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Comparison of two forms of catalytic antibody displayed on yeast-cell surface

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Abstract

Two forms of the Fab fragment of the catalytic antibody 6D9 were individually displayed on yeast-cell surface in fusion to the C-terminal half of α-agglutinin: one was 6D9 Fab1, in which the light chain of the Fab (Lc) fragment is displayed on cell surface and the heavy chain of the Fab (Fd) fragment is secreted and linked to the Lc fragment with a disulfide bond; the other was 6D9 Fab2, in which the Fd fragment is displayed on cell surface and the Lc fragment is secreted and linked to the Fd fragment with a disulfide bond. Analysis by flow cytometry indicated that some 6D9 Fab2 fragments were unable to construct an appropriate conformation, and that most of the 6D9 Fab1 fragments displayed on yeast-cell surface exhibited higher binding affinity, stability, and catalytic activity. Conformation of the surface-displayed hetero-dimeric Fab fragment mainly depended on the intermolecular disulfide bond between the Lc and Fd fragments. The conformation of 6D9 Fab1 was more stable than that of Fab2. In the reducing environment of solution containing 25 nM DTT, the function of 6D9 Fab2 was almost completely lost. The successful display of 6D9 Fab1 on yeast-cell surface provides a novel approach to the engineering of catalytic antibodies.
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Keywords: Catalytic antibodies; Yeast-cell surface; Fab fragment; Surface display; Molecular evolution

1. Introduction

Catalytic antibodies [1], like natural enzymes, are used to accelerate a chemical reaction with the binding energy of the immune system. More than 100 chemical reactions have been accelerated by catalytic antibodies, which have shown great promise for catalyzing a wide range of distinct chemical transformations [2]. However, few catalytic antibodies have efficiencies that approach those of natural enzymes. The standard approach to prepare a catalytic antibody involves immunizing animals with a stable transition-state analog for the reaction of interest. Although some catalytic antibodies have been reported with efficiencies similar to that of corresponding natural enzymes, in general it has been difficult to obtain highly active catalysts using the standard approach [3]. For this reason, an important challenge has been to find ways of boosting the catalytic activities of these antibodies. One approach to this problem is to attempt to remake original catalytic antibodies by protein engineering techniques. The system of phage display has been used to improve the catalytic activities of original antibodies [4,5]. More recently, display technologies other than phage display have been applied to construct antibody libraries, including ribosome and bacterial displays [6–8]. The system of yeast-cell surface display has also been used to enhance affinity, stability, and expression maturation of proteins [9–11].

The catalytic antibody 6D9 was generated by the standard approach with immunization of the transition-state analog (TSA) 3 in vivo [12] catalyzed hydrolysis of the chloramphenicol monoester derivative 1 to produce the active chloramphenicol 2 (Fig. 1). 6D9 has been developed

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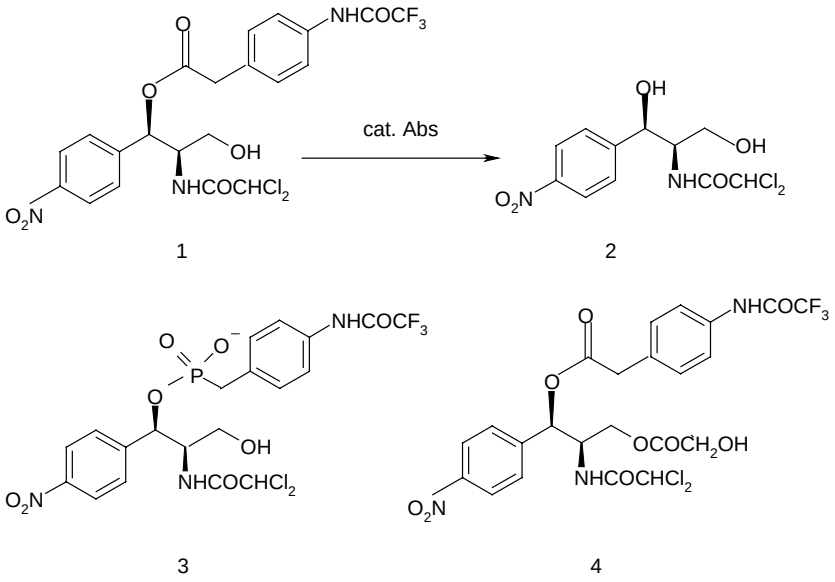

Fig. 1. 6D9 antibody-catalyzed prodrug-activation. The hydrolysis of the chloramphenicol monoester derivative 1 was catalyzed by the catalytic antibody 6D9 to produce the bioactive chloramphenicol 2. The hapten 3, a transition-state analog (TSA) of the hydrolysis, was used for immunization to generate the catalytic antibody. Substrate 4 was used for the assay of catalytic reaction.

through in vitro evolution by phage-display technology [13]. Despite much research, the expression of antibody fragment-encoding genes in *Escherichia coli* (*E. coli*) can be difficult and the yield of functional proteins is often very low [14]. Instead of the phage and *E. coli* display systems, yeast-cell surface display has been used to efficiently express a single-chain Fv and to then evolve antigen-binding affinity and stability [11]. To further apply yeast-cell surface display for the molecular evolution of catalytic antibodies, it is essential to confirm the ability to functionally

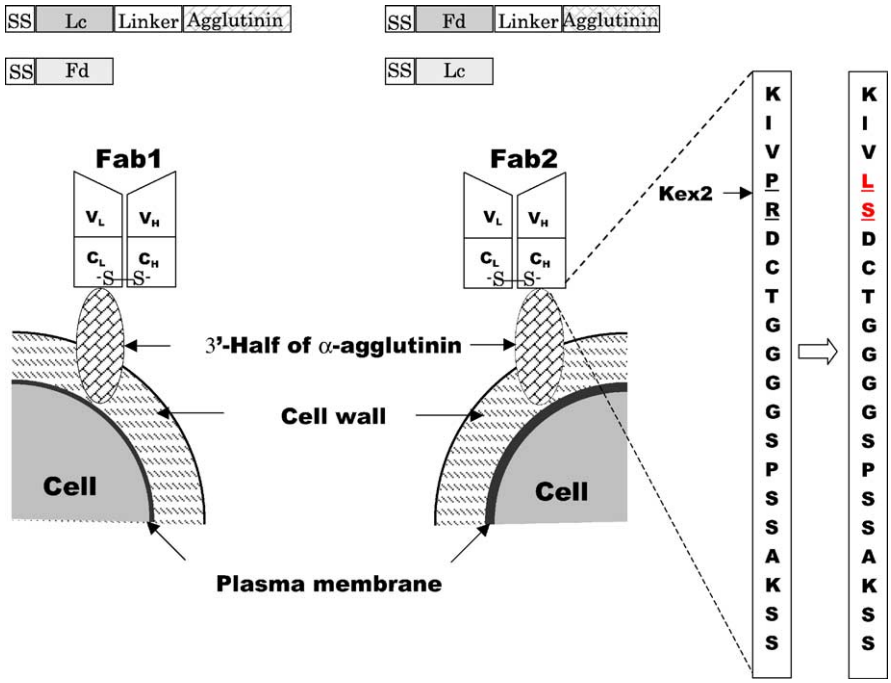

Fig. 2. Construction of 6D9 Fab by yeast display and secretory vectors. SS represents the secretion signal sequence. Left panel: 6D9 Fab1, in which the light chain of the Fab (Lc) fragment is displayed on yeast-cell surface and the heavy chain of the Fab (Fd) fragment is secreted and linked to the Lc fragment with a disulfide bond; right panel: 6D9 Fab2, in which the Fd fragment is displayed on yeast-cell surface and the Lc fragment is secreted and linked to the Fd fragment with a disulfide bond. In the yeast display vector, the Lc and Fd fragments were linked to the C-terminal half of α-agglutinin with a linker [$(Gly)_4Ser$]. In the secretory vector, the Lc and Fd fragments were placed downstream of the secretion signal sequence. By co-expression, 6D9 Fab was displayed on yeast-cell surface in two forms with an intermolecular disulfide bond between the Lc and Fd fragments.

display catalytic antibodies on yeast-cell surface, involving TSA-binding affinity and catalytic activity. The phage display of the catalytic antibody 6D9 Fab has been found more effective than scFv [15].

In the present study, we constructed two forms of the Fab fragment of the catalytic antibody 6D9 for display on yeast-cell surface and compared their binding affinities and catalytic activities on yeast-cell surface. The forms constructed were 6D9 Fab1, composed of the Lc fragment fused to the C-terminal half of α-agglutinin and the Fd fragment (a heavy chain without the constant domains C_H2 and C_H3) [16]; and 6D9 Fab2, with the Fd fragment fused to the C-terminal half of α-agglutinin and the Lc fragment (Fig. 2). We discuss here their suitability for improving the performance of catalytic antibodies on yeast-cell surface.

2. Experimental

2.1. Strains and media

Escherichia coli DH5α [F^-, *endA1*, *hsdR17* (rk^+ mk^+), *supE44*, *thi-1*, λ^-, *recAI*, *gyrA96*, *ΔlacU169*(*ϕ80lacZΔ-M15*)] was used as a host for recombinant DNA manipulation. *Saccharomyces cerevisiae* MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) was used to display the catalytic antibodies. *E. coli* was cultivated in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride). *S. cerevisiae* was grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and appropriate supplements) for the display of the catalytic antibodies.

2.2. Construction of 6D9 Fab genes and transformation in yeast

In the yeast display system, the 6D9 Lc fragment of the light chain or the 6D9 Fd fragment of the heavy chain, fused to the C-terminal half of α-agglutinin with a $(Gly)_4Ser$ linker, is anchored to yeast-cell wall. The Fd fragment or the Lc fragment is joined to the fusion protein by a disulfide linkage in the cells, resulting in the display of the 6D9 Fab fragment on yeast-cell surface (Fig. 2).

The plasmids pICAS [17] and pIRS, a derivative of pICAS, which consists of a *Sac*I–*Kpn*I section of pICAS containing the promoter (*GAPDH*), was inserted in pRS406 [18] together with the genes encoding the secretion signal sequence of the glucoamylase and the 3′-half of α-agglutinin. pICAS was digested with *Sac*II and *Kpn*I and the Fd or Lc fragment was inserted into the *Sac*II and *Kpn*I portion to construct pICAS-Fd and pICAS-Lc as secretory plasmids, respectively. DNA fragments encoding the Fd and Lc genes were amplified from pComb3-6D9 [4] by PCR. The primers used were Fd-f1 5′-CCAGGCCGCGGTGCTTGAATCTGGGGGAG-3′, Fd-r1 5′-GAGCCGGTACCACCTTAAGTACAATCCCTGGGCAC-3′; Lc-f1 5′-CATGGCCGCGGGGTGATGACCCAGACTCC-3′ and Lc-r1 5′-CTCGGTACCCACTCATTCCTGTTGAAGCTC-3′. pIRS was partially digested with *Sac*II and *Nco*I and the Lc fragment, which was obtained by PCR (primer: Lc-f1 and Lc-r2 5′-CTCGACCATGGGGCGAGCCACCGCCACCACACTCATTCCTGTTGAAGCTCTTGAC-3′) followed by *Sac*II and *Nco*I digestion, was inserted. The constructed plasmid was named pIRS-Lc. pIRS was excised with *Sac*II and *Xho*I, and a *Sac*II–*Xho*I-digesting Fd fragment amplified by PCR (primer: Fd-f1 and Fd-r2 5′-GGCGCTCGAGGGCGAGCCACCGCCACCGGTACAATCACTGAGCAC-3′) was inserted to construct pIRS-Fd. When the constructed pICAS-Fd (for secretion of the Fd fragment) and pIRS-Lc (for display of the Lc fragment) were digested with *Xba*I and *Apa*I, respectively, and co-transformed into *S. cerevisiae* MT8-1, the strain MT8-1-Lc-Fd (Fab1) was obtained. pICAS-Lc (for secretion of the Lc fragment) and pIRS-Fd (for display of the Fd fragment) were likewise digested with *Xba*I and *Apa*I,

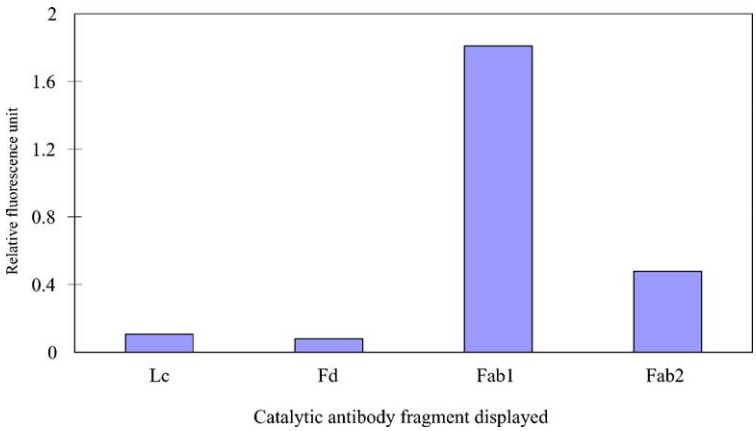

Fig. 3. Comparison of binding affinity between two forms of Fab fragment on yeast-cell surface. Cells were grown in 10 ml SD medium for 24 h (OD_{600} = 1.0). The Fab fragments (Fab1 and Fab2), Fd fragment (Fd), light chain (Lc) and as control the C-terminal half of α-agglutinin (Agg) were displayed on the respective cell surfaces. Binding affinities with FITC-labeled TSA3 were measured in relative fluorescence units (RFU) on the cell surface using a fluorometer (cells detected in 1 ml PBS buffer, pH 7.4, OD_{600} = 10).

respectively, and co-transformed into *S. cerevisiae* strain MT8-1 to obtain the strain MT8-1-Fd-Lc (Fab2).

2.3. TSA-binding affinity analysis

Cells were grown in 10 ml SD medium with shaking at 30 °C for 36 h to OD_{600} = 1.5 to check Fab display. After cultivation, cells were washed with PBS buffer containing 0.1% bovine serum albumin and incubated in 100 μl TSA3-FITC conjugate (10 g/ml in 20 mM PBS, pH 7.4) for 1 h at room temperature. Relative fluorescence units were measured with a Fluoroskan Ascent fluorometer (Labsystems OY, Helsinki, Finland). Data from fluorometer measurements of relative fluorescence units are the average of three separate experiments.

2.4. Evaluation of disulfide linkage in Fab

Cells displaying Fab were washed twice with 0.15 M NaCl and shaken for 1 h at 4 °C in 25 mM PBS (pH 7.4) containing different concentrations of dithiothreitol (DTT). Next, 100 ml of the cells was added to the TSA3-FITC conjugate solution (final concentration 10 μg/ml) and incubated for 1 h. Finally, the cells were washed and the relative fluorescence units were measured by fluorometer.

2.5. Analysis of catalytic activity

Cells were precultivated in 5 ml SD medium at 30 °C for 24 h to OD_{600} = 1.0 and transferred to 100 ml SD medium for large-scale cultivation at 30 °C for 48 h to check catalytic activity. The catalytic activity of 6D9 Fab was examined in hydrolysis of substrate 4 (Fig. 1) with 170 μl cells (OD_{600} = 560) in 50 mM Tris (pH 8.0) at 30 °C for 20 min. The reaction was initiated by adding 20 μl of a stock solution (2 mM) of substrate 4 in dimethyl sulfoxide to 170 μl of 6D9 Fab-displaying cells and 10 μl Tris buffer. Hydrolysis rates were measured by HPLC with 10 μl injection of the reaction solution. Analytical HPLC was performed on a Waters 600 unit equipped with a Waters 490 multi-wave-length detector using a YMC ODS A303 column (YMC Co. Ltd., Kyoto, Japan) eluted with CH_3CN/0.1% aqueous trifluoroacetic acid at a flow-rate of 1.0 ml/min with detection at 278 nm. The observed rate was corrected using the uncatalyzed rate of hydrolysis in MT8-1/pICAS control cells without antibody.

2.6. Analysis by flow cytometer

The binding of 6D9 Fab on the yeast cell surface with FITC-conjugated TSA was evaluated by flow cytometer. Yeast cells displaying 6D9 Fab and control cells, MT8-1 harboring pICAS, were incubated with TSA-conjugated to FITC (10 g/ml) for 1 h and washed with buffer (PBS/0.1% BSA). After washing, samples were analyzed on a FACS Calibur flow cytometer (Beckman-Coulter, Fullerton, CA) with an event rate of ≈4000 cells per second. A total of 10^5 cells was examined for each sample. The population was gated according to light scatter (size) to prevent analysis of cell clumps.

3. Results

3.1. Display of two forms of Fab fragment of catalytic antibodies on yeast-cell surface

In order to display 6D9 Fabs on yeast-cell surface, genes encoding the Lc and Fd fragments of the catalytic antibody

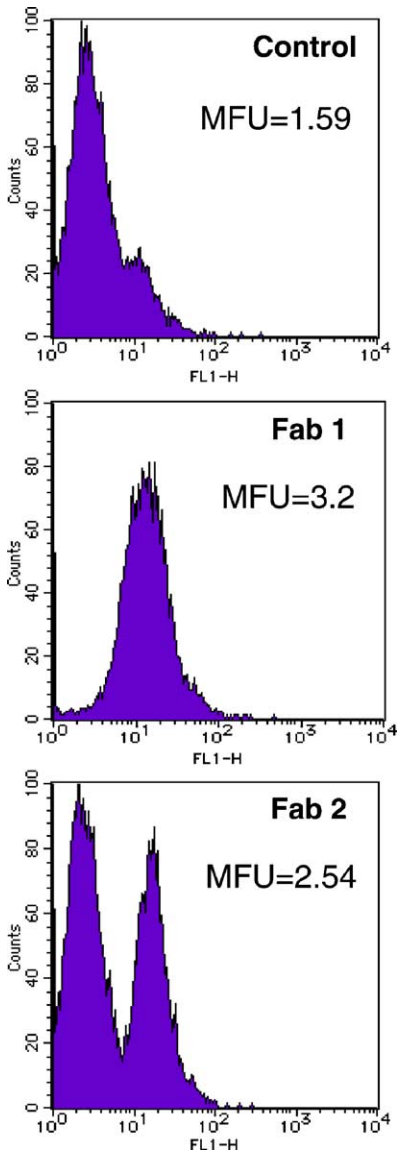

Fig. 4. Flow-cytometric histograms of 6D9 Fab fragments on yeast-cell surface bound with FITC-labeled TSA. Yeast cells displaying Fab1 and Fab2 were reacted with FITC-labeled TSA. Control cells were subjected to the same conditions of reaction. Cells were washed with PBS and analyzed on a FACS Calibur flow cytometer. MFU indicates mean of fluorescence units.

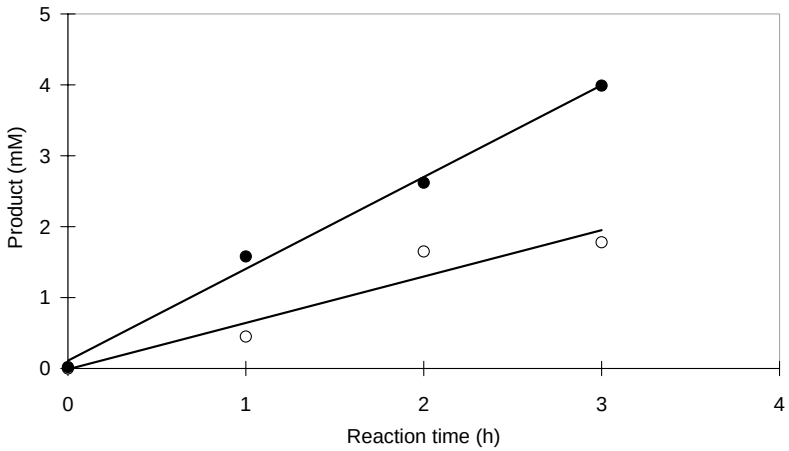

Fig. 5. Catalytic hydrolysis of chloramphenicol monoester derivative by 6D9 Fabs displayed on yeast-cell surface. Comparison between Fab1 (●) and Fab2 (○) displayed on cells was performed. All data are means of three experiments.

6D9 were individually cloned into yeast vectors for secretion and display, and co-expressed in yeast. The 6D9 Fabs were constructed by an intermolecular disulfide bond between the light chain and the Fd fragment of the heavy chain of the 6D9 Fab. The two forms of the Fab fragment displayed on yeast-cell surface were named Fab1 and Fab2 (Fig. 2). When the genes encoding the Lc fragment fused to the C-terminal half of α-agglutinin and the Fd fragment were co-expressed in yeast, Fab1 was displayed on yeast-cell surface. As a recognition site of Kex2 protease [19] was found in the C_H1 region of the Fd fragment at amino acid nos. 111 to 113, P112L and R113S mutations in the Fd fragment were introduced using PCR with the synthesized primer Fd-r2 (Experimental). The Fd mutant fragment fused with the C-terminal half of α-agglutinin and the Lc fragment were co-displayed to construct Fab2 on yeast-cell surface.

3.2. Binding affinity of Fabs displayed on yeast-cell surface

MT8-1-Lc harboring pIRS-Lc to display only the light chain of 6D9 on yeast-cell surface and MT8-1-Fd harboring pIRS-Fd to display only the Fd fragment of the heavy chain were constructed, and the ability of the various surface-displayed 6D9 fragments to bind TSA was assessed by fluorometer. The surface-displayed 6D9 Fab fragment showed higher binding affinity than the single fragments Lc or Fd, and Fab1 higher affinity than Fab2 (Fig. 3). The results were corroborated by flow cytometer results (Fig. 4).

3.3. Catalytic activity of Fabs displayed on yeast-cell surface

Comparison of catalytic activity was performed between the two forms of the 6D9 Fab fragment. An examination was made of the hydrolysis of substrate 4. Equal amounts of cells displaying 6D9 Fab1 or Fab2 were added to 200 mM substrate 4 and antibody-catalyzed reactions carried out at 30 °C in a system containing 10% DMSO/50 mM Tris (pH8.0). HPLC assay showed that surface-displayed Fab1 higher catalytic activity than Fab2 (Fig. 5).

4. Discussion

6D9 Fab1, in which the light chain of the Fab (Lc) fragment is displayed on yeast-cell surface and the heavy chain of the Fab (Fd) fragment is secreted and linked to the Lc fragment with a disulfide bond, showed more efficient catalysis than 6D9 Fab2, in which the Fd fragment is displayed on yeast-cell surface and the Lc fragment is secreted and linked to the Fd fragment with a disulfide bond. The findings suggest that Fab1 on yeast-cell surface exhibits a more appropriate conformation and greater molecular stability.

In our observations, the surface-displayed 6D9 Fab1 and Fab2 fragments showed different features in analysis by flow cytometer, with most of the cells displaying Fab1 able to bind strongly with FITC-conjugated TSA, but some of the cells displaying Fab2 failing to react with TSA and construct an appropriate conformation (Fig. 5). In the natural form of Fab in IgG molecules, a Fab molecule is a 50 kDa fragment of the 150 kDa IgG molecule with a heavy chain lacking the constant domains C_H2 and C_H3. Two heterophilic (V_L–V_H and C_L–C_H1) domain interactions determine the two-chain structure of the Fab molecule, which is further stabilized by a disulfide bridge between C_L and C_H1. In a reducing environment, the Fab fragment loses proper folding and its function. In the present study, treatment of yeast cells displaying Fab with DTT prior to the binding reaction prevented the formation of 6D9 Fab on cell surface and removed the binding ability with FITC-labeled TSA3 through reduction of the specific disulfide bonding between the light chain and the Fd fragment. It was established that Fab1 is more stable than Fab2, although the effect of two mutations must be considered. In the reaction with DTT, 25 nM DTT completely inhibited the binding ability of Fab2, but only 33% of that of Fab1 (Fig. 6).

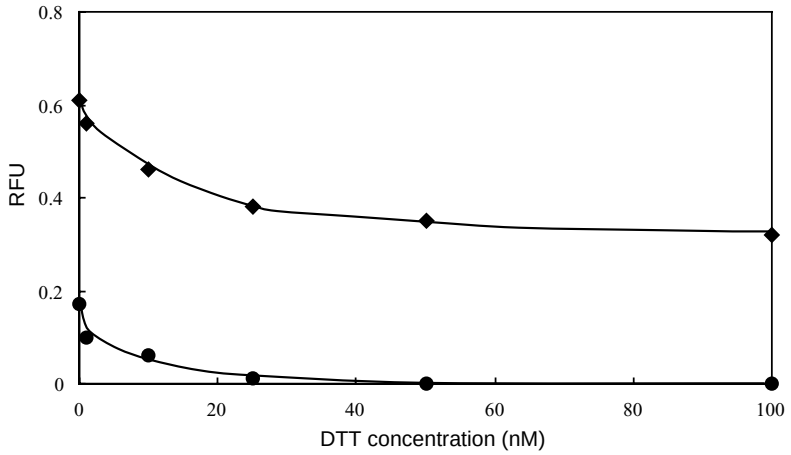

Fig. 6. Effect of dithiothreitol (DTT) on binding affinity of Fabs displayed on yeast-cell surface. Cells (OD_{600} = 5.4) were treated with various concentrations of DTT in PBS (pH 7.4) for 1 h at 4 °C. The binding affinities of Fab1 (◆) and Fab2 (●) are represented as relative fluorescence units (RFU).

In conclusion, it was demonstrated that Fab fragments of the catalytic antibody 6D9 can be expressed in functional form on yeast-cell surface. However, unlike with natural forms of IgG, the surface-display conformation of the antibody makes a difference to activity and stability by how to display on yeast-cell surface. The example of 6D9 Fab1 suggests yeast-surface display with FITC-conjugated TSA as probe provides an opportunity for directed evolution and engineering of catalytic antibodies and the construction and screening of a combinatorial library of catalytic antibodies.

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