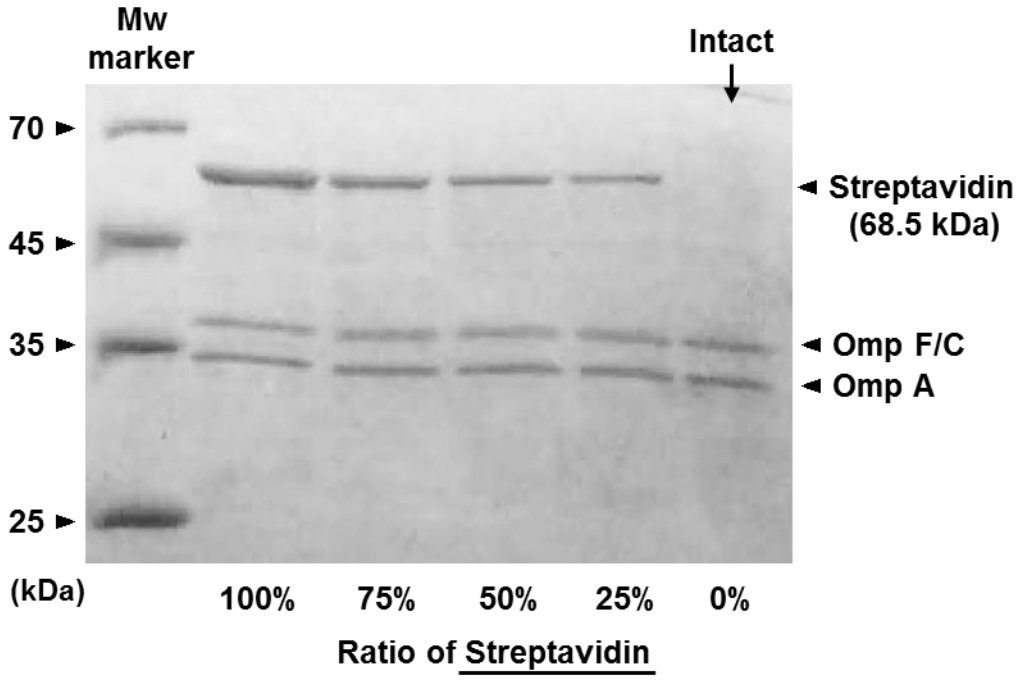

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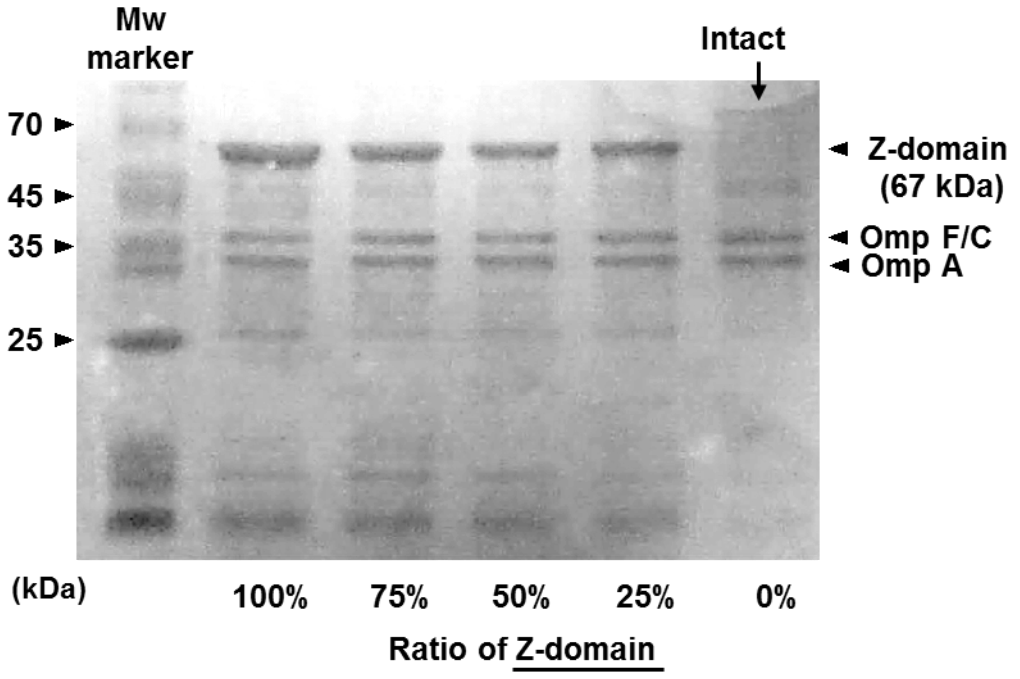

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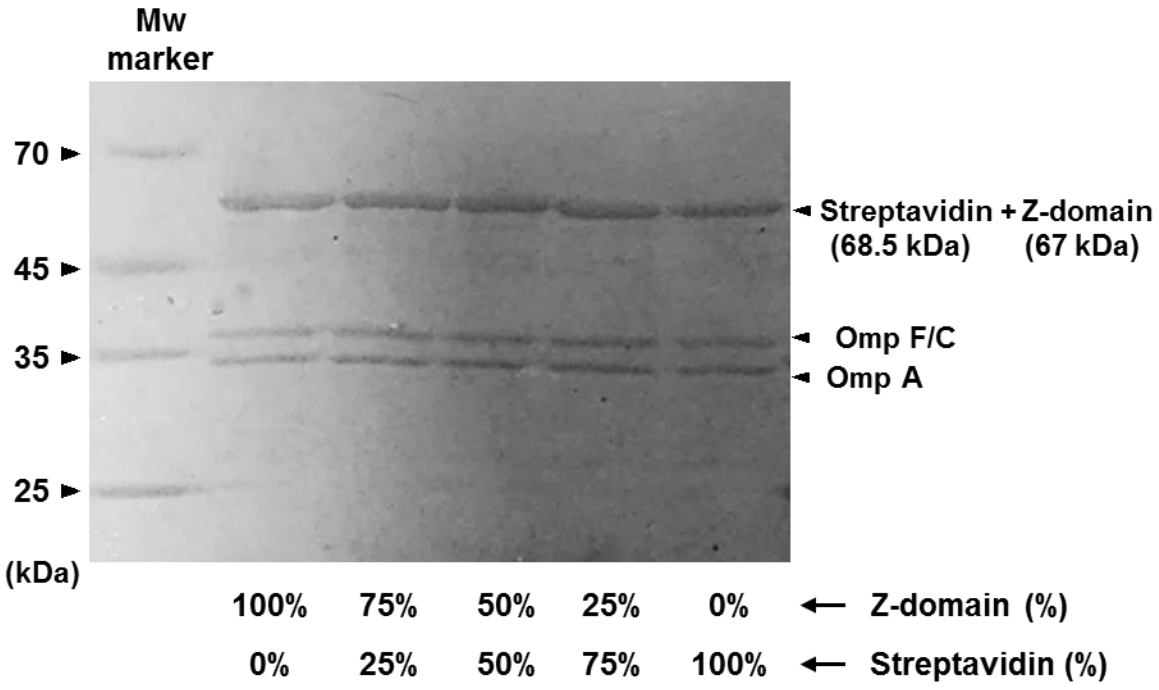

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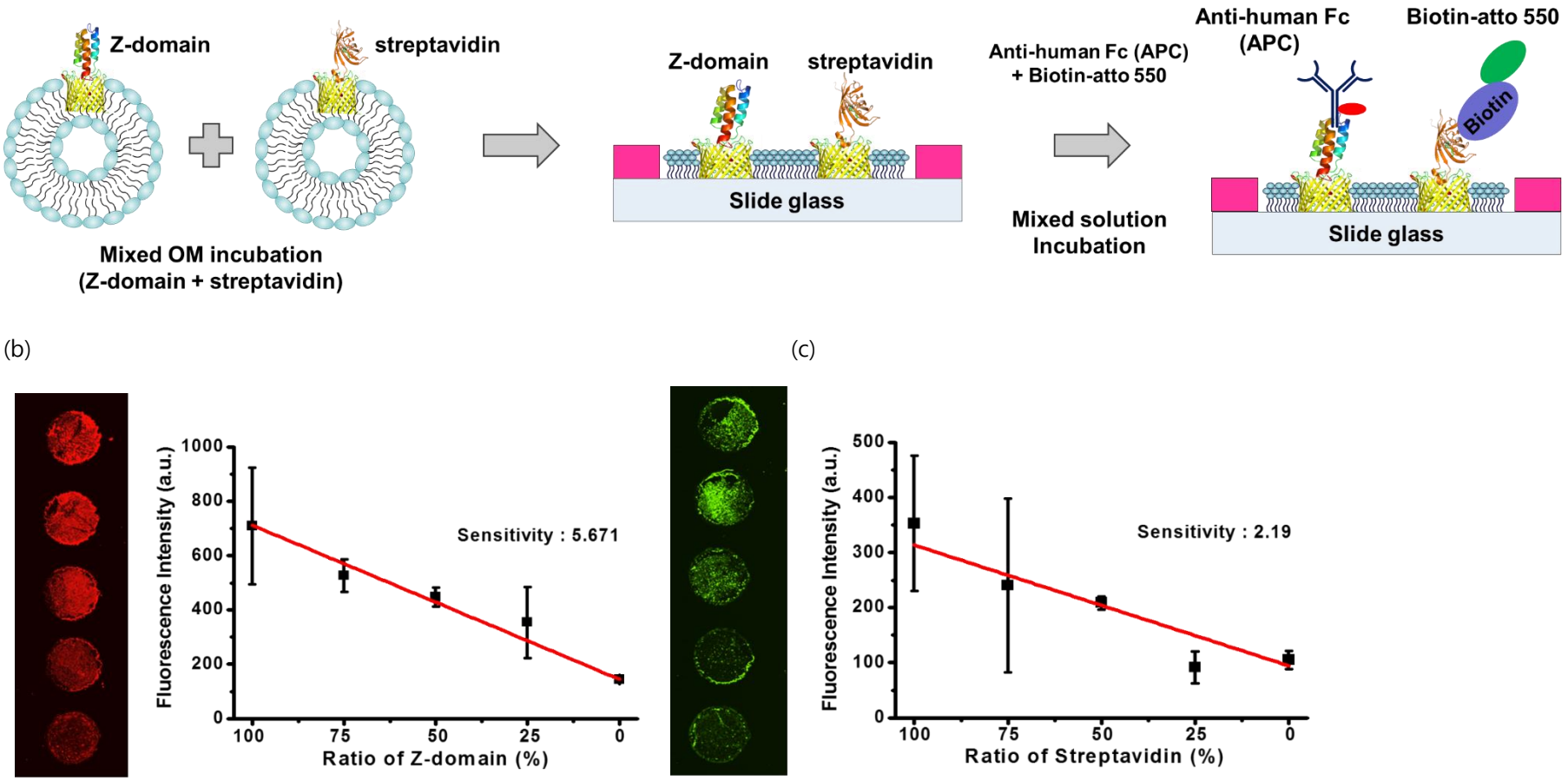

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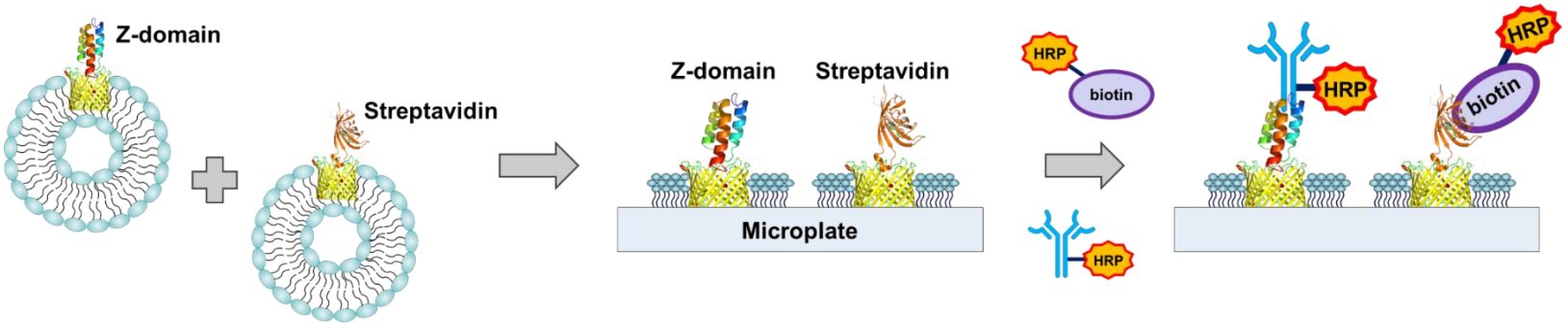

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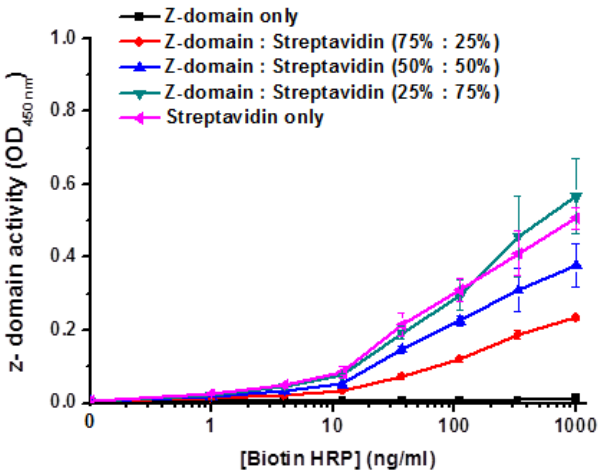

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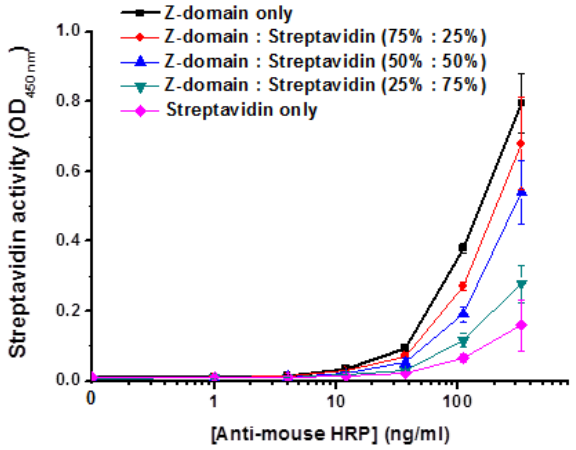

Fig. 5.

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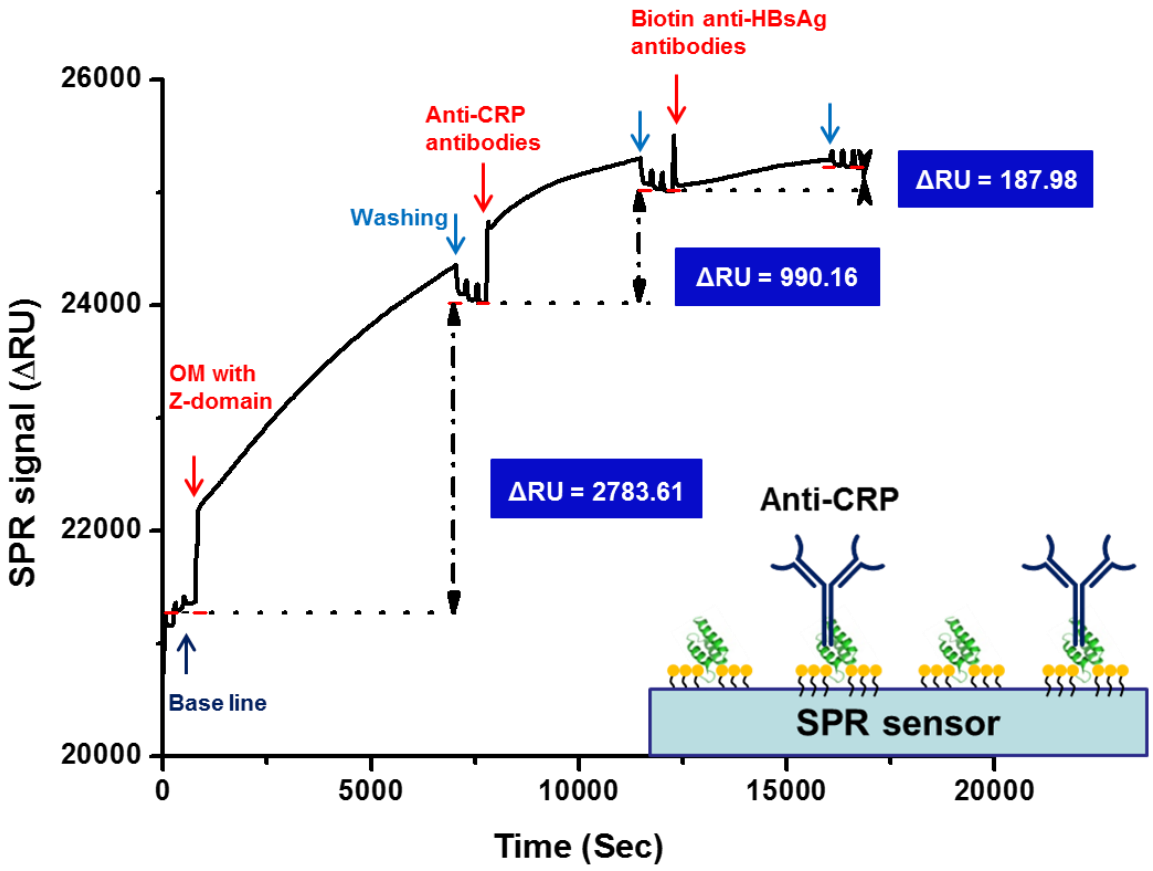

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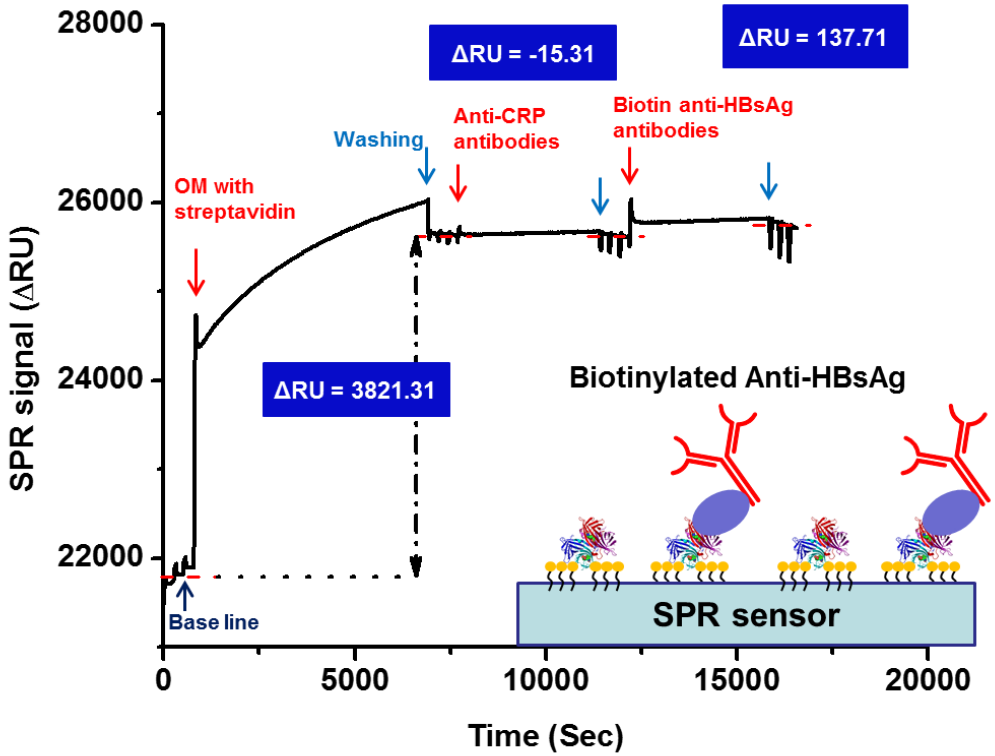

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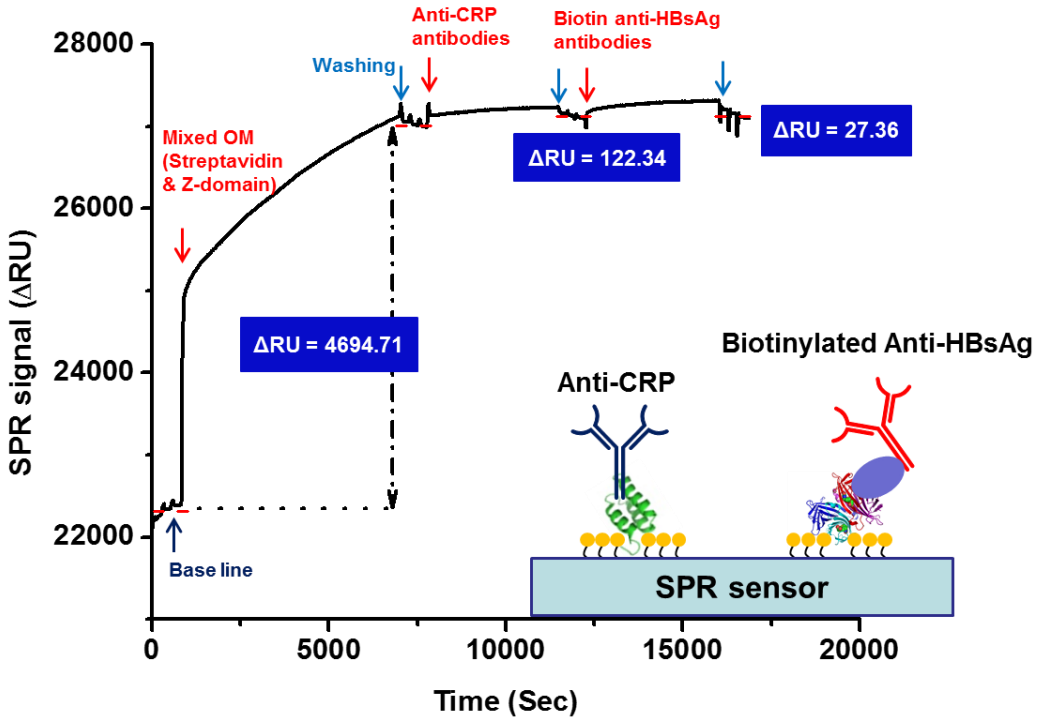
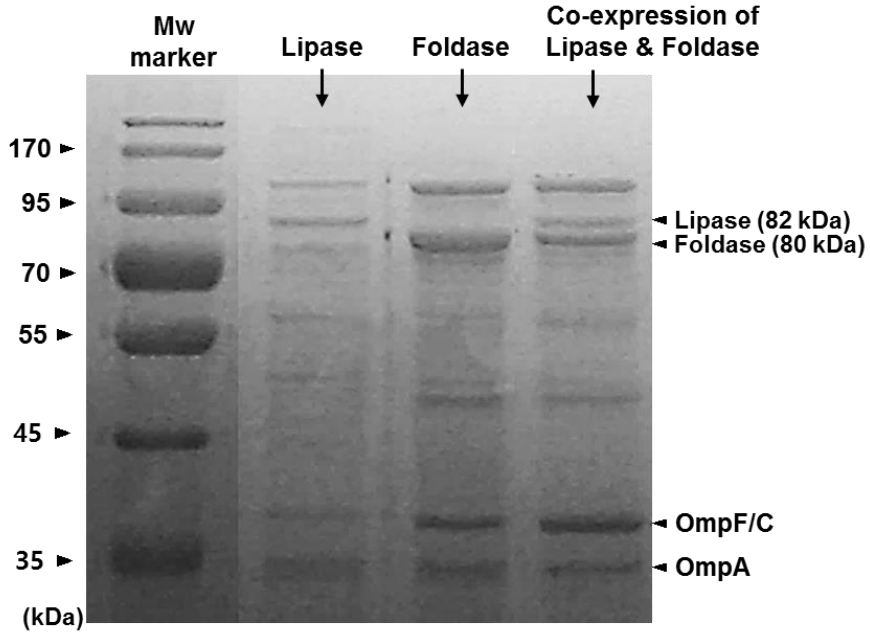


Fig. 6

(a)



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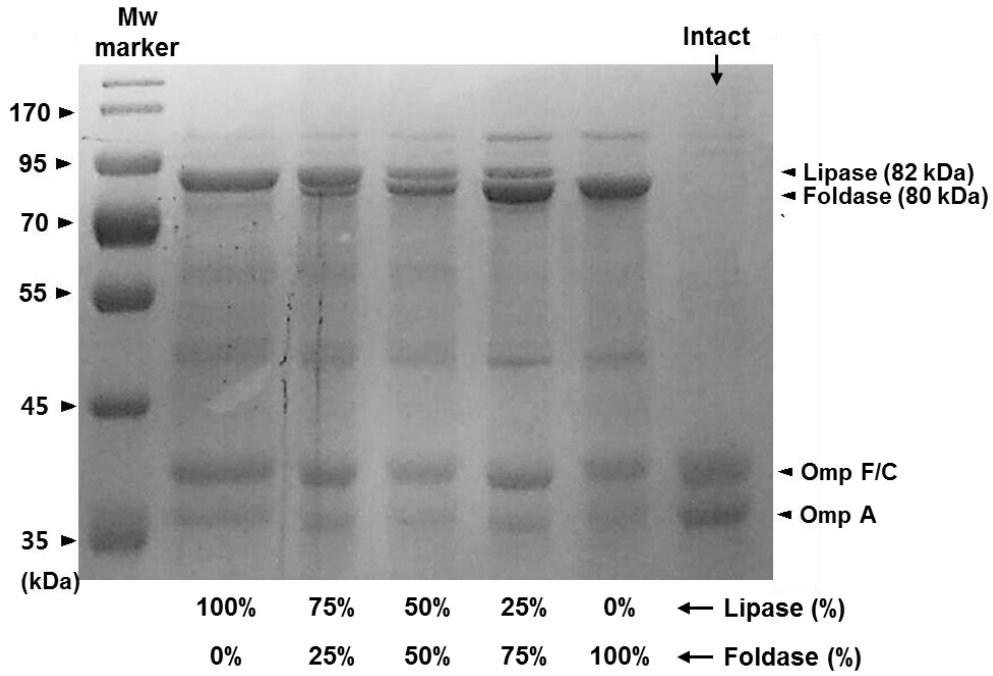

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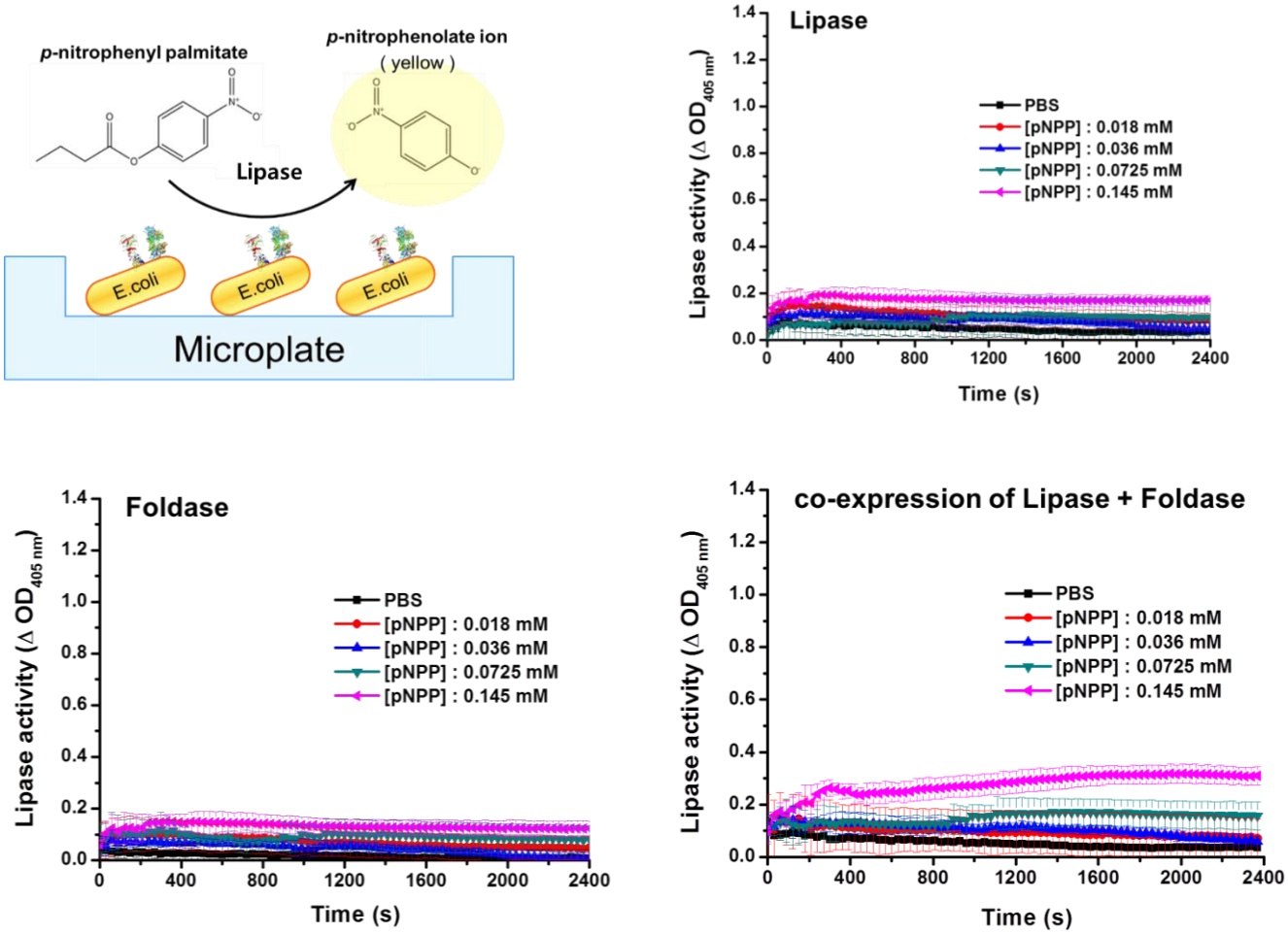

Fig. 8.

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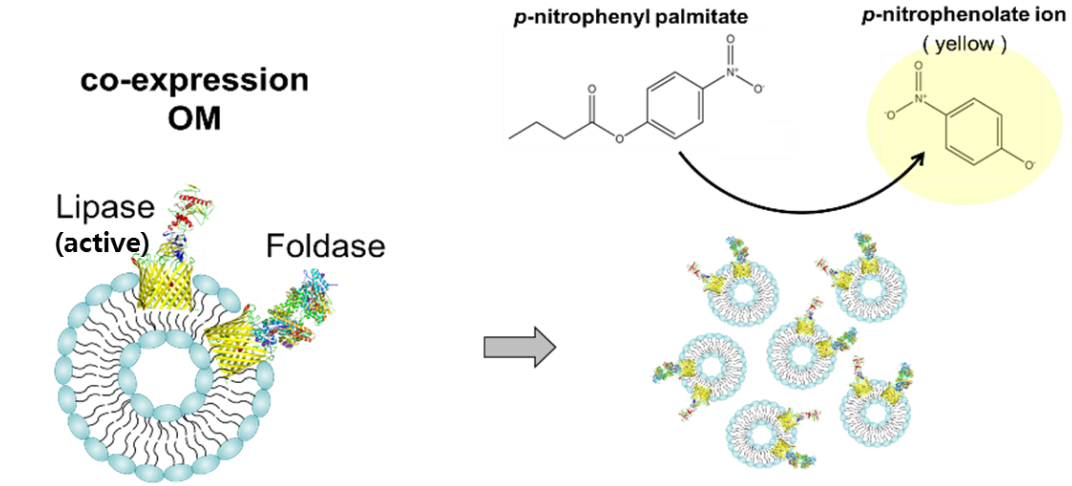

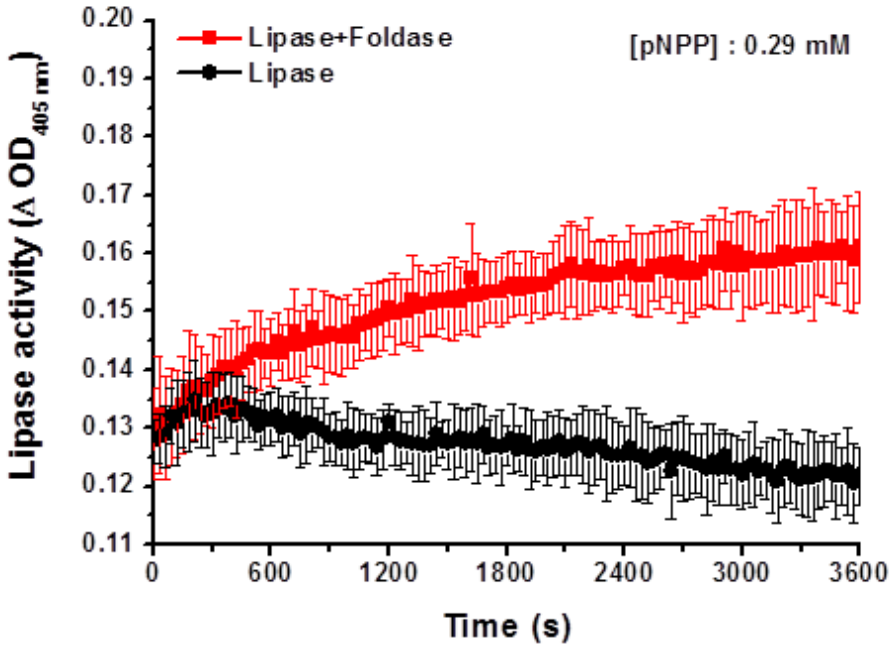

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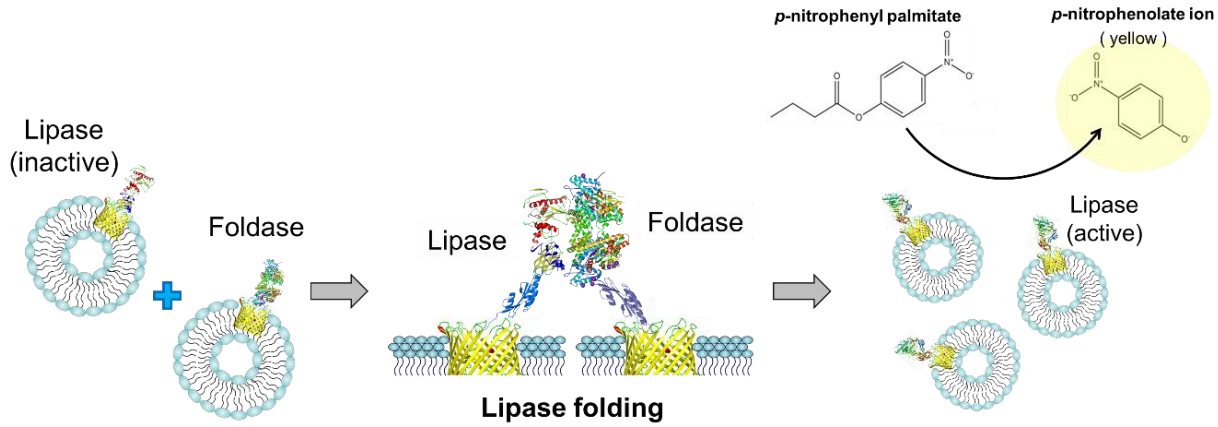

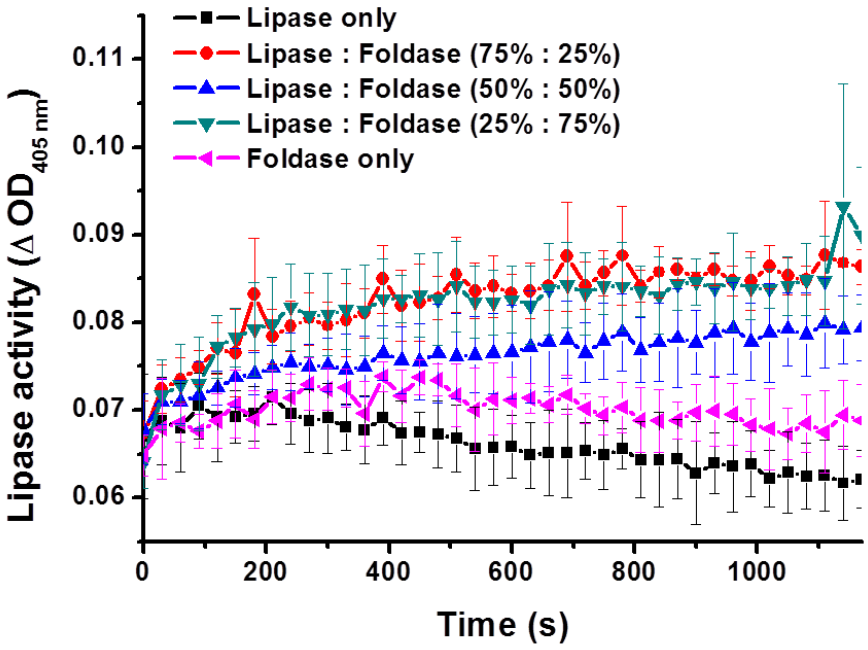

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