Co-autodisplay of Z-domains and bovine caseins on the outer membrane of *E. coli*

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**A B S T R A C T**

In this work, two proteins, Z-domains and bovine casein, were autodisplayed on the outer membrane of the same *Escherichia coli* cells by co-transformation of two different autodisplay vectors. On the basis of SDS-PAGE densitometry, Z-domains and bovine casein were expressed at 3.12 × 10⁵ and 1.55 × 10⁵ proteins/E. coli cell, respectively. The co-autodisplayed Z-domains had antibody-binding activity and the bovine casein had adhesive properties. *E. coli* with co-autodisplayed proteins were analyzed by fluorescence assisted cell sorting (FACS). *E. coli* with co-autodisplayed Z-domains and bovine casein aggregated due to hydrophobic interaction. For application to immunoassays, the Z-domain activity was estimated after (1) immobilizing the *E. coli* and (2) forming an OM layer. *E. coli* with co-autodisplayed two proteins that were immobilized on a polystyrene microplate had the same antibody-binding activity as did *E. coli* with autodisplayed Z-domains only. The OM layer from the co-transformed *E. coli* had Z-domains and bovine casein expressed at a 1:2 ratio from antibody-binding activity measurements.

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1. Introduction

Autodisplay technology is a method to express a target protein on the outer membrane (OM) of *Escherichia coli*. The target protein is expressed as a fusion protein of the β-barrel protein called adhesion involved in diffuse adherence (AIDA-1) from *E. coli*. In the fusion protein, the autotransporter domain of AIDA-1 is expressed on the OM of *E. coli* as an integral protein. The target protein is then spontaneously transported to the OM through the β-barrel of AIDA-1 [1–4]. Recently, Z-domains [5–12] and bovine casein [13–14] were reported to be autodisplayed on the OM of *E. coli*. In this work, two proteins were co-autodisplayed on the OM of the same *E. coli* cell by co-transformation with two different autodisplay vectors. The autodisplay vectors had different antibiotic-resistance genes, and co-autodisplaying *E. coli* could be cloned under selection with two antibiotics.

As reported previously, Z-domain has specific binding activity towards the F, region of antibodies (IgG), which can be used to control antibody orientation for immunoassays. *E. coli* with autodisplayed Z-domains have used the antibody-binding activity to improve the sensitivity of immunoassays by orienting the antibodies to expose the antigen-binding Fab region [5–12]. *E. coli* cells with autodisplayed Z-domains could be applied to immunoassays by (1) immobilizing them on a microplate [7–9] or (2) creating an OM layer on a solid substrate [10–12]. Bovine casein is often used as an adhesive because of its strong physical adsorption to various surfaces. The co-autodisplayed bovine casein was used as an adhesive to bind *E. coli* cells to the solid substrates, such as microplates and microbeads, for immunoassays.

In this work, two proteins, Z-domains and bovine casein, were co-autodisplayed on the OM of the same *E. coli* cell by co-transformation with two different autodisplay vectors. The co-autodisplayed proteins were analyzed by SDS-PAGE, and the amount of protein expressed was estimated by SDS-PAGE densitometry [5–8]. The antibody-binding activity was estimated by using antibodies labeled with horse-radish peroxidase (HRP). The adhesive properties of bovine casein were estimated with zeta-potential analysis and adhesion tests to surfaces with controlled hydrophilicity. The activities of the proteins co-autodisplayed on the *E. coli* OM were also analyzed by fluorescence assisted cell sorting (FACS) [15–16]. To test applicability to immunoassays, the Z-domain activity was estimated after (1) immobilizing *E. coli* and (2) forming an OM layer on microplates.

2. Materials and methods

2.1. Materials

LB broth was purchased from Duchefa (Haarlem, The Netherlands); aprotinin was purchased from Roche Korea (Seoul, Korea); and carbenicillin, kanamycin, lysozyme, DNase, bovine serum albumin
(BSA), and all other analytical grade chemicals were purchased from Sigma-Aldrich Korea (Seoul, Korea).

2.2. Co-autodisplay of Z-domains and bovine casein

_E. coli_ UT5600 (F-ara14 leuB6 azi-6 lacV1 procC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1, DomT-fepC266) were used to express autotransporter fusion proteins. Bacteria were grown at 37 °C in Luria–Bertani (LB) broth containing 100 mg/l carbenicillin, 10 μM ethylene diaminetetraacetate (EDTA) and 10 mM β-mercaptoethanol. To simultaneously express Z-domain and bovine casein, two autodisplay vectors of pET-Z-18–3 (Z-domain) and pAB002 (bovine α_s1-casein) were prepared from the pET-Adx04 plasmid, which encodes the AIDA-1 autotransporter. The passenger genes were inserted with Xhol and KpnI restriction enzymes. The bacteria were grown at 37 °C in LB broth containing 100 μg/ml carbenicillin for the pET-Z-18-3 vector and kanamycin 50 μg/ml for the pTS002 vector, 10 μM EDTA, and 10 mM β-mercaptoethanol.

2.3. Activity assays

The activity of autodisplayed Z-domains on _E. coli_ in solution was assayed with antibodies labeled with HRP (direct assay). _E. coli_ autodisplaying Z-domains at OD_{578} nm = 1.0 (1 ml) were centrifuged for 3 min at 14,000 rpm, resuspended in a solution with 1 μg/ml HRP-labeled anti-goat antibodies (500 μl), and incubated for 1 h. To remove unbound antibodies, the reaction mixture was centrifuged and resuspended in PBS with 0.1% Tween 20 by vigorous vortexing. After washing, the resuspended _E. coli_ (30 μl at OD_{578} nm = 1.0) were mixed with 100 μl of TMB solution (Pierce, NY, USA) for 5 min. The TMB reaction was quenched with 2 M sulfuric acid. The OD was measured by an ELISA reader (Molecular Devices, CA, USA) at 450 nm.

The activities of Z-domains on immobilized _E. coli_ were assayed as follows. Freshly prepared _E. coli_ at 10^9 cells/ml in PBS (100 μl) were washed three times with PBS and incubated in each well of the microplate overnight at 4 °C. The microplate was treated with 10 mg/ml BSA for 1 h then washed three times with PBS with 0.1% Tween 20. Z-domain activity was determined by adding 10, 100, or 1000 ng/ml anti-goat antibodies labeled with HRP for 1 h (n = 3). The TMB reaction was carried out for 5 min, and the reaction was quenched with 2 M sulfuric acid. The OD was measured by an ELISA reader (Molecular Devices, CA, USA) at 450 nm.

An OM solution from _E. coli_ autodisplaying proteins was prepared as previously reported to have a total protein concentration of 300 μg/ml in PBS [10–12]. Each well of a 96-well microplate was incubated with 100 μl of OM solution overnight at 4 °C. The microplate was washed three times with PBS with 0.1% Tween 20. Anti-goat antibody labeled with HRP (100 μl) at 10, 100, or 1000 ng/ml was added to each well of the microplate and incubated for 1 h at room temperature. The HRP assay was visualized by a chromogenic reaction with TMB solution (Pierce, NY, USA). After quenching with 2 M sulfuric acid solution, the OD was measured at 450 nm by an ELISA reader (Molecular Devices, CA, USA).

2.4. FACS analysis

FACS analysis was carried out with a FACSCalibur system (Becton–Dickinson, NJ, USA) at an excitation wavelength of 488 nm and filter-sterilized PBS as a sheath fluid. The threshold trigger was set on side-scatter to eliminate background noise and analyze only intact cells.

Fig. 1. Co-autodisplay of Z-domains and bovine casein. (a) Schematic of co-autodisplay of Z-domains and bovine casein. (b) SDS-PAGE of co-autodisplayed Z-domains and bovine casein. Lane 1: protein ladder, lane 2: wild-type _E. coli_, lane 3: _E. coli_ autodisplaying Z-domains alone, lane 4: _E. coli_ autodisplaying bovine casein alone, and lane 5: _E. coli_ autodisplaying Z-domains and bovine casein.
The fluorescence signal was recorded as the mean intensity [15–16]. *E. coli* autodisplaying proteins were prepared at OD$_{578}$ nm = 1.0, which was then diluted 1000-fold to $10^6$ cells/ml. Anti-goat antibody labeled with fluorescein (100 μl) at 5 μg/ml was added for 1 h. After washing with PBS with 0.1% Tween 20 twice, the fluorescence signal ($\lambda_{\text{Ex}} = 488$ nm, $\lambda_{\text{Em}} = 508$ nm) was detected in the FL1 channel (530 ± 30 nm). A density plot was made from the FL-1A (area) and FL-1 W (width) of the FL1 channel on linear mode [17–20].

![Graph and Table](https://example.com/fig2.png)

**Fig. 2.** Activity assays of co-autodisplayed Z-domains and bovine casein. (a) Antibody-binding activity of Z-domains using HRP-labeled antibodies. (b) Bovine casein adsorption to surfaces with controlled hydrophilicity.

<table>
<thead>
<tr>
<th></th>
<th>Intact <em>E. coli</em></th>
<th><em>E. coli</em> with autodisplayed Z-domains</th>
<th><em>E. coli</em> with autodisplayed bovine caseins</th>
<th><em>E. coli</em> with co-autodisplayed Z-domains &amp; caseins</th>
</tr>
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<tbody>
<tr>
<td><strong>Zeta-potential (mV)</strong></td>
<td>−50.0 ± 1.4</td>
<td>−48.0 ± 2.6</td>
<td>−1.6 ± 12.9</td>
<td>−62.0 ± 2.8</td>
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Table 1
Zeta-potential of *E. coli* with autodisplayed proteins.
Fig. 3. FACS analysis of *E. coli* co-autodisplaying Z-domains and bovine caseins. (a) Dot plot of forward scattering (FSC) vs. side scattering (SSC). (b) FACS analysis of *E. coli* after treatment with FITC-labeled antibodies (*λ*<sub>ex</sub> = 488 nm, *λ*<sub>em</sub> = 508 nm). (c) Density plot to measure the width and area distribution of *E. coli* autodisplaying proteins.
3. Results and discussion

3.1. Co-autodisplay of Z-domains and bovine casein

Recently, Z-domain [5–12] and bovine casein [13–14] were reported to be autodisplayed on the OM of E. coli. In this work, Z-domain and bovine casein were co-autodisplayed on the OM of the same E. coli cells by co-transformation of two different autodisplay vectors. As shown in Fig. 1(a), the autodisplay vectors had different antibiotic-resistance genes: carbenicillin for Z-domains and kanamycin bovine casein. Therefore, only E. coli with both vectors could be cloned from cultures grown with both antibiotics. After co-transforming E. coli with both autodisplay vectors, the proteins expression was analyzed by SDS-PAGE. As shown in Fig. 1(b), both co-autodisplayed proteins (Z-domains and bovine casein) were present at molecular weights of 55 and 75 kDa, respectively. The protein expression was quantified from 10^9 E. coli cells/ml (OD578 nm = 1.0). Densitometry of the protein bands indicated that Z-domains and bovine casein were expressed at 3.12 × 10^7 and 1.55 × 10^6 proteins/cell, respectively. These results show that Z-domains and bovine casein can be co-autodisplayed by co-transformation of two autodisplay vectors. SDS-PAGE densitometry showed that twice as much bovine casein was expressed in comparison with Z-domains.

Next, the activities of the expressed proteins were analyzed. As reported previously, Z-domains specifically bind the Fc region of antibodies, which can be used to control antibody orientation for immunoprecipitation. To estimate the antibody-binding activity of autodisplayed Z-domains, HRP-labeled antibodies were incubated with E. coli co-autodisplaying Z-domains and bovine casein. The same antibodies were incubated with wild-type E. coli and E. coli autodisplaying either Z-domains or bovine casein alone. Antibody binding was estimated from the chromogenic reaction with 3,3′,5,5′-tetramethylbenzidine (TMB). As shown in Fig. 2(a), wild-type E. coli and E. coli autodisplaying bovine casein alone had insignificant antibody-binding activity in comparison with E. coli co-autodisplaying Z-domains. E. coli co-autodisplaying Z-domains and bovine casein had 43% (n = 3) of the antibody-binding activity of E. coli autodisplaying Z-domains alone.

Bovine casein has been used as an adhesive due to its strong physical adsorption to various surfaces. To analyze the activity of autodisplayed bovine casein, the zeta-potential of E. coli, which is frequently used to analyze the surface charge of colloids, was measured [21–22]. Colloids with a zeta-potential higher than ±40 mV are well dispersed in aqueous media, and colloids with the zeta-potential of less than ±10 mV tended to aggregate. As summarized in Table 1, intact E. coli had a zeta-potential of −50 mV (n = 10), which indicates dispersion in an aqueous solution. The zeta-potential of E. coli autodisplaying bovine casein was −1.6 mV (n = 10), which indicates a tendency to aggregate. These results were expected from the adhesive properties of bovine casein. E. coli with co-autodisplaying Z-domains and bovine caseins had a zeta-potential of −62 mV (n = 10), which indicates dispersion in an aqueous solution.

The physical adsorption of autodisplayed bovine caseins was tested on various surfaces with controlled hydrophilicity. Three surfaces with contact angles of 20 ± 1°, 41 ± 1°, and 78 ± 1° were prepared from glass, silane (A-174) coated glass, and polystyrene, respectively. As shown in Fig. 2(b), the adsorption of wild-type E. coli, E. coli autodisplaying either Z-domain or bovine casein alone, and E. coli co-autodisplaying Z-domains and bovine casein were incubated on the surfaces. The slide glass, which was hydrophilic (contact angle of 20 ± 1°), had similar adsorption among all four E. coli. The relatively hydrophobic surfaces (silane coated glass [contact angle of 41 ± 1°] and polystyrene [contact angle of 78 ± 1°]) had significantly more cells adsorbed for E. coli autodisplaying bovine casein alone (7-fold compared with wild-type E. coli) and E. coli co-autodisplaying Z-domain and bovine casein (11-fold compared with wild-type E. coli). These results were due to the hydrophobic properties of autodisplayed bovine casein. The zeta-potential analysis indicated that E. coli co-autodisplaying Z-domains and bovine casein had different dispersion properties from E. coli autodisplaying bovine casein alone. However, these results show that the autodisplayed bovine casein was physically adsorptive.

3.2. Properties of E. coli co-autodisplaying Z-domains and bovine casein

E. coli autodisplaying proteins were analyzed by FACS. The size distribution of E. coli can be obtained from the forward scattering (FSC) measurement and density information from the side scattering (SSC) measurement [15–16]. As shown in Fig. 3(a), the dot plots of FSC versus SSC were obtained for E. coli autodisplaying either Z-domains or bovine casein alone and E. coli co-autodisplaying Z-domains and bovine caseins. E. coli autodisplaying Z-domains alone had similar distributions in the FSC and SSC regions. In comparison with wild-type E. coli, E. coli autodisplaying bovine caseins and E. coli co-autodisplaying Z-domains and bovine caseins had a significantly wider size distribution in the
FSC plots (X-axis) and wider density distribution in the SSC plots (Y-axis). These results could be due to aggregation through the physical adsorption, as expected from the low zeta-potential of \textit{E. coli} autodisplaying bovine casein alone (−1.6 mV, Table 1). \textit{E. coli} co-autodisplaying Z-domains and bovine casein could have aggregated through hydrophobic interactions between autodisplayed bovine caseins.

As previously described, autodisplayed Z-domains had antibody-binding activity. The antibody-binding activity of Z-domains on \textit{E. coli} with different autodisplayed proteins was analyzed by FACS. \textit{E. coli} autodisplaying either Z-domain or casein alone, \textit{E. coli} co-autodisplaying Z-domain and bovine caseins, and wild-type \textit{E. coli} as a negative control were treated with FITC-labeled antibodies. As shown in Fig. 3(b), intact \textit{E. coli} and \textit{E. coli} autodisplaying bovine casein had baseline fluorescence levels, which indicate non-specific antibody binding. Both \textit{E. coli} autodisplaying Z-domains had significantly higher fluorescence signals than did wild-type \textit{E. coli}. \textit{E. coli} co-autodisplaying Z-domains and bovine casein had lower fluorescence than did \textit{E. coli} autodisplaying Z-domains. This reduced antibody-binding activity of the co-transformed \textit{E. coli} was also observed with HRP-labeled antibodies, as shown in Fig. 2(a). The FACS analysis indicates that the fluorescence signal from \textit{E. coli} co-autodisplaying both proteins was 37\% of the mean intensity of \textit{E. coli} autodisplaying Z-domains alone.

As shown in Fig. 3(b), \textit{E. coli} co-autodisplaying both proteins had two peaks. The peak at the low fluorescence range indicated a relatively high distribution of \textit{E. coli} without antibody-binding activity. In the FACS density plot, the analyte width was determined from the time taken for the analyte to pass through the detecting area and the analyte area was measured from the integrated detector signal during the passing time. The \textit{E. coli} properties were analyzed by using the FACS density

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**Fig. 4.** Antibody-binding activity of \textit{E. coli} co-autodisplaying Z-domains and bovine casein. (a) Antibody-binding activity of immobilized \textit{E. coli} co-autodisplaying Z-domains and bovine casein on a polystyrene microplate. (b) Antibody-binding activity of OM layers on a polystyrene microplate.
plot. The density plot is usually used to analyze cell phases, such as singlet and doublet DNA states during the cell proliferation. From the density plot, the width (Y-axis) and cross-sectional area (X-axis) can be obtained simultaneously for a single analyte. As shown in Fig. 3(c), the density plot of *E. coli* autodisplaying Z-domains alone was quite different from that of *E. coli* autodisplaying both proteins. As *E. coli* autodisplaying Z-domains alone had zeta-potential high enough to indicate dispersion in an aqueous solution (∼50 mV, Table 1), and the density plot also indicated dispersion. The density plot of *E. coli* autodisplaying both proteins indicated strong aggregation due to signals outside of the dispersed region. When *E. coli* in the dispersion region were analyzed, *E. coli* autodisplaying Z-domains had a strong fluorescence signal (>10^2 AU), as shown in the enclosed graph of Fig. 3(c). *E. coli* co-autodisplaying both proteins that were in the dispersion region had baseline fluorescence signals (<10^2 AU). These results showed that the strong fluorescence (<10^2 AU) from *E. coli* co-autodisplaying both proteins in an aqueous solution (Fig. 3(b)) was mainly from aggregated cells.

3.3. Immunoassay using *E. coli* with two co-autodisplayed proteins

*E. coli* autodisplaying Z-domains have been used to improve the sensitivity of immunoassays by taking advantage of antibody-binding activity towards F_r region of immunoglobulins to orient antibodies and expose the antigen-binding Fab region [5–6]. *E. coli* autodisplaying Z-domains can be used in immunoassays by (1) immobilizing them on a microplate [7–9] or (2) isolating the OM and layering it on a solid substrate [10–12]. Conventional methods to immobilize *E. coli* on polystyrene microplates have been used after modifying the surface with positive charges. Doing so allows for effective *E. coli* binding because *E. coli* have a negatively charged outer membrane. In this work, the immobilization efficiency of *E. coli* autodisplaying bovine casein was estimated on a bare (unmodified) polystyrene surface. As shown in Fig. 4(a), *E. coli* were immobilized on a polystyrene microplate surface by physical adsorption, and the antibody-binding activity was measured with HRP-labeled antibodies. The chromogenic reaction of TMB was used to estimate the amount of bound antibody by measuring the optical density at 450 nm. As shown in Fig. 4(a), intact *E. coli* and *E. coli* autodisplaying bovine casein had baseline chromogenic signals across the whole range of antibody concentrations. *E. coli* co-autodisplaying Z-domains and bovine caseins had nearly the same sensitivity as *E. coli* autodisplaying Z-domains alone. These results occurred due to the different abilities to adsorb to the polystyrene surface. As shown in Fig. 2(c), *E. coli* co-autodisplaying both proteins had far higher adsorption than did wild-type *E. coli* or *E. coli* autodisplaying Z-domains. *E. coli* autodisplaying both proteins were estimated to bind up to 10 times more than were wild-type *E. coli* or *E. coli* autodisplaying Z-domains. These results show that a polystyrene microplate with immobilized *E. coli* co-autodisplaying both proteins could have the same antibody-binding activity as *E. coli* autodisplaying Z-domains. Therefore, *E. coli* co-autodisplaying Z-domains and bovine casein could be effectively used after immobilization on a bare polystyrene surface.

*E. coli* autodisplaying Z-domains have also been used for immunoassays by using a layer of OM autodisplaying Z-domains on isolated particles [7–9]. The OM layer can also control antibody orientation and improve the sensitivity of immunoassays. As shown in Fig. 4(b), the OM fraction of *E. coli* co-autodisplaying Z-domains and bovine caseins was isolated and layered on a microplate. The antibody-binding activity of the OM layer co-autodisplaying Z-domains and bovine caseins was estimated by mixing different ratios of OM particles from *E. coli* autodisplaying either Z-domains or bovine casein alone. The antibody-binding activity of the OM layer was estimated with HRP-labeled antibodies. As shown in Fig. 4(b), the antibody-binding activity of the OM layer co-autodisplaying Z-domains and bovine casein was the same as the OM layer made by mixing OM particles autodisplaying Z-domains and bovine casein alone in a 1:2 ratio. These results can be explained by the decreased antibody-binding activity of *E. coli* co-autodisplaying both proteins (43% of *E. coli* autodisplaying Z-domains alone in Fig. 2(a)). When the mixing ratio was increased to 2:1, the antibody-binding activity approached that of the OM layer autodisplaying Z-domain alone. These results show that the co-transformed *E. coli* expressed Z-domains and bovine casein at about a 1:2 ratio.

4. Conclusions

Z-domains and bovine casein were co-autodisplayed on the OM of the same *E. coli* by co-transformation of two different autodisplay vectors. SDS-PAGE densitometry indicated that Z-domains and bovine casein were expressed at 3.12 × 10^5 and 1.55 × 10^5 proteins/cell, respectively. *E. coli* co-autodisplaying Z-domains and bovine caseins had the antibody-binding activity of Z-domains and the adhesive properties of bovine caseins. *E. coli* autodisplaying the proteins were analyzed by FACS. *E. coli* co-autodisplaying Z-domains and bovine casein aggregation due to hydrophobic interactions. The antibody-binding activity of *E. coli* co-autodisplaying both proteins was estimated to be 37% of *E. coli* autodisplaying Z-domains alone. The Z-domain activity was estimated after (1) immobilizing *E. coli* and (2) forming an OM layer from *E. coli* co-autodisplaying both proteins. After immobilization on a polystyrene microplate surface, *E. coli* co-autodisplaying both proteins had the same antibody-binding activity as that of *E. coli* autodisplaying Z-domains alone. The OM layer from co-transformed *E. coli* had an estimated 1:2 ratio of Z-domain to bovine casein expression.

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