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ORIGINAL RESEARCH PAPER

Surface display of active lipase in *Pichia pastoris* using Sed1 as an anchor protein

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Abstract A *Pichia pastoris* cell-surface display system was constructed using the Sed1 anchor system that has been developed in *Saccharomyces cerevisiae*. *Candida antarctica* lipase B (CALB) was used as the model protein and was fused to an anchor that consisted of 338 amino acids of Sed1. The resulting fusion protein CALBSed1 was expressed under the control of the alcohol oxidase 1 promoter (p*AOX1*). Immunofluorescence microscopy of immunolabeled *Pichia pastoris* revealed that CALB was displayed on the cell surface. Western blot analysis showed that the fusion protein CALBSed1 was attached covalently to the cell wall and was highly glycosylated. The hydrolytic activity of the displayed CALB was more than 220 U/g dry cells after 120 h of culture. The displayed protein also exhibited a higher degree of thermostability than free CALB.

Keywords *Candida antarctica* lipase B · Immunofluorescence microscopy · Lipase · *Pichia pastoris* · Yeast surface display

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Introduction

Lipases (EC 3.1.1.3) are widely used in various fields including the synthesis of organic compounds, the production of food and detergents, and in the paper industry (Jaeger and Reetz 1998). The extracellular lipase of *Candida antarctica*, lipase B (CALB), which is an efficient catalyst of hydrolysis, esterification and transesterification reactions, has been used in the alcoholysis of oils, for example in the production of biodiesel (Torres et al. 2004; Modi et al. 2006), the synthesis of short-chain flavor esters (Larios et al. 2004; Han et al. 2009), and for kinetic resolution (Fransson et al. 2006; Lou and Zong 2006). However, the high cost of the enzyme is a limiting factor.

One promising area of application is based on the development of novel reaction processes using cells that display enzymes on their surface. The yeast cell-surface display system allows active enzymes of various sizes and conformations to be displayed on the cell surface. The use of different anchor proteins can significantly influence the activities of the displayed proteins (Van der Vaart et al. 1997). The anchor proteins, Flo1 and α-agglutinin, have mainly been used to display lipases on the surface of *Saccharomyces cerevisiae* (Kondo and Ueda 2004). The anchor protein, Cwp2, has been also used similarly (Liu et al. 2009). However, to enable their utilization in large-scale manufacturing processes, the productivity of enzyme-displaying yeast cells must be improved. In

addition, it is necessary to improve the stability of enzymes displayed on the cell surface of yeast.

To address these issues, we have used the anchor protein, Sed1, to display CALB on the surface of *Pichia pastoris*. The coding sequence of CALB was fused to that of the Sed1 anchor protein, and then the fusion protein CALBSed1 was expressed under the control of the alcohol oxidase 1 promoter (p*AOX1*). The thermostability of CALB displayed on whole cells was then investigated.

Materials and methods

Strains, plasmids and medium

Escherichia coli Top10 was used as the host for DNA manipulations, and was grown in LB medium (1% w/v tryptone, 0.5% w/v yeast extract, and 1% w/v NaCl). *Pichia pastoris* strain GS115 was grown in MD medium [1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 2% dextrose, 2% agar], BMGY (1% yeast extract, 2% peptone, 50 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) or BMMY medium (1% yeast extract, 2% peptone, 50 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% methanol). *P. pastoris* and the vector pPIC9K were purchased from Invitrogen Corporation (Carlsbad, CA). The plasmid pKFS-CALB, which contained the mature CALB cDNA, was constructed in our laboratory. *Taq* DNA polymerase, restriction enzymes, T4 DNA ligase, and modification enzymes were purchased from TaKaRa Biotechnology Co., Ltd (Dalian, China).

Construction of plasmids and expression

The mature CALB-encoding fragment was amplified from plasmid pKFS-CALB using the primers, 5′-ATTCGAATTC*GATTACAAGGACGATGACGATAAG*GCCACTCCTTTGGTGAAGCG-3′ (where the *Eco*RI site is underlined and the sequence encoding the FLAG tag is in italics) and 5′-ATAACGCGTTCAGGGGGTGACGATG-3′ (where the *Mlu*I site is underlined). The PCR was carried out using KOD-plus DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The amplified fragment was digested with *Eco*RI and *Mlu*I, and introduced into the *Eco*RI and *Mlu*I sites of the plasmid pPIC9K. The resulting plasmid was named p9K-CALB. The *Sed1* gene was amplified from the genomic DNA of *S. cerevisiae* by PCR using the following primers: *Mlu*I-Sed1 (5′-ATCTACGCGTAAATTATCAACTGTCCTATTATC-3′) and *Not*I–Sed1 (5′-ATTAGCGGCCGCTTATAAGAATAACATAGCAACAC-3′). The amplified fragment was digested with *Mlu*I and *Not*I, and inserted into the *Mlu*I and *Not*I sites of p9K-CALB. The resulting plasmid construct was named p9KSed1-CALB.

Yeast transformation and cultivation conditions

P. pastoris was transformed with *Sac*I-digested p9KSed1-CALB using a Pichia Easy-Comp Transformation Kit (Invitrogen) according to the protocol specified by the supplier. Transformants were isolated by incubation at 30°C for 48 h on MD plates. Then the transformants were precultured in BMGY medium at 30°C. After 24 h, the culture was centrifuged at 6,000×*g* for 5 min and resuspended in BMMY medium containing 1% (v/v) methanol. To maintain the induction of the fusion protein CALBSed1, methanol was added to the culture every 24 h to give 1% (v/v).

Measurement of lipase activity in yeast cells

Transformants were inoculated onto tributyrin agar plates [1% (v/v) tributyrin and methanol were added to MD plates], and the activities of the displayed lipases were estimated from the size of the halos that formed around the colonies. The lipase activity was measured by spectrophotometrically using *p*-nitrophenyl butyrate (*p*NPB) as substrate (see Tanino et al. 2007). The reaction was carried out in 1 ml 50 mM Tris/HCl buffer (pH 8.0) for 5 min at 45°C after the addition of 10 µl cell suspension. One unit of enzyme activity is defined as the amount of enzyme that released 1 µmol *p*-nitrophenol per min.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as reported previously (Kobori et al. 1992). Immunostaining was performed as follows. Induced cells were washed twice in ice-cold water and resuspended at 4°C in phosphate buffered saline (PBS; pH 7.4) supplemented with 1 mg BSA/ml. They were then incubated

with an antibody against FLAG at a dilution of 1:1,000 in a total volume of 1 ml on a rotator at room temperature for 2 h. The cells were then washed with PBS (pH 7.4) and exposed to the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H + L), which was diluted 1:300 in a total volume of 300 µl, for 1 h at room temperature. After three washing steps, the cells were examined using a fluorescence microscope.

Isolation of the cell wall and protein extraction

Glucanase extraction was carried out as described previously (Matsumoto et al. 2002). Cells were harvested by centrifugation at 6,000×*g* and washed three times with iced 10 mM Tris/HCl buffer (pH 7.8) that contained 1 mM PMSF. The cells, buffer and glass beads (0.5 mm diameter) were mixed at 1:2:1 (wet wt/vol/wt) in a microcentrifuge tube and agitated vigorously using a vortex mixer at maximum speed for 1 min five times, with incubation on ice for 1 min between each step. Cell walls were collected by centrifugation for 5 min at 6000×*g*/w, and washed three times with the above-mentioned buffer. Cell walls from 10^9 cells were treated with 0.2 U laminarinase (as β-1,6-glucanase; Sigma) in 200 µl 50 mM sodium acetate (pH 5.4) and incubated overnight at 37°C. After treatment, the extracts were separated by centrifugation for 10 min at 10,000×*g*.

Western blot analysis of CALBSed1

SDS-PAGE was carried out using an 8% (v/v) gel. The protein was blotted onto a PVDF membrane. CALB-Sed1 was detected by binding of the primary rabbit anti-FLAG IgG antiserum, which reacted with the FLAG tag (eight amino acids) at the *N*-terminus of CALB, and the secondary goat anti-rabbit antibody, which was conjugated with horseradish peroxidase (HRP). The protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China).

Measurement of the thermal stability of lipase on yeast cells

The thermostability of the lipase CALBSed1 on the yeast cells was measured as follows. Yeast cells from culture broth were washed twice with distilled water, resuspended in 50 mM potassium phosphate buffer (pH 8.0) and then held at 60°C with shaking at 180 rpm. Aliquots of the cell suspension were removed and washed twice with distilled water and the residual lipase activity on the cells was measured at 45°C. The residual activity was determined using *p*NPB as the substrate, as described above.

Results and discussion

Construction of the expression plasmid

The plasmid for the cell surface display of CALB was constructed as shown in Fig. 1. In pKSed1-CALB, the mature cDNA for CALB (951 bp) was fused to the sequence that encoded the *C*-terminal amino acids of Sed1 and expressed under the control of the p*AOX1* promotor. A FLAG tag (eight amino acids) at the *N*-terminus of CALB was used for immunofluorescent detection.

Enzyme activity

Transformants that harbored the plasmid pKSed1-CALB showed clear halos around their colonies, whereas no halo was observed around the control

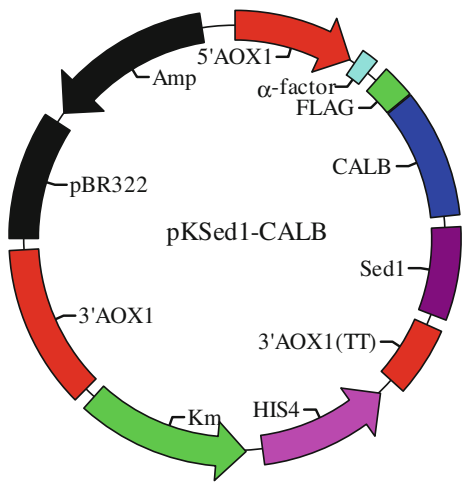

Fig. 1 Schematic diagram of the plasmid encoding the CALBSed1 fusion protein. The sequence encoding CALB was fused at the *N*-terminus to a FLAG tag and at the *C*-terminus to the sequence encoding Sed1 and inserted into pPIC9K after digestion with *Eco*RI and *Not*I. The fusion protein was expressed under the control of the p*AOX* promotor

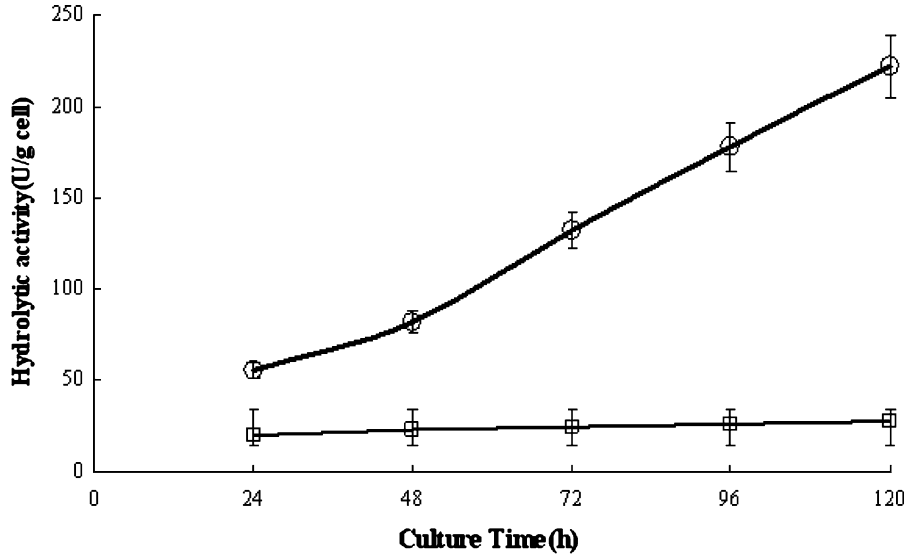

Fig. 2 Time course of the lipase hydrolytic activity in whole *P. pastoris* cells that displayed CALB fused with the Sed1 anchor protein. *Open circle* GS115/Ksed1-CALB, *open square* GS115/KSed1. The data points represent the mean of three independent experiments

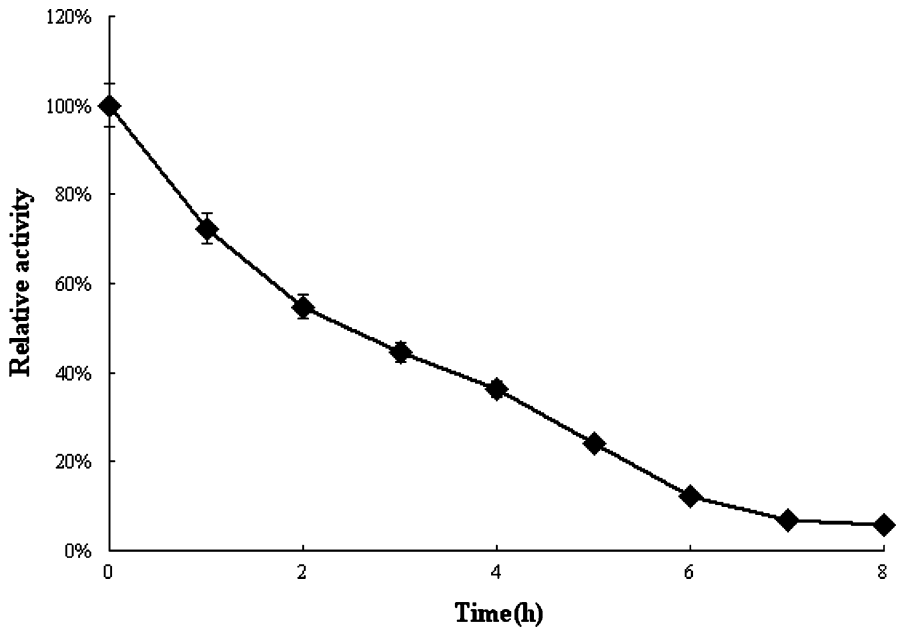

Fig. 3 The thermostability of displayed CALB. *P. pastoris* cells displaying CALB were incubated at 60°C for 1–8 h in 50 mM Tris/HCl buffer (pH 8.0). The residual lipase activity was measured spectrophotometrically. Residual activity was calculated by taking the value at 45°C as 100%. The data points represent the mean of three independent experiments

cells. This suggested that the displayed lipase was functionally active.

The hydrolytic activity of whole GS115 cells that carried pKSed1-CALB was approx. 220 U/g dry cells after 120 h at 45°C in BMMY medium containing 1% (v/v) methanol (see Fig. 2) whereas the activity in the supernatant was negligible. This suggested that the fusion proteins were not secreted into the culture medium but were anchored on the cell surface.

Thermal stability of the lipase on yeast cells

The thermal stability of the lipase displayed on GS115/pKSed1-CALB cells that had been induced for 120 h in BMMY medium containing 1% (v/v) methanol was evaluated (Fig. 3). Whole GS115/pKSed1-CALB cells retained approx. 57% of the original activity at 60°C for 2 h but lost nearly all activity after 7 h. In contrast, free CALB retained

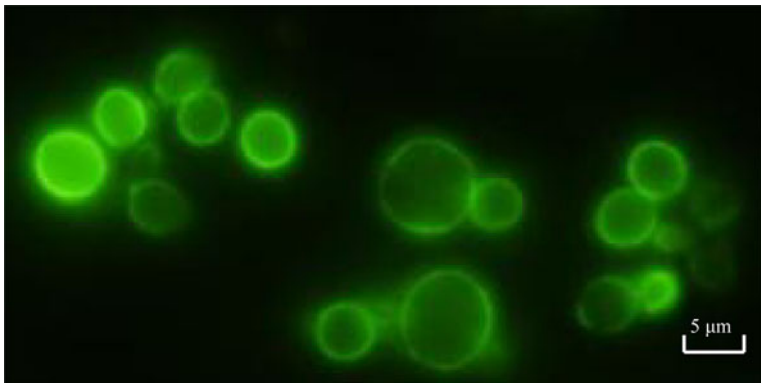

Fig. 4 Fluorescence micrograph *of P. pastoris* cells displaying the FLAG-tagged CALB fusion protein after immunofluorescent labeling. Cells were labeled with an anti-FLAG antibody and Alexa Fluor 488 anti-mouse IgG. The fluorescence emitted at 519 nm after excitation at 495 nm was observed by fluorescence microscopy. Marker bar = 5 μm

only approx. 15% of its original activity at 60°C for 10 min (data not shown). The results suggested that the thermostability of CALB displayed on the cell surface of *P. pastoris* was considerably higher than that of free CALB.

Immunofluorescence microscopy

CALB on the surface of the GS115/pKSed1-CALB cells was detected by immunofluorescence microscopy. The cells were induced for 120 h in BMMY medium containing 1% (v/v) methanol. Green fluorescence was observed around the edge of the GS115/pKSed1-CALB cells (Fig. 4). In contrast, no green fluorescence was observed on the control cells. These results indicated that the fusion protein CALBSed1 was anchored and was displayed on the surface of the GS115/pKSed1-CALB cells.

Localization of the CALBSed1 fusion protein

To determine the localization of the fusion protein CALBSed1, SDS-extracted fractions from cells that had been induced for 120 h in BMMY medium containing 1% methanol were subjected to SDS-PAGE. The fractions were then blotted onto a PVDF membrane and immunostained. As shown in Fig. 5, CALBSed1 was detected in the glucanase-extracted fraction (lane 1), which indicated that the fusion protein was attached covalently to the cell wall. The molecular mass of CALBSed1 was larger than the calculated value of 65 kDa. This was probably due to glycosylation of the protein, which has been described

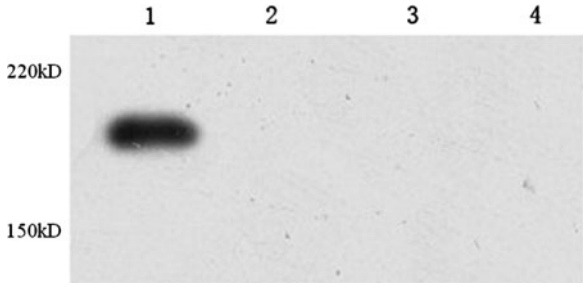

Fig. 5 Immunoblotting of extracts from the *P. pastoris* cell surface and the culture medium. *Lane 1*, glucanase-extracted fraction from GS115/pKSed1-CALB cells; *lane 2*, glucanase-extracted fraction from GS115 cells; *lane 3*, culture medium from GS115/pKSed1-CALB cells; and *lane 4*, culture medium from GS115 cells

previously (Bony et al. 1997; Tanino et al. 2004). However, other modifications might also be associated with the incorporation of Sed1 into the cell wall. In addition, no CALBSed1 was detected in the culture medium (Fig. 5, lane 2).

Conclusions

We have displayed an active lipase on the cell surface of *P. pastoris* using Sed1 as the anchor protein. van der Vaart et al. (1997) had demonstrated that the Sed1 anchor protein could be used in *S. cerevisiae*. The fusion protein CALBSed1 was attached non-covalently to the cell wall of *P. pastoris* although no CALBSed1 was detected in the culture medium (Fig. 5). The thermal stability of CALB displayed on

the cell surface of *P. pastoris* was higher than that of free CALB**.** Therefore, the displayed CALB might be suitable for industrial use as a whole-cell catalyst and provide a new cost-saving substitute for free lipases in industry.

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