

# Improvement in organophosphorus hydrolase activity of cell surface-engineered yeast strain using Flo1p anchor system

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**Abstract** Organophosphorus hydrolase (OPH) hydrolyzes organophosphorus esters. We constructed the yeast-displayed OPH using Flo1p anchor system. In this system, the N-terminal region of the protein was fused to Flo1p and the fusion protein was displayed on the cell surface. Hydrolytic reactions with paraoxon were carried out during 24 h of incubation of OPH-displaying cells at 30°C. *p*-Nitrophenol produced in the reaction mixture was detected by HPLC. The strain with highest activity showed 8-fold greater OPH activity compared with cells engineered using glycosylphosphatidylinositol anchor system, and showed 20-fold greater activity

than *Escherichia coli* using the ice nucleation protein anchor system. These results indicate that Flo1p anchor system is suitable for display of OPH in the cell surface-expression systems.

**Keywords** Cell surface engineering · Flo1p anchor system · GPI anchor system · Organophosphorus hydrolase

## Introduction

Organophosphorus compounds (OPs) such as pesticides, fungicides, and herbicides are highly toxic but are nevertheless used worldwide. The continued use of OPs increases the possibility that delayed cholinergic toxicity and neurotoxicity are induced (Tuovinen et al. 1994). Developed countries inspect imported agricultural food products for toxic OPs, and the growing public concern about their safety in modern agriculture has stimulated the development of methods for OPs detection. Current analytical techniques, such as chromatography, immunoassay, and the use of enzyme biosensors based on inhibition of cholinesterase activity, are highly sensitive but have disadvantages such as high cost or lengthy procedures (Cortada et al. 2009; Lee et al. 2003).

Organophosphorus hydrolase (OPH) effectively hydrolyzes OP esters, including pesticides, such as

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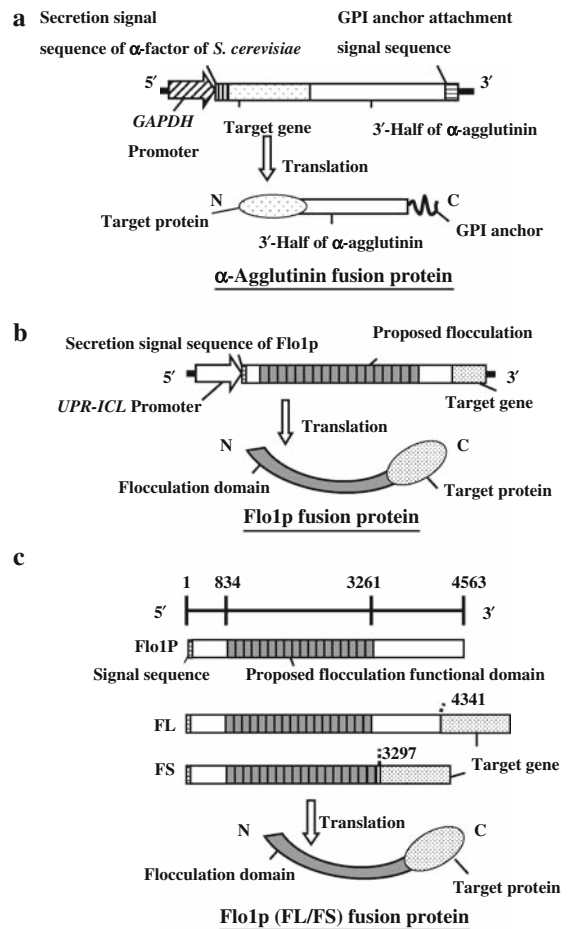
parathion (Tuovinen et al. 1994). We previously constructed a yeast strain that displayed OPH from *Flavobacterium* species; the cell surface-engineered yeast-displayed OPH using glycosylphosphatidylinositol (GPI) anchor system (Takayama et al. 2006). Details of this system are shown in Fig. 1a. Yeasts displaying OPH exhibited greater activity than *Escherichia coli* displaying OPH using the ice nucleation protein (INP) anchor system (Shimazu et al. 2001). However, it is possible that OPH activity might be suppressed in yeast strains when using GPI anchor system. Because the OPH active site is located near the C-terminus, the 3'-half of  $\alpha$ -agglutinin of the GPI anchor might be sterically hindered (Matsumoto et al. 2002). Therefore, we adopted an optimal anchor system for the location of the OPH active site.

In this study, yeasts displaying OPH using Flo1p anchor system were constructed. Flo1p is a lectin-like cell-wall protein in *Saccharomyces cerevisiae*. Detail of Flo1p are shown in Fig. 1b. Using Flo1p anchor system, a recombinant lipase having its active site near the C-terminus was displayed on the yeast and it showed higher activity than that obtained using GPI anchor system. Using Flo1p anchor system, we attempted to construct OPH-displaying yeasts that exhibited greater activity compared with that obtained using GPI anchor system.

## Materials and methods

### Strains and media

*E. coli* strain, DH5 $\alpha$  [ $F^-$ , *endA1*, *hsdR17* ( $r_K^-$ ,  $m_K^+$ ), *supE44*, *thi-1*,  $\lambda^-$ , *recA1*, *gyrA96*, *AlacU169* ( $\phi 80lacZ \Delta M15$ )], was used as a host cell for recombinant DNA manipulation. *Saccharomyces cerevisiae* MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) (Tajima et al. 1985), was used to display OPH from *Flavobacterium* species on its cell surface. LB medium with 50  $\mu\text{g}$  ampicillin  $\text{ml}^{-1}$  was used for bacterial growth and plasmid amplification. The yeast cells were grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) polypeptone, and 2% (w/v) glucose]. Yeast recombinant transformants were selected on plates containing SD-W medium [0.67% (w/v) yeast nitrogen base without amino acids (Difco, MI, USA), 2% (w/v) glucose, 0.002% (w/v) adenine sulfate, 0.002% (w/v) L-histidine-HCl, 0.003% (w/v) L-leucine, and 0.002% (w/v) uracil].



**Fig. 1** The  $\alpha$ -agglutinin fusion gene and Flo1p fusion gene for display of protein on the yeast cell surface. **a**  $\alpha$ -Agglutinin fusion protein. The secretion signal sequence of  $\alpha$ -agglutinin and the GPI anchor attachment signal sequence are genetically fused to the C-terminal region of the target protein. **b** Flo1p fusion protein. The N-terminal regions of target proteins such as OPH are fused to the Flo1p flocculation functional domain. **c** Design of Flo1p (FL/FS) fusion gene for display of protein on the yeast cell surface

HEPES was added to the liquid SD-W medium at a concentration of 200 mM (pH 7.0). Cell growth in the culture broth was measured based on absorbance at 600 nm.

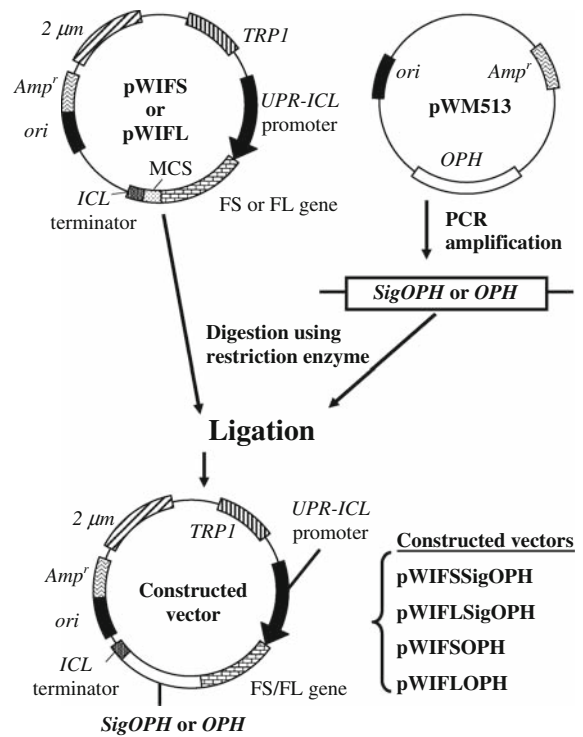
### Construction of plasmids for cell surface display

Plasmids for OPH display using Flo1p anchor system were constructed as follows. The gene encoding OPH was amplified using PCR with pWM513 (Takayama et al. 2006) as the template (Fig. 2). Two forward

primers were used. For the gene encoding OPH, the forward primer was 5'-GGTAGATCTTCGATCGG CACAGG CCATCGGATCAAT-3' (*Bgl*III site, italic). To amplify the signal sequence domain of OPH, we used the forward primer SigOPH-5': 5'-GGTAGATCTATGCAAACGAGAAGGGTTGTGCTCAAG-3' (*Bgl*III site, italic). In this sequence, the upstream region of OPH signal sequence is underlined. The OPH-3' reverse primer was 5'-ACTGCTCGAGCCTCCAG CCTTGTCATCGTCATCCTTGTAATCAGATCCA CCTGACGCCCGCAAGGTC GGTGACAA-3' (*Xho*I site, italic). pWIFS and pWIFL were the plasmid vectors used for expressing OPH on the yeast cell surface (Fig. 2). The FL gene comprised almost all the sequence of Flo1p and the FS gene consisted of part of Flo1p without 1,044 bp of 3' downstream of the FL gene (Fig. 1c) (Matsumoto et al. 2002). The amplified fragment was digested and introduced in each plasmid vector (Fig. 2). The resulting plasmids were named pWIFSSigOPH, pWIFLSigOPH, pWIFSOPH, and pWIFLOPH (Fig. 2). OPH DNA was analyzed using a DNA sequencer (Applied Biosystems, Japan, Tokyo, Japan). The plasmids were transformed into *S. cerevisiae* MT8-1 by the lithium acetate method.

### Measurement of OPH activity

The hydrolytic activities of OPH-displaying cells were assayed by measuring *p*-nitrophenol (PNP) produced during hydrolysis of Paraoxon (Sigma) as the substrate. Cells were precultured in 5 ml SD-W medium [0.1 mM CoCl<sub>2</sub> (Nacalai tesque, Kyoto, Japan)] for 24 h at 20°C and were further cultured for 75 h. Cells were harvested by centrifugation at 3,000×*g* for 5 min at 25°C and washed three times with 50 mM HEPES buffer (pH 8.0) containing 100 μM CoCl<sub>2</sub>. Washed cells were resuspended in 50 mM HEPES buffer (pH 8.0) containing 100 μM CoCl<sub>2</sub> and adjusted to OD<sub>600</sub> = 1. An OD<sub>600</sub> value of 1 = 0.313 mg dry cells/ml<sup>-1</sup> (Horii et al. 2009). Next, 50 μl of 20 mM Paraoxon in methanol was added and reactions were carried out for 24 h at 30°C. After incubation, cells were removed by filtration using a 0.22 μm Ultrafree-MC filter unit (Millipore). PNP in the filtrated solution was detected by HPLC using Hitachi L-7100 and TSKgel for ODS-80Ts (75 × 4.6 mm i.d.) (Tosoh, Tokyo, Japan) at 30°C. The eluent was 90% (v/v) acetonitrile in 50 mM HEPES buffer (pH 8.5) at 0.5 ml min<sup>-1</sup> at



**Fig. 2** Scheme for the construction of plasmids for display of OPH on the yeast cell surface using Flo1p anchor system. The gene encoding OPH is amplified by PCR with pWM513, as the template. pWIFS and pWIFL are the plasmid vectors for expressing the OPH on the yeast cell surface. The amplified fragment is digested and introduced in each plasmid vector. The resulting plasmids are named pWIFSSigOPH, pWIFLSigOPH, pWIFSOPH and pWIFLOPH

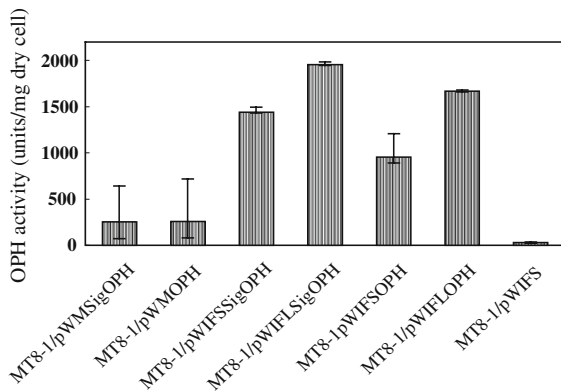
30°C and the eluate was monitored at 415 nm. The retention time of PNP was 12 min. One enzyme unit is defined as the formation of 1 nmol PNP per min per mg dry cells (Takayama et al. 2006).

## Results and discussion

### Effect of the anchor system on OPH activity

As shown in Fig. 3, MT8-1/pWIFLOPH using Flo1p anchor system showed 6-fold greater OPH activity compared with MT8-1/pWMSigOPH or MT8-1/pWMOPH using GPI anchor system, indicating the effective display of OPH with an active site located near the C-terminus.

We investigated the effect of Flo1p length on OPH activity. The optimal whole cell activity of MT8-1/pWIFSOPH was only 960 units/mg cell dry wt



**Fig. 3** Comparison of OPH activity of yeast cells transformed with various plasmids. The y axis shows the OPH activity, which expressed as formation of 1 nmol of PNP per mg dry cells (units/mg dry cell). The x axis shows the strains. Strains MT8-1/pWIFSSigOPH, MT8-1/pWIFLSigOPH, MT8-1/pWIFSOPH, and MT8-1/pWIFLOPH are constructed using Flo1p anchor system (present study). MT8-1/pWM-SigOPH and MT8-1/pWMOPH that were constructed previously (Takayama et al. 2006), are positive controls using GPI anchor system. MT8-1/pWIFS is a negative control using Flo1p anchor system, which was not inserted OPH gene to the vector. Data are averages based on three independent measurements

whereas, MT8-1/pWIFLOPH had 1,600 units/mg dry cell (Fig. 3). Matsumoto et al. (2002) demonstrated that the yeast cells displaying FL-anchor protein had higher flocculation ability than that of the FS-anchor protein and that the flocculation ability of the FL-anchor protein was sufficient to absorb on the cell surface. Therefore, a large amount of the FL-OPH fusion protein would be displayed stably on the cell surface compared with the FS-OPH fusion protein, and MT8-1/pWIFLOPH would exhibit high activity. This result shows that the C-terminal region of the FL-anchor protein (consisting of 3,297–4,341 bp of DNA sequence) was a significant domain for flocculation of the Flo1p-OPH fusion protein on the cell surface.

#### Effect of the OPH secretion signal on OPH activity

Next, the effect of the OPH secretion signal on OPH activity was investigated. MT8-1/pWIFSOPH and MT8-1/pWIFLOPH contained only the secretion signal sequence of Flo1p in the 5'-upstream region of Flo1p-OPH sequence in each transformed plasmid, whereas, MT8-1/pWIFSSigOPH and MT8-1/pWIFL-

SigOPH contained two secretion signal sequences for OPH in the 5'-upstream region of OPH and a secretion signal sequence for Flo1p in the 5'-upstream region of Flo1p in each transformed plasmid. MT8-1/pWIFS-SigOPH showed high activity (1,440 units/mg dry cell) compared with MT8-1/pWIFSOPH (960 units/mg dry cell) (Fig. 3). While the flocculation ability of the FS-anchor protein was insufficient, in contrast to the FL-anchor protein (Matsumoto et al. 2002), the function of the OPH secretion signal might contribute to the efficient expression of OPH, and would complement its activity. Furthermore, MT8-1/pWIFLSig-OPH showed highest activity (1,920 units/mg dry cell). A synergy between the FL/FS anchor and the secretion signal of OPH would be occurred toward activity of OPH on the yeast cell surface.

#### Conclusions

We succeeded in displaying OPH on the yeast cell surface using Flo1p anchor system, which resulted in higher OPH activity than GPI anchor system. In particular, MT8-1/pWIFLSigOPH showed 8-fold higher OPH activity than MT8-1/pWMSigOPH or MT8-1/pWMOPH. Microbial biosensors for OPs have been recently developed using recombinant *E. coli* intracellularly expressing the OPH gene, but the response was slow because of the mass-transport limitation of substrates and products across the cell membrane (Ranina et al. 1996). Therefore, *E. coli* was engineered to express OPH on the cell surface and the INP anchor was used to improve OPH display on the surface of *E. coli* (Shimazu et al. 2001). In the present study, we constructed a biocatalyst for OPs detoxification and detection based on the yeast-displayed OPH using Flo1p anchor system. The OPH activity of this strain was about 20-fold more efficient than that obtained with *E. coli* using the INP anchor system. The strains we used may not only be useful for the detoxification of OP nerve agents but also for the rapid detection of OPs. For example, the fluorescence intensity of GFP depends on pH; GFP displayed on *E. coli* showed 65% attenuation in fluorescence for a pH change from 8 to 6 (Shi and Su 2001). To detect OPs by monitoring pH changes in the fluorescence of GFP based on the protons generated by the hydrolysis performed by OPH,

novel strains that co-display GFP with OPH on the yeast surface are now being developed.

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