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Full Length Research Paper

Characterization and stability of D-amino acid oxidase and catalase within the permeabilized recombinant *Pichia pastoris* cells

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An effort was devoted to the detailed characterization and stability analysis of D-amino acid oxidase (DAAO) and catalase (CAT) in the permeabilized cells, involved in a summary of our previous works. It was observed that the culture conditions, the permeabilization treatments, the enzyme leakage and pH value of solution except the enzyme expression levels, displayed a significant effect on the enzyme stability. About 96% of the CAT activity in the cetyltrimethylammonium bromide (CTAB)-permeabilized cells was inhibited when treated with sodium azide (0.8 M, 30 min), while no visible effect on the activity and stability of DAAO was detected. Though, almost 100% of the CAT activity in the CTAB-permeabilized cells could be eliminated by the alkali treatment (pH 11.5, 30 min), both the activity and stability of DAAO were slightly impaired. The half-life of DAAO could be up to 15.5 days by treating the CTAB-permeabilized cells with 1% glutaraldehyde (GLA). Additionally, it was noted that the gene sources and expression hosts might play an important role on the stability of DAAO and CAT in permeabilized cells.

Key words: *Pichia pastoris,* D-amino acid oxidase, catalase, stability, permeabilized cells.

INTRODUCTION

D-Amino acid oxidase (DAAO, EC 1.4.3.3) is a significant flavoprotein that catalyzes the O_2-dependent transformation of α-amino acid substrates into the corresponding α-keto acids, hydrogen peroxide (H_2O_2) and NH_3 (Pilone and Pollegioni, 2002; Tishkov and Khoronenkova, 2005; Nidetzky et al., 2007). This enzyme has been exploited in several different processes with respect to biocatalytic synthesis and analytical purposes (Pilone and Pollegioni, 2002; Tishkov and Khoronenkova, 2005). Both in the presence and absence of catalase (CAT), DAAO has several biotechnological applications, including the production of α-keto acids (Brodelius et al., 1982; Buto et al., 1994; Upadhya et al., 1999), 7-aminocephalosporanic acid (7-ACA) (Parmar et al., 1998), resolution of racemic mixture of amino acids (Tu and McCormick, 1972) as well as the analytical determination of D-amino acids (Sacchi et al., 1998).

DAAO from the microorganisms, especially *Trigonopsis variabilis*, are the prime candidate for commercial applications because it displays good activity toward cephalosporin C and is reasonably resistant to the oxidants (O_2, H_2O_2) (Pilone and Pollegioni, 2002; Pollegioni et al., 2004; Tishkov and Khoronenkova, 2005). For large-scale industrial enzyme production, DAAO genes from *T. variabilis* have been isolated and

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Abbreviations: DAAO, D-Amino acid oxidase; **CAT,** catalase; **7-ACA,** 7-aminocephalosporanic acid; **CPC,** cephalosporin C; **CTAB,** cetyltrimethylammonium bromide; **GLA,** glutaraldehyde; **DCW,** dry cell weight.

constructed with high expression system in other strains for example, *Schizosaccharomyces pombei* (Isoai et al., 2002), *Pichia pastoris* (Yu et al., 2002; Zheng et al., 2006) and *Escherichia coli* (Alonso et al., 1999; Lin et al., 2000; Kim et al., 2008), for large-scale industrial enzyme production. The recombinant host cells are generally natural and robust producers of CAT. Therefore, use of whole-cell catalysts containing DAAO and CAT would demonstrate disadvantage in some bioconversions where CAT produces negative effects, for example, production of 7-ACA by conventional two-enzyme process (Luo et al., 2004 a,b; Tan et al., 2006). However, it is no doubt suitable for other bioconversions, in particular for the enzymatic production of α-keto acids (Brodelius et al., 1982; Upadhya et al., 1999) or the production of 7-ACA by new three-enzyme process (Lopez-Gallego et al., 2005; Tan et al., 2010).

Immobilization of DAAO on carriers can enhance the enzyme's persistence against inactivation and facilitate continuous re-use of this enzyme (Fernandez-Lafuente et al., 1999; Betancor et al., 2003), but this suffers many complicated processes, which inactivated DAAO seriously, rendering poor activity recovery in the immobilized derivatives. The carrier-immobilized DAAO is relatively expensive and the cost of per unit of activity was evaluated to be at least 10-fold more than that of the whole-cell catalysts (Vicenzi and Hansen, 1995).

Whole-cell catalysts are great promise for enzyme application. Nevertheless, whole cells generally exhibit comparatively low activities of intracellular enzymes because of the permeability barriers of the cell wall/membrane to substrates and products. To remove the permeability barriers, various methods have been proposed (Vicenzi and Hansen, 1995; Upadhya et al., 1999; Sekhar et al., 1999; Moreno et al., 2004). Permeabilized cells display autolytic process leading to solubilization of cell components and hence decrease in the stability of the intracellular enzymes (Flores et al., 1996). To improve this problem, cell immobilization by some means was recommended previously (Brodelius et al., 1982; Jirku and Turkova, 1987; Flores et al., 1996; Upadhya et al., 2000; Beck et al., 2003; Nahalk et al., 2008).

In our previous work, DAAO and CAT exhibited the noteworthy stability in the permeabilized *P. pastoris* cells (Tan et al., 2010) and had a good conversion behavior toward cephalosporin C (CPC) and D-phenylalanine (aromatic D-amino-acid) (Tan et al., 2006, 2007). In this study, we focused on detailed characterization and analysis of the stability of the two cellular enzymes.

MATERIALS AND METHODS

D-Alanine, cetyltrimethylammonium bromide (CTAB) and sodium azide were purchased from Fluka Co. 2,4- Dinitrophenylhydrazin and glutaraldehyde (GLA) were obtained from Merck (Germany). All other chemicals were of analytical grade. The recombinant *P. pastoris* strain was kindly donated by Professor Yuan. The components of culture media of this strain were described in a previous paper (Yu et al., 2002).

Cell culture and enzyme expression

The *P. pastoris* cells were inoculated to 100 ml of the buffered glycerol-complex medium in a 500 ml flask and incubated with shaking at 30°C (approximately 18 h). The seed culture was used as inoculums for fermentation in shaking flask and a 1.0 L fermentor. Expression of DAAO and CAT in shaking flask was carried out by inoculating the seed culture (10%, v/v) to 200 ml of the basal salt medium in a 500 ml flask. After culture at 30°C for 24 h with shaking, the cells were collected and added to 200 ml of the inductive basal salt medium, where the glycerol was replaced entirely by 5 ml of methanol. Periodically, the cell cultures were fed with methanol to a final concentration of 5% each day and withdrawn for enzyme assays. The enzyme expression in the fermentor has been well described previously (Tan et al., 2007).

Cell treatments

Ultrasonic disruption and permeabilization treatments of cells were performed by the methods reported previously (Tan et al., 2007, 2010). Treatment of the permeabilized cells with sodium azide was carried out by the following procedure: 0.31 g (dry cell weight, DCW) of the CTAB-permeabilized cells was suspended in 10 ml of cold pyrophosphate buffer (50 mM, pH 8.5) with sodium azide at different concentration. The treated cells were collected by centrifugation at intervals and washed twice with the cold buffer. Then, these cells were used for enzyme assays. Treatment of the CTAB-permeabilized cells with GLA was performed by the addition of the cells (0.31 g DCW) to 10 ml of GLA solution with different concentration (0.1 to 2%, aqueous w/v) in 25 mM potassium phosphate buffer (pH 7.0). After incubation at 4°C for 30 min with constant shaking, the collected cells were washed twice with the same buffer and used for enzyme assays.

Analytical procedures

The stability of DAAO and CAT within the permeabilized cells was assayed by the following methods: the cell suspensions were incubated at different temperature (4 or 30°C). Aliquots of the cell suspension were withdrawn at different time intervals and the cells were collected for assays of enzymatic activities.

DAAO activity was determined with the permeabilized cells as a described method (Brodelius et al., 1982; Yu et al., 2002). One unit of DAAO activity corresponded to the formation of 1 µmol min^{-1} of pyruvate at 37°C. CAT activity was assayed spectroscopically at 240 nm (Aebi, 1984). One unit of CAT activity is defined as the amount of enzyme required to degrade 1 µmol of H_2O_2 per min under standard conditions.

RESULTS AND DISCUSSION

The stability of cellular DAAO and CAT with different expression levels

DAAO and CAT in the cells with different expression levels were sampled from the fermentor fermentation at two different periods. After treatment with CTAB, the stability of the cellular enzymes was assayed. As shown in Figure 1, there was no significant change in the stability of the cellular DAAO or CAT. The half-lives of

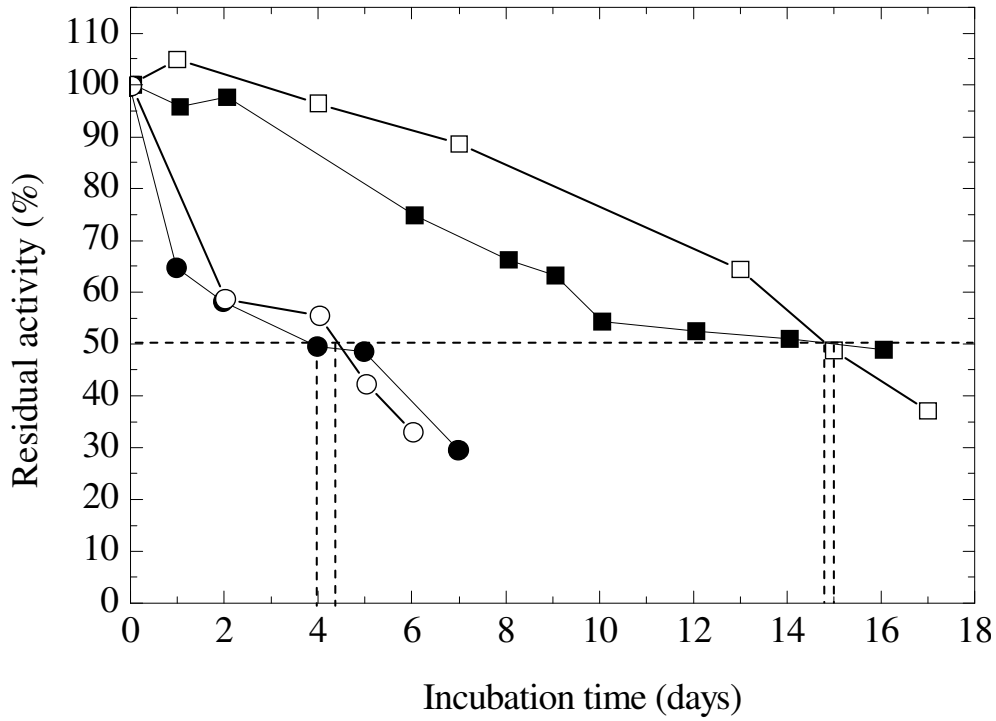

Figure 1. Stability of the cellular DAAO and CAT with different expression levels. The cells were cultured in a 1 L fermentor and the cell cultures were withdrawn at about 50th and 60th h, obtaining 156.8 U/g DCW of DAAO (□) and 11385 U/g DCW of CAT (○), 293 U/g DCW of DAAO (■) and 15,984 U/g DCW of CAT (●) in the CTAB-permeabilized cells, respectively. The permeabilized cells were stored at 30°C for enzyme assays.

DAAO and CAT in both samples were around 14 and 4 days, respectively. This might suggest that the different enzyme expression levels under the same culture conditions were not the significant impact factor of their stability. It was possible that the binding force between enzyme proteins and peroxisomal structures (peroxisomes were regarded as the location of DAAO and CAT *in vivo* (Upadhya et al., 2000) would be immobile as long as enzyme molecules were generated in the cell, regardless of the enzyme amount.

The stability of cellular DAAO and CAT expressed in the flask and fermentor

The expression of DAAO and CAT was conducted at the shaking flask and a 1 L fermentor. Figure 2 shows the relationship among fermentation time, biomass and enzyme activities. The cells were harvested after 60 h of the fermentor fermentation for obtaining the highest DAAO activity (Tan et al., 2007). On the other hand, the maximum enzymatic activities were achieved after 72 h of the shaking flask fermentation (2872 U/l of DAAO, 116906 U/l of CAT, along with 10.3 g DCW/l of biomass). Thus, comparing with the fermentor fermentation, it appeared that the shaking flask fermentation had a litter delay in occurrence of the highest enzymatic activities.

Furthermore, the stability of the cellular enzymes was assayed by incubating these permeabilized cells at 30°C. The results are shown in Figure 3. The half-lives of cellular DAAO and CAT expressed in the fermentor were 14.5 and 4 days, respectively, compared with 5 days and 16 h of those expressed in the shaking flask. It was suggested that the culture conditions exhibited a significant effect on the stability of cellular enzymes. This result implied that the stability of enzymes in permeabilized cells can be improved by the optimization of the culture conditions.

Effect of the permeabilization treatments on the enzyme stability

In our previous study, it was found that the permeabilization treatments displayed an important effect on the stability of cellular enzymes (Tan et al., 2010). The CTAB-permeabilized exhibited the highest activity and the lowest stability of DAAO, while the frozen-melted cells presented the highest stability and the lowest activity of DAAO. There was a negative relationship between the stability and the apparent enzymatic activities. These results might demonstrate a trend that the enzyme

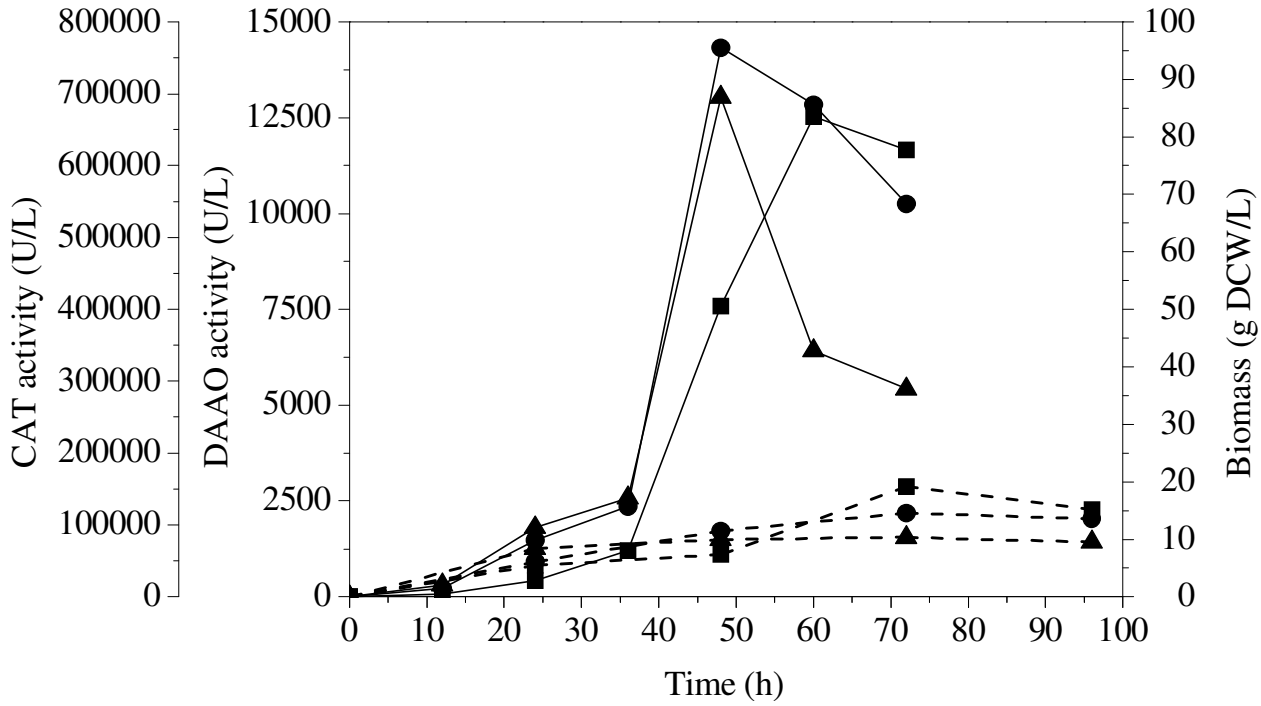

Figure 2. Expression of DAAO and CAT in the shaking flask (the broken line) and fermentor (the solid line). In this study, the enzymatic activities here presented were assayed with the CTAB-permeabilized cells. DAAO (■); CAT (●); Biomass (▲).

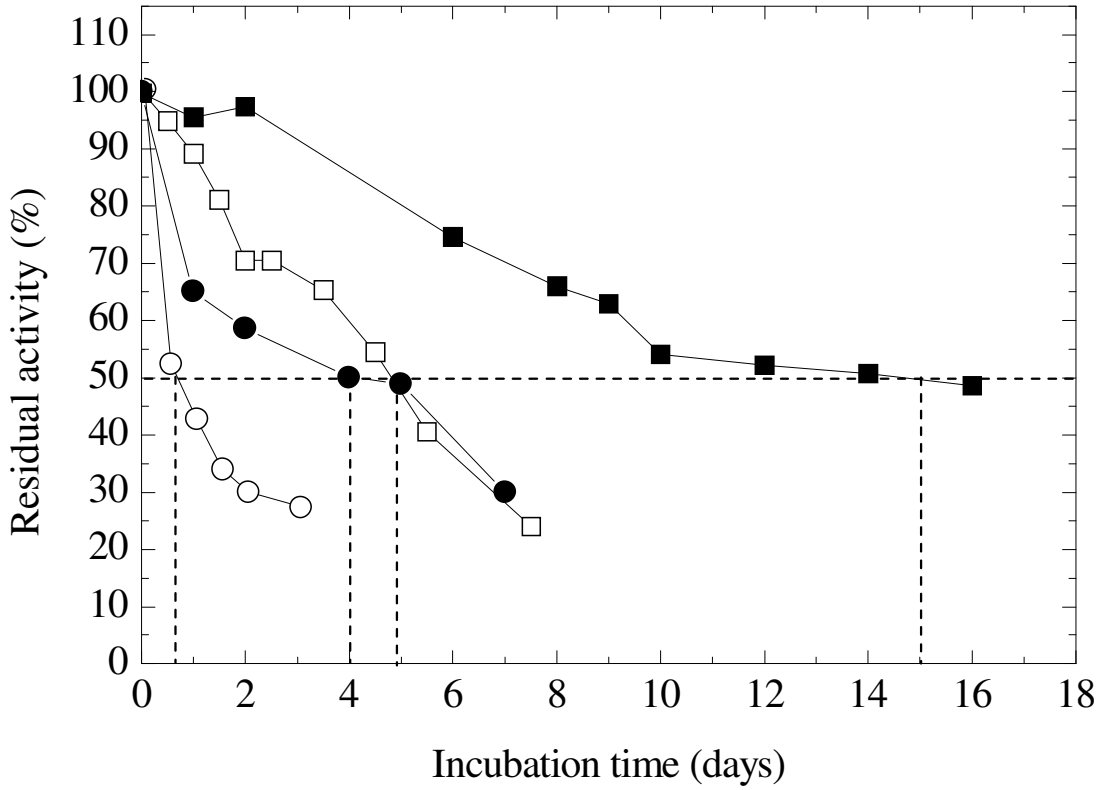

Figure 3. Stability of the cellular DAAO and CAT expressed in the shaking flask and fermentor. The enzymatic activities were assayed with the CTAB-permeabilized cells under incubation at 30°C. Expression in the shaking flask: 279 U/g DCW of DAAO (□); 11350 U/g DCW of CAT (○). Expression in the fermentor: 293 U/g DCW of DAAO (■); 15,984 U/g DCW of CAT (●).

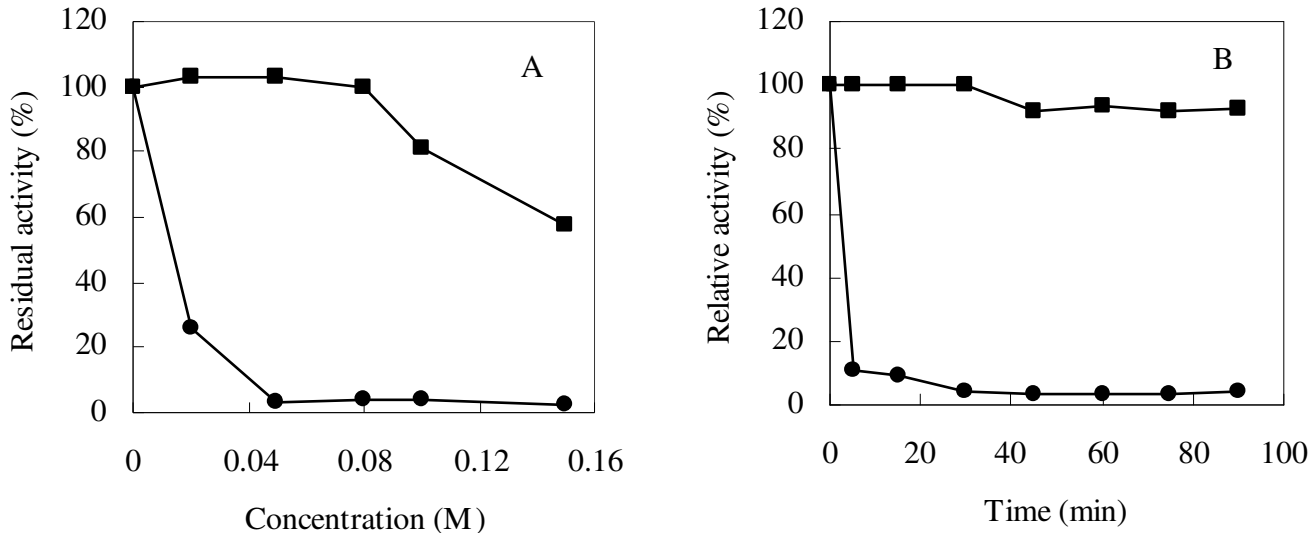

Figure 4. Effect of concentration of sodium azide (A) and treatment time (B) on the activities of DAAO (■) and CAT (●) in the CTAB-permeabilized cells. In A, the permeabilized cells was suspended in sodium azide solution with different concentration for 30 min. Similarly, in B, the cell suspendsion was treated with 0.8 M sodium azide solution for different time.

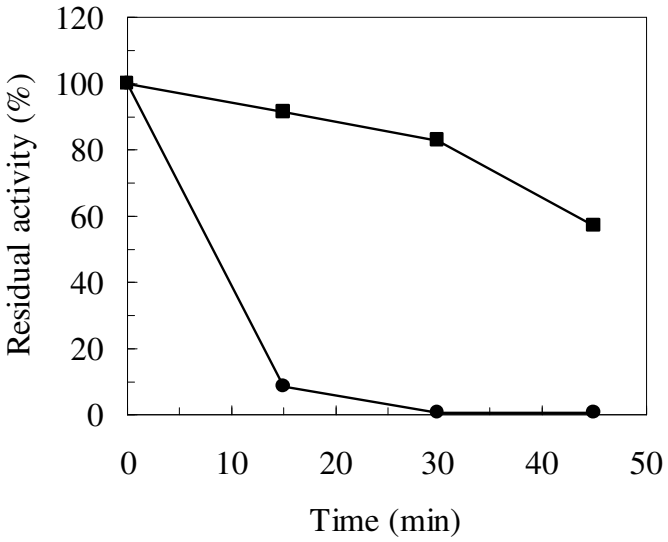

Figure 5. Effect of alkali treatment on the activities of DAAO (■) and CAT (●) in the CTAB-permeabilized cells. In this study, the cell suspension was treated with base solution at pH 11.5 for different time.

stability could be improved by decreasing in the apparent activity of the permeabilized cells.

Inhibition of CAT activity by sodium azide

CAT is an unacceptable enzyme for the bioconversion of CPC to 7-ACA by the two- enzyme process. As described previously, CAT activity can be inhibited by the addition of carbon monoxide, formate ion, acetate ion, fluoride ion or azide ion (sodium azide). But among these, the azide ion was recommended because it is the strongest inhibitors in this case (Nikolov and Danielsson, 1994; Vicenzi and Hansen, 1995). In our study, optimization of sodium azide treatment was performed. It was observed at 0.8 M sodium azide of the treatment concentration for 30 min that the residual CAT activity was 3.9% of the original, while no activity loss of DAAO was found (Figure 4). Furthermore, there was no visible impairment on the thermostability of DAAO (14.5 days of the half-life at 30℃) in the treated permeabilized cells. However, it is disadvantageous that sodium azide can reacts with one or more cephalosporinic derivatives in the medium (Nikolov and Danielsson, 1994).

Inactivation of CAT by alkali treatment

The toxicity of sodium azide makes it undesirable for use in large-scale production of 7-ACA. Alternatively, CAT can be inactivated irreversibly under the strong alkali conditions. After treating with alkali solution (pH 11.5, 30 min), the CTAB-permeabilized cells lost almost 100% of CAT activity, whereas about 83% of DAAO activity was still retained (Figure 5). The half-life of DAAO was observed in the treated permeabilized cells for 12.2 days at 30℃ compared with 14.5 days of the untreated permeabilized cells. The alkali treatment exhibited a slight effect on the stability of the cellular DAAO (Tan et al., 2006). It was inferred that the integrality of peroxisomes was slightly impaired during the process of inactivating CAT *in vivo*. As a result, the binding strength of DAAO molecules integrated with other peroxisomal structures was weakened, leading to a decrease in the enzymatic

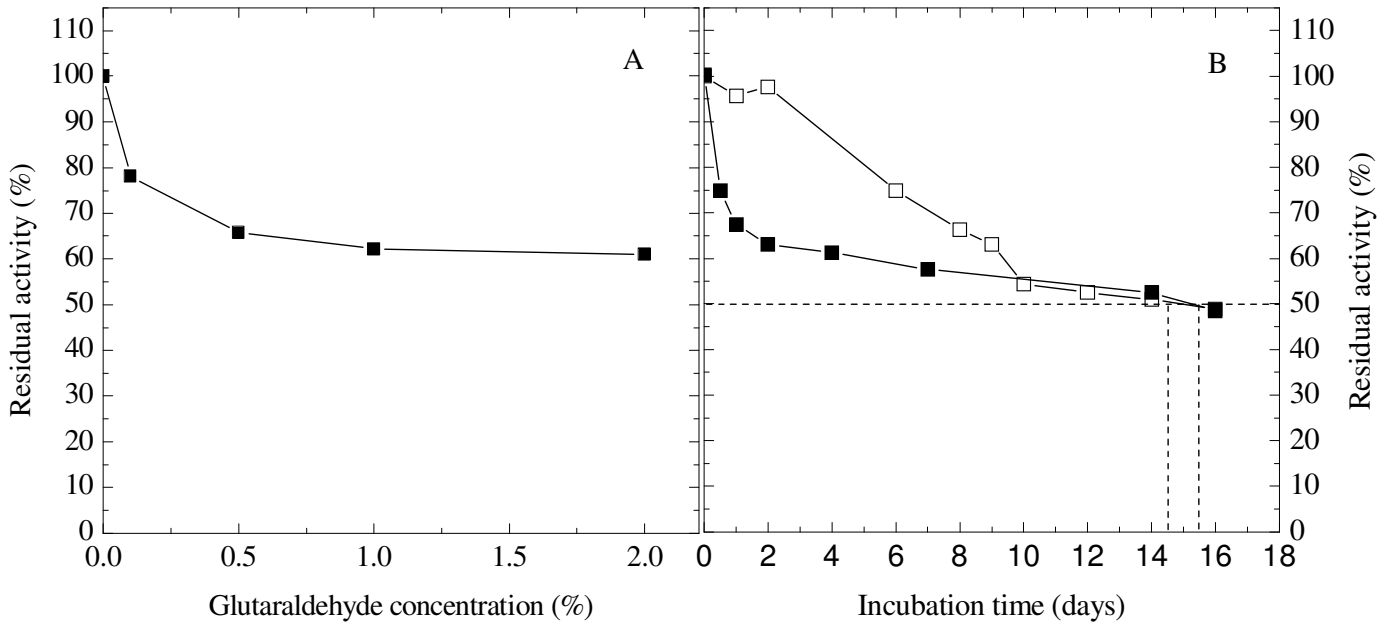

Figure 6. Effect of GLA treatment on the activity (A) and the stability of DAAO (B) in the CTAB-permeabilized cells. In A, the permeabilized cells were added to GLA solution with different concentration for 30 min. In B, the permeabilized cells were treated with 1% of GLA. DAAO in the GLA-treated cells (■); DAAO in the untreated cells (□).

stability.

Effect of the enzyme leakage and pH on the enzyme stability

The study of enzyme leakage was performed previously by storing the CTAB-permeabilized cell suspension at low temperature in the present of β-mercaptoethanol (stabilized agent of DAAO) (Pollegioni et al., 1992; Tan et al., 2007). Periodically, these cells were collected by centrifugation and the enzymatic activities present in the supernatant were assayed. The leakage amount of CAT was almost 17-fold more than that of DAAO after 144 h of observation (Tan et al., 2007). This may be one of the reasons to explain why cellular CAT exhibited a relatively low stability. It may be inferred from the result that the binding strength of CAT proteins attached with other cell components was comparatively weak compared with DAAO. Moreover, the pH value of cell suspension played a significant effect on the stability of cellular DAAO. According to the results, DAAO in the permeabilized cells was relatively unstable at high alkaline conditions and the highest enzyme stability occurred at pH 7.0 (Tan et al., 2006). This may be explained by a fact that the leakage rate of DAAO from the permeabilized cells was increased gradually with increase in pH value of the suspension. This fact hinted that the persistence of DAAO in the permeabilized cells would be weakened or the autolysis of the permeabilized cells would be accelerated at high alkaline condition.

Effect of GLA treatment on the enzyme stability

It is inevitable that a further disruption of the cell/membrane in the permeabilized cells is trigged by an autolysis process. So, it is favorable to further immobilize permeabilized cells for retaining enzymes inside cells if possible. In this study, the permeabilized cells were treated with GLA at different concentration under the described conditions, and the stability of DAAO in the treated cells was detected. As shown in Figure 6, DAAO was inactivated seriously with the increase in the GLA concentration (Figure 6a). When GLA concentration was up to 1%, about 38% of the original activity was lost. Meanwhile, a light improvement in the stability of cellular DAAO could be obtained. The half-life of DAAO in the GLA-treated permeabilized cells was 15.5 days, compared with 14.5 days in the untreated permeabilized cells at 30°C (Figure 6b). According to the results, enzyme suffered a serious inactivation with the exception of being stabilized effectively. Thus, the results obtained here were not similar to those reported previously (Upadhya et al., 2000).

Stability of DAAO and CAT in permeabilized cells of different yeasts

The stability of DAAO and CAT in permeabilized cells of different yeasts has been documented previously (Tan et al., 2007). The stability of cellular DAAO and CAT in this study was much higher than those in permeabilized cells

of *Rhodotorula gracilis* and *T. variabilis*, even in more severe conditions (Vicenzi and Hansen, 1995; Upadhya et al., 1999; Moreno et al., 2004). Additionally, it has been reported that soluble DAAO from *T. variabilis* (TvDAAO) was more stable than that from *R. gracilis* (RgDAAO) (Betancor et al., 2003; Pollegioni et al., 2004). According to this study, it appeared that the TvDAAO enzyme in permeabilized cells was more stable than RgDAAO enzyme. These results might indicate that expression hosts and gene sources possessed an important role in the stability of DAAO in permeabilized cells.

Conclusion

This study investigated the impact factors on the stability of DAAO and CAT in the permeabilized *P. pastoris* cells. It was indicated that the enzyme expression levels might not exhibit a visible effect on their stability. However, the culture conditions and the permeabilization treatments had a significant effect on the stability of the cellular enzymes. These results could permit us to methodically improve the enzyme stability by optimization of the culture conditions or decrease the apparent activities of permeabilized cells by a relatively gentle treatment.

Additionally, both the activity and stability of cellular DAAO were not damaged when the CAT activity (about 96% of the original) was inhibited by treating the permeabilized cells with an inhibitor-sodium azide, but they were slightly impaired during the CAT inactivation by alkali treatment. The leakage studies demonstrated that the intercellular DAAO was gradually unstable with increase in pH value of cell suspension. Treatment with 1% GLA did not as expected bring about a significant enhancement on the stability of the cellular DAAO. From the documents about the stability of DAAO and CAT in permeabilized cells of different yeasts, it may be inferred that expression hosts and gene sources displayed an important effect on the stability of the two cellular enzymes.

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