

Quantitative evaluation of *Candida antarctica* lipase B displayed on the cell surface of a *Pichia pastoris* based on an FS anchor system

Xing-xiang Liang · Bei-bei Wang · Yu-fei Sun · Ying Lin · Shuang-yan Han · Sui-ping Zheng · Tang-bing Cui

Received: 25 August 2012 / Accepted: 1 November 2012 / Published online: 10 November 2012
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Abstract A new approach is described to quantify the number of enzyme molecules, such as *Candida antarctica* lipase B, that are displayed on the cell surface of *Pichia pastoris*. Enhanced green fluorescent protein (EGFP) and *Candida antarctica* lipase B (CALB) were fused and displayed on the surface of *P. pastoris* by linking to the anchor flocculation functional domain of FLO1p from *Saccharomyces cerevisiae*. Confocal laser scanning microscopy, flow cytometry, and fluorescence spectrophotometry were used to monitor the fluorescence intensity of fused EGFP. Combined with the corresponding protein concentration detected in the medium, a standard curve describing the relationship between the fusion protein concentration and fluorescence intensity were obtained and could be used to number CALB displayed on the cell surface. The results showed that approx. 10^4 molecules of CALB molecules were immobilized on the single *P. pastoris* cell wall based on FS anchor system.

Keywords EGFP · FLO1 · Lipase · *Pichia pastoris* · Protein quantification · Yeast surface display

Introduction

Recently, the development of yeast surface display system heralds a new era of combinatorial bioengineering by virtue of characteristics of well developed cultural technique, post-translational modification as well as the industrial application in the field of biotechnology (Kondo and Ueda 2004). In yeast surface display technology, proteins or enzymes are immobilized on the cell surface by genetic operation and could be used for a wide variety of biotechnological applications (Tanaka et al. 2010), such as the delivery of live vaccines (Georgiou et al. 1997; Liljeqvist et al. 1997), immobilized biocatalysts used bioconversions (Li et al. 2008; Lee et al. 2004; Tanino et al. 2009), bioremediation of heavy metals (Samuelson et al. 2000; Kuroda and Ueda 2006), and as biosensors (Li et al. 2008). As a common host, *Pichia pastoris* proved its competitive ability for appropriate *N*-glycosylation for heterologous protein, inducible expression regulated tightly by alcohol oxidase 1 promoter (pAOX1) as well as little own protein secretion in the supernatant when compared to other common yeasts such as *Saccharomyces cerevisiae* (Wang et al. 2010; Tanino et al. 2006).

To some extent, the efficiency of protein display or enzyme anchoring on the yeast surface which has not been accurately evaluated hinders the practical

X. Liang · B. Wang · Y. Sun · Y. Lin · S. Han (✉) · S. Zheng · T. Cui (✉)
Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Bioscience and Bioengineering, South China University of Technology, Higher Education Mega Center, Guangzhou 510006, People's Republic of China
e-mail: syhan@scut.edu.cn

T. Cui
e-mail: fetbcui@scut.edu.cn

application of cell surface display systems. Shibasaki et al. (2001) calculated the number of enhanced green fluorescent protein (EGFP) displayed as α -agglutinin fused protein on the *S. cerevisiae* cell surface by measuring the fluorescence intensity of the cell pellet suspended in PBS buffer (pH 7.4). But few reports have described the quantification of foreign proteins/enzyme displayed on the *P. pastoris* cell surface for any anchor. In addition, due to differences between the two hosts, the method suggested by Shibasaki et al. (2001) was insufficiently accurate for quantifying surface displaying proteins.

FLO1p from *S. cerevisiae* is a lectin-like anchor protein that plays an important role in flocculation. FLO1p contains 1,200 amino acids and is composed of the following four domains: (1) secretion signal, (2) flocculation functional domain, (3) glycosylphosphatidylinositol (GPI)-anchor attachment signal, and (4) membrane-anchoring domain (FS anchor) (Kondo and Ueda 2004). The FS anchor of approx. 874 amino acids here contains the *N*-terminal flocculation functional domain (FFD) which can recognize and adhere noncovalently to cell-wall components such as mannan carbohydrates (Han et al. 2009; Kondo and Ueda 2004). Moreover, the FS anchor is especially suitable for displaying the enzymes which has their active sites near the C terminus such as *Rhizopus oryzae* lipase (ROL), *Rhizomucor miehei* lipase, RML (Han et al. 2010), so that it is becoming a popular anchor for yeast surface display (Matsumoto et al. 2002; Han et al. 2010). Tanino et al. (2006) displayed the *Rhizopus oryzae* lipase including a pro sequence (ProROL) on *P. pastoris* with FS as anchor, and demonstrated that the FS anchor system in *P. pastoris* is effective in the same manner as in *S. cerevisiae* or even more competitive when taking account of the factor that *P. pastoris* can be cultivated at a higher cell density.

In this study, we used the FS anchor to display a fusion protein of EGFP and *Candida antarctica* lipase B (CALB) on the surface of *P. pastoris*. CALB catalyzes many esterification and trans esterification reactions (Jaeger and Eggert 2002). Here we examined the relationship between the fluorescence intensity of leaked fusion protein and its protein concentration in the culture medium of recombinant EGFP-CALB/CALB-EGFP displaying *P. pastoris*. Using the relationship between fluorescence intensity and protein concentration, we quantified the number of fusion displayed on the *P. pastoris* cell surface. This method

can be extend to other CALB yeast surface displaying systems involving other anchor proteins, thus facilitating more efficient CALB displaying systems and enabling accurate evaluation of displaying efficiency of single host cell.

Materials and methods

Strains and media

Escherichia coli strain Top 10 was used as a host for recombinant DNA manipulation. *P. pastoris* strain GS115 was used to display the fuse protein of EGFP and CALB. *E. coli* cells were grown in LB medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl and 0.1 % glucose). *P. pastoris* was grown in BMGY medium (1 % yeast extract, 2 % peptone, 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.0), 1.34 % yeast nitrogen base without amino acids (YNB), 4×10^{-5} % biotin, and 1 % v/v glycerol), and BMMY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34 % yeast nitrogen base, 4×10^{-5} % biotin, and 0.5–2 % v/v methanol).

Construction of expression plasmids

The fluorescence positive control plasmid pKFS-EGFP was constructed as follows: a *MluI*–*EcoRI* fragment of the EGFP gene was prepared using PCR (primers 5'-ATGAACGCGTATGGTGAGCAAGG GCGAGG-3' and 5'-CTGAGAATTCCTTGTACAG CTCGTCATGC-3') using pEGFP-N1 (GenBank Accession No. U55762) as a template, and inserted into the *MluI*–*EcoRI* site of the integration-type yeast cell-surface-displaying cassette vector pKFSR (stored in our laboratory; Han et al. 2009). The resulting plasmid was referred to as pKFS-EGFP. The pKFS-EGFP-CALB plasmid was constructed as follows: a *MluI*–*ApaI* fragment of the EGFP gene was prepared using PCR (primers 5'-ATGAACGCGTATGGTGA GCAAGGGCGAGG-3' and 5'-TAGTGGGCCCC TTGTACAGCTCGTCCATGC-3') using pEGFP-N1 (GenBank Accession No. U55762) as a template, and an *ApaI*–*EcoRI* fragment of the CALB gene was prepared using PCR (primers, 5'-ATGTGGGCCCCCT ACCTCCGGTTCG-3' and 5'-GTATGAATTCGG GGGTGACGATGC-3') using pKNS-CALB (stored in our laboratory; Su et al. 2010) as a template. The

two genes were linked through *ApaI* and inserted into the *MluI–EcoRI* site of the integration-type yeast cell-surface-displaying cassette vector pKFSR. The resulting plasmid was referred to as pKFS-EGFP-CALB. A pKFS-CALB-EGFP plasmid was constructed as follows: an *ApaI–EcoRI* fragment of the EGFP gene was prepared using PCR (primers, 5'-ACTTGGG CCCATGGTGAGCAAGGGCGAG-3' and 5'-CTGA GAATTCCTTGTACAGCTCGTCCATGC-3') using pEGFP-N1 (GenBank Accession No.U55762) as a template, and an *MluI–ApaI* fragment of the CALB gene was prepared using PCR (primers 5'-ATTG ACGCGTCTACCTTCCGGTTCG-3' and 5'-ATTA GGGCCCGGGGGTGACGATGC-3') using pKNS-CALB as a template. The two genes were linked through *ApaI* and inserted into the *MluI–EcoRI* site of the integration-type yeast cell-surface-displaying cassette vector pKFSR. The resulting plasmid was referred to as pKFS-CALB-EGFP. The plasmids pKFS-EGFP, pKFS-EGFP-CALB, and pKFS-CALB-EGFP were transformed into *P. pastoris* using the lithium chloride method.

Fluorometric, microscopy and flow cytometry (FLC) assays

A fluorescence spectrophotometer (Tecan Infinite M200, San Jose, CA, USA) was used to measure the fluorescence of samples in Tris–HCl buffer (50 mM, pH8.0) on a 96-well plate. A filter with excitation at 480 nm and a filter for emission at 520 nm were used to detect fluorescence intensity. Fluorescence microscopy was carried out using an L710 confocal laser scanning microscope (CLSM) (Shibasaki et al. 2001). The 488 nm line of an argon/krypton ion laser was used for excitation. To measure the fluorescent signal of EGFP, the beam splitter and filter were set to 500–530 nm. The flow cytometer used for the analysis was a Flow Cytometry Quanta SC equipped with an argon ion laser (488 nm wavelength) capable of GFP excitation (Bahl et al. 2004).

Analysis of lipase activity

Lipase activity was measured using 4-nitrophenyl caprylate (*p*NPC; Sigma) and following the release of *p*-nitrophenol, at 405 nm. Free lipases and cell surface-displayed lipases were dissolved in Tris–HCl

buffer (50 mM, pH 8.0), mixed with substrate solution (0.0625 mM *p*NPC, 0.1 % Triton X-100 in lipase assay buffer), and reacted for 5 min before measuring the A_{405} using a kinetic microplate reader. After centrifugation at 10,000×*g* for 1 min, 200 μl of the supernatant was added to a 96-well plate and absorbance was measured. Product yields were determined based on a standard curve prepared using *p*-nitrophenol as a standard. One unit of activity was defined as the amount of enzyme that released 1 μmol *p*NP per min from *p*NPC under the assay conditions. Each sample was assayed in triplicate and the average value was determined (Han et al. 2009).

Western blot analysis and protein analysis

SDS-PAGE using an 8 % (v/v) gel was carried out. The protein extracts separated on the gel were electroblotted on a nitrocellulose (NC) membrane (PALL Corporation, Port Washington, NY, USA) and allowed to react with the anti-GFP HRP-DirecT (MBL International, Woburn, MA, USA). A Bradford protein assay kit was used to determine protein concentration (Tanino et al. 2006; MBL International; Afanassiev et al. 2000).

Results

Detection the EGFP fluorescence intensity on cell surface

Using CLSM and FLC, the fluorescence emitted from the transformants GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP cultivated in BMGY and BMMY liquid medium were investigated. As a control, cells harboring the plasmid pKFS-EGFP (GS115/pKFS-EGFP) were used. After cultivation, all three strains showed obvious fluorescence when evaluate using CLSM and FLC. The light spots of GS115/pKFS-EGFP were much denser and brighter than the other two strains, GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP detected using CLSM (Fig. 1—2a, 2b, 2c). Additionally, the intensity of the light spots from GS115/pKFS-EGFP-CALB was similar to that of GS115/pKFS-CALB-EGFP (Fig. 1—2c), and the distribution of the light spots from the two strains were identical. Upon further analysis using FLC, the fluorescence histograms show the same trend

as observed using CLSM; the signal of GS115/pKFS-EGFP was much stronger than for the other two plasmids (approx. 10 times), and the signal of the other two strains were similar to each other (Fig. 1—3a, 3b, 3c).

Detection of CALB activity

The CALB displayed on recombinant yeasts GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP were analyzed. As a negative control, GS115/pKFS-EGFP showed no CALB activity (data not shown) and the CALB activity displayed on the cell surface of GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP were similar (Table 1). The highest activity of the two strains obtained at 120 h induced by methanol was consistent (approx. 500 U/g). These results agree with those of Tanino et al. (2006) who showed that some protein leaked into the culture medium. Notably, leakage into the medium for both the GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP appeared to approach the same extent since the CALB activities in the supernatants were similar (Table 1, approx. 14 U/mL). Moreover, the OD_{600} of GS115/pKFS-EGFP-

CALB and GS115/pKFS-CALB-EGFP revealed similar growth rates.

These results suggest that the position between *EGFP* and *CALB* in the fusion protein does not affect significantly the activity of CALB displayed on the cell surface or in the medium. A correlation between fluorescence intensity of the cells and lipase activity on the cell wall were expressed as Fig. 2. The fluorescence intensity and lipase activity went up at the same slope with the culture time increased. Besides, when the fluorescence intensity of the displaying yeast increased within the range of 4×10^7 to 10^8 RFU/g, the corresponding lipase activity displayed on the cell surface showed a coincident rise from 150 to 500 U/g dry cell wt.

SDS-PAGE and Western blot analysis

Proteins of interest were extracted from the yeast cell wall and medium and analyzed using SDS-PAGE and Western blot (Fig. 3). The result in SDS-PAGE showed that only one band appeared in the medium and it is of the same molecular size as the main band of extracts from recombinant yeast cell wall. When

Fig. 1 Confocal laser scanning microscopy and flow cytometry assay of *P. pastoris* recombinants strains (cultivated for 120 h): GS115/KFS-EGFP (a), GS115/KFS-EGFP-CALB (b) and GS115/KFS-CALB-EGFP (c). In FLC results, the left histogram shows the negative control of the flow cytometry assay is GS115 and the right histogram represents the *P. pastoris* recombinants strains

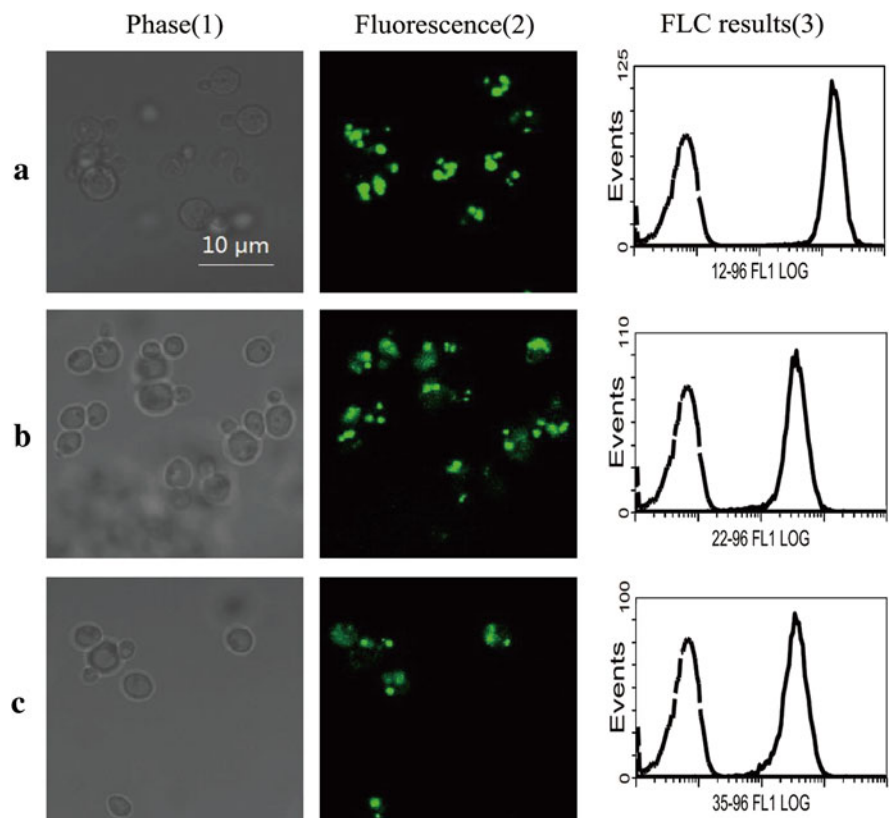


Table 1 Lipase activity of the two fused EGFP-CALB strains

Cultivation time (h)	GS115/pKFS-EGFP-CALB			GS115/pKFS-CALB-EGFP		
	Displayed ^a (U/g)	Medium ^b (U/mL)	OD ₆₀₀	Displayed (U/g)	Medium (U/mL)	OD ₆₀₀
24	165.3	3.29	51.2	187.2	3.32	48.1
48	267.4	5.22	54.9	260.6	5.68	51.1
72	326.9	7.43	55.0	332.2	8.40	50.8
96	366.7	8.22	53.6	385.1	9.33	51.1
120	480.7	12.46	56.7	495.8	14.45	52.9

^a Activity on the cell surface (U/g dry cell)

^b Activity in the medium

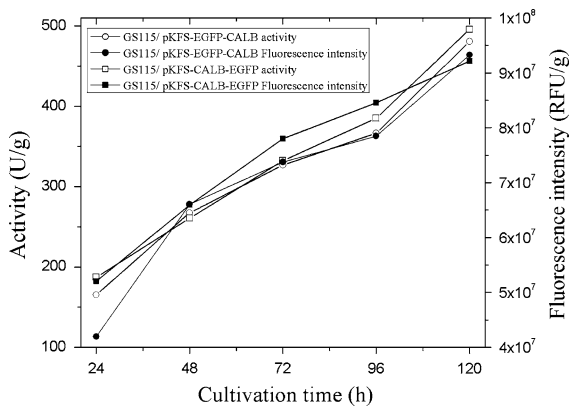


Fig. 2 Correlation between fluorescence intensity and lipase activity (dry cell) in the cell wall

performed with Western Blot, both these proteins of same molecular mass recognized anti-EGFP antibody as well. As mentioned in section of ‘Detection of CALB activity’, we also detected certain activity of CALB and certain fluorescence of EGFP in the medium and on the cell wall. Since no other protein existed in the medium, the protein in the medium must have the peptides or structures of both CALB and

EGFP. Taking the other factors that FS has been fused with CALB and EGFP by genetic engineering and FS is non-covalently adherent to the cell wall as well as the leakage in the medium based on FS surface displaying, the protein appeared in medium could be referred as same as that immobilized on the cell wall. The molecular weights of all fusion proteins displayed on the cell surface or leaked into the medium were similar (approx. 220 kDa) regardless of what strains were used; GS115/pKFS-EGFP, GS115/pKFS-EGFP-CALB as well as GS115/pKFS-CALB-EGFP. This high molecular weight may have been due to glycosylation events in *P. pastoris* (Ardo et al. 2012). Proteins displayed by FS often have *O*-glycosylated or have high-mannose and/or hybrid structures linked to FS fused protein so the molecular mass of the fusion protein is larger than the calculated value (Goossens et al. 2011; Tanino et al. 2006).

Fluorescence and quantification of protein in the medium

Since no other protein detected, this protein concentration in the supernatant was attributed to the fusion

Fig. 3 **a** SDS-PAGE of the protein displayed on the cell surface and leaked in the medium; **b** Western blot results (*M* marker (TaKaRa, Shiga, Japan; D532A), 1 GS115, 2 GS115/KFS-EGFP, 3 GS115/KFS-EGFP-CALB, 4 GS115/KFS-CALB-EGFP, 5 protein from the supernatant)

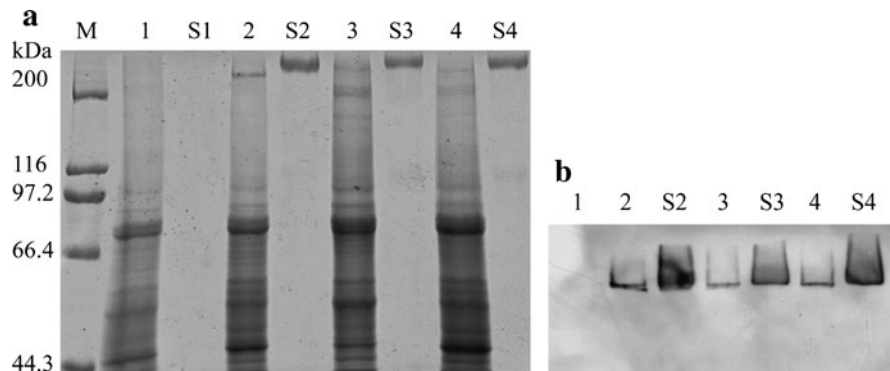


Table 2 Fluorescence intensity and protein concentration in the medium of two fused EGFP-CALB strains

Cultivation time (h)	GS115/ pKFS-EGFP-CALB		GS115/ pKFS-CALB-EGFP	
	Fluorescence (RFU/mL)	Protein concentration ($\mu\text{g}/\text{mL}$)	Fluorescence (RFU/mL)	Protein concentration ($\mu\text{g}/\text{mL}$)
24	3.40×10^5	82.00	2.70×10^5	72.99
48	7.21×10^5	110.37	6.40×10^5	103.62
72	9.39×10^5	127.04	10.70×10^5	116.68
96	11.08×10^5	143.25	11.36×10^5	147.30
120	13.67×10^5	169.37	13.65×10^5	168.92

protein. The protein concentration of GS115/pKFS-EGFP-CALB always kept accordant to that of GS115/pKFS-CALB-EGFP during the regular culture process (Table 2). Additionally, fluorescence spectrophotometry indicated that the increased fluorescence intensity of the two strains were similar as well. In addition, the fluorescence intensity of the two strains increased proportionately and respectively with increased protein concentration.

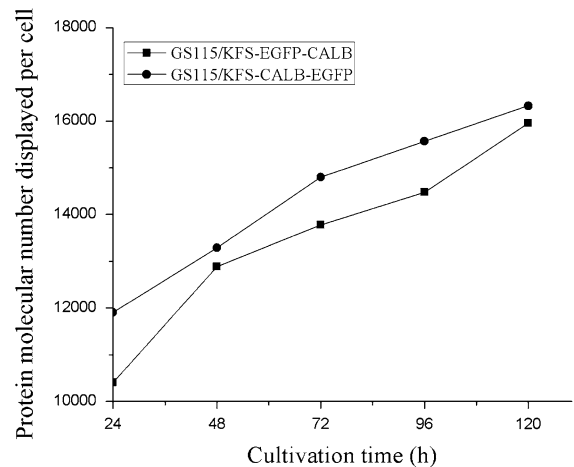
Quantification of molecular number displayed on the cell surface

From the result indicated by Fig. 2, the relationship between the fluorescence intensity and protein concentration for corresponding strain could be described as the following linear formula. The linear formula of GS115/pKFS-EGFP-CALB is $y = 50.85 + 8.44 \times 10^{-5}x$, the linear formula of GS115/pKFS-CALB-EGFP is $y = 48.53 + 8.76 \times 10^{-5}x$, where x is fluorescence intensity (RFU/mL) and y is protein concentration ($\mu\text{g}/\text{mL}$).

In view of the alignment between the fusion protein anchored on the cell wall and the leakage into the medium, we extrapolated the formula above to quantify the protein quantification and calculate the number of molecules on the cell surface.

Paus et al. (2002) indicated that $1\text{OD}_{600} = \sim 5 \times 10^7$ *P. pastoris* cells. Therefore from Avogadro's number, one dalton (Da) has a value of 1.66053×10^{-27} kg; thus, the number of fused protein molecules displayed per cell is $y \times 6.02 \times 10^{23} / (M/N)$, where M is molecule weight (kDa) of fusion protein which is deemed to be 220 kDa because of glycosylation, N is cell number and y is protein concentration ($\mu\text{g}/\text{mL}$).

The numbers and trend of the fusion protein displayed on the *P. pastoris* cell surface during the

**Fig. 4** Number of fusion protein molecules displayed on *P. pastoris* per cell

fermentation process are indicated in Fig. 4. Compared with the number of displayed EGFP molecules per cell calculated by Shibasaki et al. (2001), an approx. order of magnitude as much as 10^4 per cell for the fusion protein displayed on *P. pastoris* surface based on FS anchor system could be obtained.

Discussion

Currently, yeast cell surface display systems have become effective tools for protein evolution and enzyme catalyst development. Many yeast surface systems have been developed using *P. pastoris* as a host (Kondo and Ueda 2004). However, there is no suitable method for quantifying displayed protein; thus, the efficiency of display systems cannot be enhanced and better enzyme display systems using yeast whole cell catalysts cannot be explored. Shibasaki et al. (2001) used *S. cerevisiae* as a host and a GPI-anchor system

such as α -agglutinin. However, a new method for quantifying other displayed proteins in other display systems, particularly for *P. pastoris*, is necessary. In this work, we proposed a method for quantifying molecular numbers of a model enzyme such as CALB displayed on the cell surface of *P. pastoris* based on the FS anchor system.

In this study, we constructed strains GS115/pKFS-EGFP, GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP which were then used to display proteins on the *P. pastoris* cell surface. EGFP was used as a monitoring protein. The results from CLSM and FCL showed that the fluorescence of GS115/pKFS-EGFP is much brighter than that of GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP, but the fluorescence of GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP were similar. CALB activity and protein concentration analysis showed a similar trend for both GS115/pKFS-EGFP-CALB and GS115/pKFS-CALB-EGFP, which reached the same maximum value. Thus, the location of EGFP and CALB in the fused gene does not result in a distinct difference in CALB display.

SDS-PAGE and western blot results demonstrated that the protein displayed on the cell surface and that leaked into the medium had the same molecular weight of approx. 220 kDa and could recognize the EGFP antibody. Thus, the protein displayed on the cell surface and that leaked into the medium were the same and should have the same properties. Additionally, the results of SDS-PAGE combined with those of western blot analysis showed that only the target protein existed in the culture medium, so the fluorescence and protein concentration detected in the medium were due to EGFP-CALB leaked into the medium. During extraction and purification, the EGFP-CALB protein anchored to the cell wall may degenerate and emit green fluorescence. Thus, quantification of CALB displayed on the *P. pastoris* directly is quite difficult.

The relationship between fluorescence and the protein concentration of the fusion protein leaked into culture medium was linear. Using our designs and results above, the correlation between fluorescence intensity and protein concentration in the medium was found to be also appropriate for fused EGFP-CALB displayed on the cell surface. Based on our linear formula, we developed a method for quantifying protein displayed on the *P. pastoris* cell surface.

Compared to the results of Shibasaki et al. (2001), the number of the fusion proteins displayed on the

surface of *P. pastoris* is at least in the same order of magnitude as the number of EGFP displayed on the surface of *S. cerevisiae*). Shibasaki et al. (2001) only observed a single EGFP protein without regard to whether this molecule was part of a fusion protein. Additionally, compared with *S. cerevisiae*, *P. pastoris* has more advantages, such as ease of growth to very high cell density and limited presence of other endogenous proteins in the culture medium (Monika et al. 2009). *P. pastoris* surface display systems appear more promising for industrial application. Quantification of proteins displayed on the *P. pastoris* surface may provide insight into the development of more efficient yeast surface display systems.

Acknowledgments This work was supported by Supported by the grants from National Natural Science Foundation of China (30900017).

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