Short Communication

Constitutive cell surface expression of ZZ domain for the easy preparation of yeast-based immunosorbents

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Kohei Katsurada1,2, Masahiro Tominaga1, Misato Kaishima3, Hiroko Kato1, Toshihide Matsuno2, Chiaki Ogino3,4, Akihiko Kondo1,3,4,5, Jun Ishii1,4,*, Katsumi Takayama2

1 Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
2 Department of Chemistry and Biology, National Institute of Technology (KOSEN), Fukui College, Geshi, Sabae, Fukui 916-8507, Japan
3 Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
4 Engineering Biology Research Center, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
5 Center for Sustainable Resource Science, RIKEN, 1-7-22 Suehiro, Tsurumi, Yokohama 230-0045, Japan

*Corresponding author: Jun Ishii
Engineering Biology Research Center, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
Tel: +81-78-803-6356; Fax: +81-78-803-6192; E-mail: junjun@port.kobe-u.ac.jp

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Easy preparation of yeast immunosorbents
**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; HSA, human serum albumin; IgG, immunoglobulin G; standard deviation, SD.
Abstract
We describe a novel expression cassette that enables efficient and constitutive expression
of the ZZ domain derived from *Staphylococcus aureus* protein A on the yeast cell surface
to easily prepare yeast-based immunosorbents. Using this expression cassette containing
the *PGK1* promoter, a secretion signal derived from α-factor, and a Flo1-derived anchor
protein, we successfully created a yeast-based immunosorbent for human serum albumin.

Keywords: cell surface display, immunosorbent, protein A, yeast
Yeast cell surface display of the ZZ domain, a protein derived from *Staphylococcus aureus* protein A that binds to immunoglobulin G (IgG), has previously been used to create immunosorbent assays [Fukuda et al., 2007; Nakamura et al., 2001; Shibasaki et al., 2007]. The ZZ protein interacts with IgG from various species, including humans and rabbits [Kronvall and Williams, 1969], and therefore yeast *Saccharomyces cerevisiae* cells that display the ZZ domain could potentially be used in immunosassays and affinity purification as an immunosorbent [Shibasaki and Ueda, 2014].

Previously, the ZZ protein was displayed on the yeast cell surface using the 3'-half of α-agglutinin anchor and an exogenic secretion signal peptide derived, respectively, from *S. cerevisiae* Sag1 cell-wall protein and *Aspergillus oryzae* glucoamylase [Fukuda et al., 2007; Nakamura et al., 2001; Shibasaki et al., 2007]. Inducible promoters, such as *GAL1* and *UPR-ICL*, were used to efficiently express the ZZ protein on the surface of the yeast. However, an extensive effort was required to optimize the incubation conditions needed to attain maximum expression levels. In the case of *UPR-ICL* (induced by glucose depletion), a longer incubation time (120 h) was required for efficient ZZ expression [Nakamura et al., 2001]. Using the *GAL1* system (induced by galactose but strongly repressed in the presence of glucose), efficient ZZ expression was obtained for only a limited time [Shibasaki et al., 2007]. In contrast, ZZ expression was found to be less efficient when a constitutive promoter was used with an aim to easily prepare the ZZ-displaying yeast cells [Fukuda et al., 2007]. Thus, the promoters are important for ZZ expression, while the anchor proteins and the secretion signals would also have significant effects on the performance of yeast immunosorbents. The exchange of these elements is therefore necessary for the efficient, constitutive ZZ display on the yeast cell surface.

In this study, we developed a novel expression cassette that mediates the efficient display of ZZ protein on the surface of *S. cerevisiae* cells, even when using a constitutive promoter. The expression cassette includes the *PGK1* constitutive promoter and a fusion protein consisting of the ZZ protein, a Flo1-derived Flo428 anchor protein [Sato et al., 2002], and the α-factor derived secretion signal peptide (including pre-pro region); all components of the cassette including the secretion signal originate from the host organism *S. cerevisiae*, allowing for easy and efficient cell surface expression of ZZ. Besides, the Flo428 anchor permits an easier access of macromolecule (IgG) to the ZZ displayed on the outer cell surface than the 3'-half of α-agglutinin anchor [Sato et al., 2002; Ishii et al., 2016]. Our yeast strain may be used for the detection of albumin in clinical samples, such as human serum and human blood plasma.

First, we constructed the expression plasmid for the ZZ protein, which contains a fusion protein that consists of the α-factor signal peptide and Flo428 fused N- and C-terminally to the ZZ protein, respectively (Figure 1A). The fusion gene was under the control of the *PGK1* promoter and terminator. The *PGK1* gene encodes a...
3-phosphoglycerate kinase that is a key enzyme in glycolysis, and, therefore, its promoter ($P_{PGK1}$) could drive the constitutive ZZ protein expression. To prepare this plasmid, the gene encoding the ZZ domain was inserted into the previously constructed pFGKII426 vector [Suzuki et al., 2015]. The resulting plasmid (pFGKII426-EZZ) and other plasmids to be compared were introduced into the S. cerevisiae BY4741 strain. We then estimated the antibody binding capacity of the resultant yeast displaying ZZ protein on its cell surface using an immunofluorescent assay as described previously [Fukuda et al., 2007]. In short, the yeast cells cultured in the glucose-containing media (SDC) up to 96 h, except the cells harboring the GAL1-inducible plasmid (pGUZZ) [Nakamura et al., 2001]. After 24 h of cultivation in the SDC medium, the cells with pGUZZ were transferred into the galactose-containing medium (SGC) for induction and cultured for an additional 36 h. The cells were recovered and incubated with a fluorescein isothiocyanate (FITC)-labeled chicken anti-protein A antibody [Fukuda et al., 2007]. The FITC-fluorescence of the cell was subsequently measured using a BD FACSCanto II flow cytometer to quantify the anti-protein A antibody bound onto the yeast cell surface (Figure 1B). Yeast harboring the pFGKII426-EZZ constitutive expression plasmid showed a high binding of the antibody with ZZ protein, with a moderate decrease during the late phase of cultivation. The maximal binding of the antibody was 2.4-fold higher compared with yeast harboring a conventional constitutive expression plasmid (pUMGPZZ) [Fukuda et al., 2007], and comparable to those harboring the GAL1-inducible pGUZZ [Fukuda et al., 2007].

We next investigated whether yeast harboring the new expression plasmid could be used as an immunosorbent to immobilize rabbit IgG (Figure 2A). Sixty or eighty micro-liters of yeast cells (OD$_{600}$=10, 1.8×10$^7$ and 2.4×10$^7$ cells, respectively [Day et al., 2004]) displaying ZZ were incubated for 10 or 20 min with different concentrations of rabbit IgG. The amount of immobilized rabbit IgG was determined using an enzyme-linked immunosorbent assay (ELISA) as described previously [Nakamura et al., 2001]. The sigmoid curves were obtained with a series of diluted rabbit IgG solutions (1 - 10$^6$ ng/mL); the detection range of rabbit IgG was 10$^1$ - 10$^4$ ng/mL, which was comparable with a previous study [Nakamura et al., 2001].

Because the ZZ protein interacts with IgG from a number of species (e.g., human and rabbit), with some exceptions such as goat [Kronvall and Williams, 1969], ZZ-displaying yeast could be applied to a sandwich ELISA for the detection of various antigens by using rabbit- and goat-derived IgG. We chose human serum albumin (HSA) as the target antigen, and ZZ-displaying yeast cells (80 µL, 20 min, OD$_{600}$=10) were applied to the sandwich ELISA using an anti-HSA rabbit IgG and horseradish peroxidase (HRP)-conjugated anti-HSA goat IgG (Figure 2B), as described previously [Nakamura et al., 2001]. The detection range of the HSA concentration was 10$^1$ - 10$^4$.
ng/mL, which was comparable with a previous study using the strong, inducible UPR-ICL promoter [Nakamura et al., 2001].

To further explore the practical uses of the immunosorbent developed here, the HSA concentration in the crude samples needed to be monitored. Thus, we assessed the concentration of HSA in human serum and human plasma using sandwich ELISA with the ZZ-displaying yeast. Using the data in Figure 2B as a standard curve, the HSA concentrations were calculated from the absorbance of HRP activity (Table 1). The HSA concentrations determined using this method were roughly comparable with those obtained with high-performance liquid chromatography (HPLC) and a Shodex Protein KW-803 column (Table 1). Although further study is required to evaluate the accuracy of our method, we could demonstrate that the ZZ-displaying yeast prepared in this study can be applied to rapid and simple measurements of HSA concentration in crude samples.

In this study, we have described a novel expression cassette that enables the easy preparation of an immunosorbent to estimate HSA concentrations in crude samples. This was achieved by incorporating the strong, constitutive PGK1 promoter, the Flo428 anchor (428 a.a.), which is longer than the previously used 3’-half of α-agglutinin (320 a.a.) and promotes easier antibody access, and the endogenous α-factor secretion signal peptide for ZZ protein display. Although we have not determined which elements have substantial effects on the ZZ protein display efficiency, the resultant expression cassette resulted in a significantly increased ZZ protein display efficiency compared with the relatively weak ZZ protein expression obtained when using the previous pUMGPZZ constitutive expression plasmid. Compared with inducible systems, our constitutive ZZ protein expression system additionally simplifies the yeast culture conditions. This system could be easily applied to measuring various antigens in crude samples by changing antibodies.

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Declaration of competing interest
The authors declare that there are no conflicts of interest.

Author contributions
KK and JI conceived and designed the experiments through discussion with CO, AK and KT. KK performed the experiments and acquired data with assistance from all
authors. MT and MK contributed to genetic works. HK contributed to analytical works. TM assisted genetic works and data acquisition. AK, JI and KT supervised the research. KK, MT and JI wrote the manuscript. All authors have approved the final manuscript.

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Figure 1. ZZ domain expression on yeast cell surfaces using the constitutive PGK1 promoter, Flo428 anchor, and α-factor secretion signal. A. Diagram of expression plasmids. B. Quantitative estimation of antibody binding capacity of yeast harboring novel and conventional ZZ domain expression cassettes using a FITC-labeled chicken anti-protein A antibody. Up until 24 h, all yeast strains were cultured in SDC (glucose) media. After 24 h, yeast strain harboring pGUZZ was cultured in SGC (galactose) medium for induction, while other strains were continuously cultured in SDC media. Data are presented as the average and standard deviation (SD) of three independent transformants. α: Secretion signal sequence from α-factor peptide, Flo: Flo428 anchor, agg: 3’-half of α-agglutinin anchor, Gss: Secretion signal sequence from Aspergillus oryzae glucoamylase.
Figure 2. Quantitative immunoassays for IgG and HSA using ZZ displaying yeast (harboring pFGKII426-EZZ). (A) ELISA method to detect rabbit IgG and (B) sandwich ELISA method to detect HSA using yeast cells displaying ZZ domain. Shown in inset is the standard curve for HSA ($R^2 = 0.99399$). ELISA, Enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, immunoglobulin G; HSA, human serum albumin. Data are presented as the average and standard deviation (SD) of three independent experiments.
Table 1. Quantification of HSA in human serum and human blood plasma using sandwich ELISA with ZZ-displaying yeast or using HPLC.

| Sample              | Sandwich ELISA (mg/mL)  | HPLC (mg/mL)  |
|---------------------|-------------------------|---------------|
| Human serum         | 37.3±3.2                | 61.9±2.5      |
| Human blood plasma  | 36.7±6.6                | 60.1±2.0      |

- Samples were diluted with various dilution factors and analyzed in independent (biological) triplicates (n=3).
- HSA concentrations were estimated using the standard curve with a linear concentration range from 25 to 500 ng/mL (Fig. 2B). Average and standard deviation (SD) of the estimated HSA concentrations with three different dilution factors are presented.
- HSA concentrations were estimated using the standard curve with a linear concentration range from 50 µg/mL to 5 mg/mL (data not shown). Average and SD of the estimated HSA concentrations with five different dilution factors are presented.