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Properties of alkaline protease genetically engineered on cell surface of the yeast *Yarrowia lipolytica*

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ALP2 gene encoding alkaline protease cloned from *Aureobasidium pullulans* HN2-3 was ligated into the surface display plasmid and expressed in the cells of the yeast *Yarrowia lipolytica*. The expressed alkaline protease was immobilized on the yeast cells. The activity of the immobilized enzyme with 6× His tag was found to be significantly higher than that of without 6× His tag. The immobilized enzyme showed lower optimal temperature and a lower affinity for azocasein than the free enzyme purified from *A. pullulans* HN2-3. The thermal stability of the immobilized enzyme enhanced and the pH stability decreased, compared to that of the free enzyme.

Keywords: Yeast surface display, Alkaline protease, *Aureobasidium pullulans, Yarrowia lipolytica*, Marine yeast

Enzymes can be immobilized on various supports either by physical adsorption, covalent binding, ionic interactions or by entrapment. Immobilized enzymes has several advantages over the free one such as enhanced stability, reusability, ease of product separation, greater control over catalysis and process economics[1]. However, changes in the structure or the characteristics of immobilized proteins often occur, due to severe treatment and there are many difficulties in the determination of the immobilization reaction conditions[2].

In recent years, yeast cell surface display of the enzymes has been receiving increased attention[2,3], as it can overcome limitations of the physical and chemical immobilization. The proteins can be regenerated according to the activation of the promoter and are 'naturally' immobilized on the cell surface. The displayed proteins have wide range of biotechnological and industrial applications in cell adhesion, molecular recognition, immobilized biocatalyst, bioconversion, bioremediation, change of cell function, signal transduction, biosensor, live vaccine development and ultra-high-throughput screening for the identification of novel biocatalysts[2,4-6].

So far, amylase, lipase, cellulase, haemolysin, peptide libraries, green fluorescent proteins (GFP), antigen and antibody have been genetically immobilized on the yeast cell surface[3]. The reduction in the activity of the enzyme is an important issue[7] in the development of whole-cell biocatalysts by cell-surface display. Compared with their free forms, surface anchored α-galactosidase, lipase, cutinase and β-lactamase show reduced catalytic activities. This may be steric hindrance, incomplete exposure, unfolded or misfolded structure and repulsion of substrate by the hydrophobicity of the cell wall. In addition, the occurrence of artifacts caused by cell envelope changes is another important problem in cell-surface display. It seems that the length of the cell-wall-spanning region of the carrier protein must exceed a critical length to allow efficient folding of the passenger protein[8].

Earlier, we have found that *Aureobasidium pullulans* HN2-3 can produce alkaline protease[9]. In this study, some properties of the alkaline protease immobilized on cell surface of the yeast *Yarrowia lipolytica* have been investigated and compared with its free form purified from *A. pullulans* HN2-3.

Materials and Methods

Strains and media

The *Y. lipolytica* yeast strain Po1h (genotype: *MatA*, *ura3-302*, *xpr2-322*, *axp1-2*; phenotype: *Ura*$^-$, *AEP*, *AXP*, *Suc*$^+$)[10] was used for cell surface display. The yeast strain was grown in YPD [1.0% (*w*/*v*) yeast extract, 2.0% (*w*/*v*) bactopeptone, 2.0% (*w*/*v*) glucose). The yeast transformants were selected on

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YNB-N5000 [0.17% (*w*/*v*) yeast nitrogen base without amino acids and ammonium sulfate, 1.0% (*w*/*v*) glucose, 0.5% (*w*/*v*) ammonium sulfate]. The PPB medium was used to produce the immobilized alkaline protease by yeast transformants[11].

The *E. coli* strain DH5α [*F⁻endA1 hsdR17* (*rK⁻/mK⁺*) *supE44 thi-1λ⁻ recA1 gyr96ΔlacU169* (φ*80lacZΔM15*)] used in the study for plasmid recovery and cloning experiments was grown in Luria broth (LB). The *E. coli* transformants were grown in LB medium with 100 μg/ml ampicillin or 30 μg/ml kanamycin. The marine yeast strain HN2-3, which could produce a large amount of extra cellular alkaline protease was isolated from sediment of sea saltern in Yellow Sea, China and identified as *Aureobasidium pullulans*[9]. This yeast strain was grown in YPD medium. The medium for alkaline protease production contained 2.5% (*w*/*v*) soluble starch and 2.0% (*w*/*v*) $NaNO_3$, *p*H 6.0.

Plasmids and gene

The surface display vector pINA1317-YlCWP110 which contained the C-terminal end of *YlCWP1* from *Y. lipolytica* was constructed in this laboratory[3]. pMD19-T and pMD-19T simple vectors were purchased from TaKaRa (Japan). The alkaline protease gene (*ALP2*) was cloned from the marine yeast strain *A. pullulans* HN2-3[12].

Alkaline protease display on cells of *Y. lipolytica*

In order to amplify the cDNA*ALP2* gene encoding alkaline protease by PCR, the forward primer used was: P1*Sfi* I ATATGGCCGTTCTGGCC GCTCCTGTTCCTCAGGAT (underlined bases encode *Sfi* I restriction site) and the reverse primers were P2 *Hind* Ⅲ AAGCTTGTGATGGTGATGGT GATGACGACCGCTGTTGTTGTAAAC (underlined bases encode *Hind* III restriction site and bold bases encode 6×His tag) and P3 *Hind* Ⅲ AAGCTTACGACCGCTGTTGTTGTAAAC (underlined bases encode *Hind* III restriction site), respectively. The gene amplification by PCR, the gene expression and alkaline protease display on cells of *Y. lipolytica* were performed as described previously[3]. The surface display vector used in the study was pINA1317-YlCWP110[3] and the resulting plasmid carrying cDNA*ALP2* gene was designated as pINA1317-YlCWP110-*ALP2*. The cells of *Y. lipolytica* Po1h carrying only yeast cassette without cDNA*ALP2* gene were used as controls.

Determination of alkaline protease activity

The cells of the positive transformants carrying cDNA*ALP2* gene and *Y. lipolytica* Po1h carrying only yeast cassette without cDNA*ALP2* gene were grown in the PPB medium for 96 h, respectively. The cultures were washed three times with sterile saline water by centrifugation at 6,000 × *g* and 4°C for 5 min. The recombinant alkaline protease activity immobilized on the yeast cells and the purified protease activity were determined as described previously[13] with minor modifications. One unit of protease activity was defined as the increase of 0.001 absorbance unit at 440 nm.

Cell dry weight of the cultures was determined as described previously[11]. The specific alkaline protease activity was defined as units per g of cell dry weight or units per mg of protein. Protease activity was also estimated using the double plate in which upper medium was the PPB medium (pH 7.4) and bottom medium contained 2.0% (*w*/*v*) milk. Different colonies of the transformants were transferred to the double plates and incubated at 28°C for 3 days. The clear zones around the colonies were observed and photographed.

Alkaline protease production and purification

Two loops of the cells of the marine yeast strain were transferred to 50 ml of YPD medium prepared with seawater in a 250-ml flask and aerobically cultivated for 24 h. Five ml of the cell culture with a final concentration of 10^5 cells/ml was transferred to 45 ml of the production medium and grown by shaking at 180 rpm and 24.5°C for 30 h. Enzyme purification was carried out at 4°C according to the methods described earlier[11]. One liter of the culture grown aerobically for 30 h was used as the starting material for the enzyme purification. The purity and molecular mass of the purified enzyme were analyzed in non-continuous denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[14] using an electrophoresis system (Amersham, Biosciences, Sweden) and stained with Coomassie brilliant blue R-250[15].

Effects of pH and temperature on alkaline protease activity

The effect of *p*H on activity of the alkaline protease immobilized on the yeast cells and the purified enzyme was determined between *p*H 4.0 and 11.0 using the standard assay condition[11]. The *p*H stability was tested for 12 h pre-incubation in appropriate buffers having the same ionic concentrations at different *p*H values ranging from 4.0 to 11.0 at 4°C.

The remaining activity of the alkaline protease was estimated immediately after this treatment with the standard method as mentioned above.

The optimal temperature for activity of the enzyme immobilized on the yeast cells and the purified enzyme was determined between 25 and 65°C at 5°C intervals in the same buffer as described above. Temperature stability was tested by pre-incubating at different temperatures ranging from 0 to 45ºC during 24 h and residual activity was estimated as described above immediately. The relative activity of the pre-incubated sample at 4°C was considered as 100%.

Effect of different metal ions and protein inhibitors on alkaline protease activity

To examine the effect of different metal ions/protease inhibitors, the enzyme immobilized on the yeast cells and the purified enzyme were pre-incubated with the respective compound for 30 min at 4°C. The enzyme activity was assayed in the reaction mixture as described above with various metal ions/protease inhibitors at a final concentration of 5.0 mmol/L. The activity assayed in the absence of metal ions/protease inhibitors was defined as control. The relative activity assayed in the absence of the metal ions/protease inhibitors was considered as 100%.

Determination of kinetics parameter

The K_m of alkaline protease for azocasein was determined according to the method described previously[11].

Results and Discussion

Effects of 6×His Tag on the alkaline protease activity immobilized on the yeast cells

The *ALP2* genes encoding the alkaline protease from *A. pullulans* HN2.3 were amplified from the plasmid carrying cDNA*ALP2* gene with the reverse primers with the bases encoding 6×His tag and without the bases encoding 6×His tag, respectively. After the genes were cloned into the surface display plasmid, they were expressed in the cells of yeast *Y. lipolytica* and the alkaline protease was immobilized on the yeast cells. The activity of the alkaline protease with 6×His tag was significantly higher than that of without 6×His tag. Most of the transformants immobilizing the alkaline protease with 6×His tag and without 6×His tag had the specific activity of over 600 and lees than 400 U/g of cell dry wt, respectively (data not shown). Also, the colonies carrying the alkaline protease with 6×His tag could form bigger clear zones on the double plates than those without 6×His tag (Fig. 1). Thus, the transformants immobilizing the alkaline protease with 6×His tag were used for the subsequent studies.

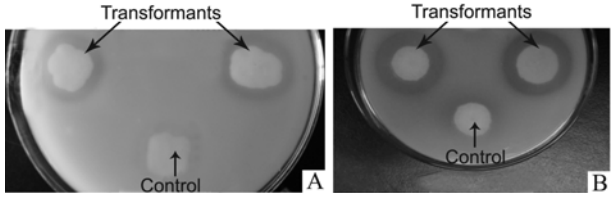

Fig. 1—Clear zones formed on the double plates in which upper medium was the PPB medium and bottom medium contained milk protein. (A): the colonies immobilizing alkaline protease without 6×His tag on their cells; and (B): the colonies immobilizing alkaline protease with 6×His tag on their cells

The activity of the surface-immobilized lipase was reported to increase from 0.8 to 83 units per mg lipase as the spacer length varied from 10 to 92 amino acids[8]. It was believed that the length of cell-wall-spanning region of the carrier protein must exceed a critical length to allow efficient folding of the passenger protein. Therefore, 6× His tag as the spacer used in this study might also play an important role in the efficient folding of the alkaline protease on the yeast cells, resulting in the increased enzyme activity. However, it is still unclear, if the longer His tag could further increase the alkaline protease activity.

Optimum temperature and thermal stability

The alkaline protease immobilized on the yeast cells and the free enzyme showed optimal temperature of 40°C (Fig. 2A) and 52°C (Fig. 2B), respectively,

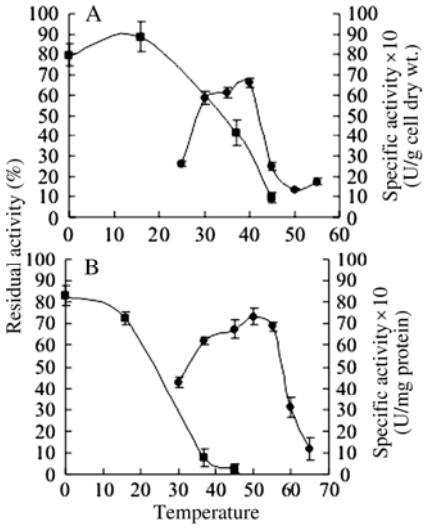

Fig. 2—Effect of different temperatures on the activity (•) and thermal stability (■) of alkaline protease immobilized on the yeast cells (A) and the free alkaline protease purified from *A. pullulans* HN2-3 (B). Data are given mean ± SD, n=3

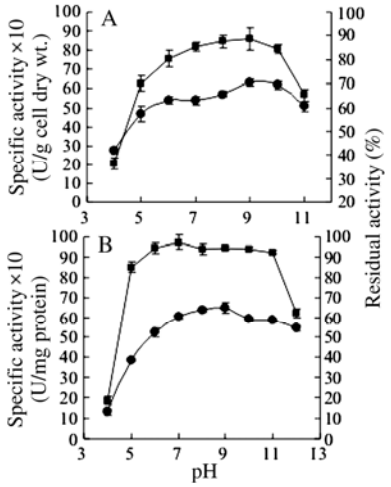

Fig. 3—Effect of different pH on the activity (•) and pH stability (■) of alkaline protease immobilized on the yeast cells (A) and the free alkaline protease purified from *A. pullulans* HN2-3 (B). Data are given mean ± SD, n=3

indicating that the optimal temperature decreased on immobilization. A decrease in the optimum temperature was also observed for immobilized inulinase as compared with the free form, possibly due to changes in physical and chemical properties of the enzyme on immobilization[16]. However, the exact mechanism for the decrease in the optimal temperature of the immobilized alkaline protease is still not known.

Immobilized enzyme after treatment at 16°C for 24 h had the residual enzyme activity of 88.6% of the control, indicating that it was stable up to 16°C. It was inactivated rapidly at temperature higher than 16°C and was completely inactivated at 45°C within 24 h (Fig. 2A). However, the free enzyme (Fig. 2B) showed only 71.4% residual activity after the treatment at 16°C for 24 h, indicating that thermal stability of the enzyme increased on immobilization. The increase of thermal stability on immobilization due to the delayed enzyme activity decay has been reported for several enzymes[16,17]. However, the exact mechanism for increase of thermal stability of the alkaline protease immobilized on the yeast cells is still not clear.

Optimum pH and pH stability

Both the alkaline protease immobilized on the yeast cells, as well as free enzyme showed the maximum activity at pH 9.0 (Fig. 3A, B), indicating that optimal pH did not change on immobilization. The enzyme was stable from pH 5.0 to pH 10.0 (Fig. 3 A), especially in the range of pH 7 and 9.0 and the activity decreased significantly, when pH value was higher than 10.0 or lower than 5.0; the residual activity was over 70.0% after the treatment at pHs from 5.0 to 10.0 for 12 h. In contrast, the free enzyme retained 85.1% of the activity of the control after the treatment in the range of pHs from 5 to 11.0 for 12 h (Fig. 3B), suggesting that the pH stability of the enzyme decreased on immobilization. The exact mechanism of the decreased pH stability of the immobilized alkaline protease is still not clear.

Table 1—Effect of different cations and protease inhibitors (final conc, 5 mmol/L and of control 0 mmol/L) on activity of alkaline protease

[Values represent mean ± SD, n=3]

Ion and inhibitors	Relative activity (%)	
	Enzyme immobilized on yeast cells	Free enzyme
Control	100.0 ± 1.8	100.0 ± 2.6
$ZnCl_2$	103.9 ± 2.5	110.8 ± 2.4
$CaC\ l_2$	101.2 ± 1.9	106.9 ± 1.1
$MgCl_2$	105.8 ± 3.5	135.1 ± 4.1
LiCl	97.7 ± 2.8	100.4 ± 1.1
NaCl	100.4 ± 1.1	137.5 ± 4.3
$CuSO_4$	52.9 ± 2.1	95.4 ± 2.9
$FeCl_2$	74.9 ± 3.5	65.6 ± 3.4
$FeCl_3$	67.9 ± 2.0	44.4 ± 1.4
$HgCl_2$	17.6 ± 2.6	14.3 ± 2.0
$MnCl_2$	105.0 ± 2.9	139.0 ± 5.1
$CoCl_2$	72.0 ± 3.0	63.3 ± 3.1
KCl	99.2 ± 2.7	98.8 ± 3.2
$AgNO_3$	47.5 ± 3.5	34.8 ± 3.1
EDTA	54.2 ± 4.1	48.2 ± 3.0
Iodoacetic acid	91.0 ± 1.0	90.8 ± 3.1
PMSF	5.7 ± 2.8	3.4 ± 2.1

Effect of different cations and protease inhibitors on activity of the alkaline protease

The Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, K^+, Na^+ and Li^+ had no effect on activity of the immobilized enzyme, whereas Mg^{2+}, Mn^{2+}, and Na^+ had activating effect on the purified enzyme, while Ca^{2+}, Zn^{2+}, K^+ and Li^+ had no effect on the free enzyme. The Fe^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Ag^+ and Hg^{2+} acted as inhibitors of both immobilized and free enzymes with Hg^{2+} showing the highest inhibition in both cases (Table 1). This suggested that some biochemical characteristics of the immobilized enzyme were different from those of the free enzyme.

The EDTA inhibited the activity of the immobilized enzyme, suggesting that it was metalloenzyme[18]. The enzyme activity was also strongly inhibited by PMSF, indicating that Ser residues were essential for the enzyme active sites[15]. Iodoacetic acid had a negative effect on the enzyme

activity, suggesting that Cys residues were important for active sites of the enzyme[11]. EDTA, PMSF and iodoacetic acid had the same effect on the activity of free enzyme (Table 1).

Kinetics parameter

The Lineweaver-Burk plot showed that apparent K_m of the immobilized enzyme cells was higher than that of the free enzyme (data not shown). The activity of immobilized enzyme is lowered and the Michaelis constant increases, compared with the free enzyme[19]. These alterations might be due to the structural changes in the enzyme by the immobilization and the creation of a microenvironment in which the enzyme works, different from the bulk solution.

Conclusion

The activity of immobilized alkaline protease with 6× His tag was found to be significantly higher than that of without 6× His tag. The immobilized enzyme showed lower optimal temperature and a lower affinity for azocasein than the free enzyme. The thermal stability of immobilized enzyme enhanced and the pH stability decreased, compared to that of the free enzyme.

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References

1 Vaidya B K, Ingavle C, Ponrathnam S, Kulkarni B D & Nene S N (2008) *Biores Technol* 99, 3623–3629
2 Ueda M & Tanaka A (2000) *Biotechnol Adv* 18, 121–140
3 Yue L X, Chi Z M, Wang L, Liu J, Madzak C, Li J & Wang X H (2008) *J Microbiol Meth* 72, 116-123
4 Won H, Lee S H, Lee K J, Park J, Kim S, Kwon M H & Kim YS (2006) *Biochem Biophy Res Commun* 346, 896–903
5 Becker S, Schmoldt H U, Adams T M, Wilhelm S & Kolmar H (2004) *Curr Opin Biotechnol* 15, 323–329
6 Zhu K L, Chi Z M, Li J, Zhang F L, Li M J, Yasoda H N & Wu L F (2006) *Vaccine* 24, 6046–6052
7 Lee S Y, Choi J H & Xu Z H (2000) *Trends Biotechnol* 21, 45-52
8 Strauss A & Gotz F (1996) *Mol Microbiol* 21, 491–500
9 Madzak C, Gaillardin C & Beckerich J M (2004) *J Biotechnol* 109, 63–81
10 Jolivalt C, Madzak C, Brault C A, Caminade E, Malosse C & Mougin C (2005) *Microbiol Biotechnol* 66, 450–456
11 Ma C L, Ni X M, Chi Z M, Ma L Y & Gao L M (2007) *Mar Biotechnol* 9, 343–351
12 Ni X M, Chi Z M, Ma C L & Madzak C (2008) *Mar Biotechnol* 10, 319–327
13 Inamura H, Nakai T & Muroga K (1985) *Bull Jp Soc Sci Fish* 51, 1915–1920
14 Laemmli UK (1970) *Nature* 227, 680–685
15 George V & Diwan A M (1983) *Anal Biochem* 132, 448–481
16 Catana R, Eloy M, Rocha J R, Ferreira B S, Cabral J M S & Fernandes P (2007) *Food Chem* 101, 260–266
17 Mateo C, Palomo J M & Fernandez-Lorente G (2007) *Enzy Microb Technol* 40, 1451–1463
18 Ramirez-Zavala B, Mercado-Flores Y, Hernadez-Rodriguez C & Villa-Tanaca C L (2004) *FEMS Microbiol Lett* 235, 369–375
19 Krajew B (2004) *Enzy Microb Technol* 35, 126–139