World J Microbiol Biotechnol (2010) 26:145–152
DOI 10.1007/s11274-009-0153-9

ORIGINAL PAPER

Single-pot conversion of cephalosporin C to 7-aminocephalosporanic acid in the absence of hydrogen peroxide

Qiang Tan · Yewang Zhang · Qingxun Song · Dongzhi Wei

Received: 18 December 2008 / Accepted: 5 August 2009 / Published online: 19 August 2009

Abstract In this study, D-amino acid oxidase (DAAO) and catalase (CAT) in the permeabilized recombinant *Pichia pastori* cells were well investigated. It appeared that their thermal stability was negatively correlated with the apparent enzymatic activities. The frozen-melted cells presented the best stability and the lowest apparent activities of DAAO and CAT, whereas the cetyltrimethylammonium bromide (CTAB) permeabilized cells displayed the weakest stability and the highest apparent activities of the two enzymes. Simultaneous action of DAAO and CAT in the CTAB-permeabilized cells and glutaryl-7-aminocephalosporanic acid acylase (GA) immobilized on carrier contributed to the conversion of cephalosporin C (CPC) to 7-aminocephalosporanic acid (7-ACA) with a yield of 76.2%. During such a reaction cycle, no visible activity loss occurred at the immobilized GA, whereas the loss rates of DAAO and CAT activities were about 0.029 and 1.13 U min^{-1}, respectively. Nevertheless, this problem could be easily solved by continuous feeding of the new permeabilized cell suspension at the rate of 6 ml h^{-1} to the reactor. Following such a fed-batch strategy, these permeabilized cells and the immobilized GA could be efficiently reused for 6 and 15 reaction cycles, respectively, yielding around 76% 7-ACA at each reaction cycle.

Keywords Cephalosporin C · D-Amino acid oxidase · Glutaryl-7-aminocephalosporanic acid acylase · Catalase · 7-Aminocephalosporanic acid

Q. Tan · Y. Zhang · Q. Song · D. Wei
State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, People's Republic of China
e-mail: tanqiang2003@gmail.com

Q. Tan
Faculty of Pharmacy, Guangxi Traditional Chinese Medical University, Nanning 530001, People's Republic of China

Y. Zhang
Faculty of Pharmaceutical Engineering Department, Jiangsu University, Zhenjiang 212013, People's Republic of China

D. Wei (✉)
Institute of Biochemistry, East China University of Science and Technology, Shanghai 200237, People's Republic of China
e-mail: dzhwei@ecust.edu.cn

Introduction

As a significant intermediate for making semisynthetic cephalosporin antibiotics, 7-aminocephalosporanic acid (7-ACA) is prepared by a two-step enzymatic transformation performed in two or three reactors prevalently (Bianchi et al. 1998; Parmar et al. 1998). The first step involves that cephalosporin C (CPC) is converted by D-amino acid oxidase (DAAO) into α-ketoadipyl-7-ACA (AKA-7-ACA) and hydrogen peroxide (H_2O_2), and then the two intermediate products spontaneously reacts each other to render 7-β-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA). GL-7-ACA is further hydrolyzed by GL-7-ACA acylase (GA) to 7-ACA in the second step.

Extensive attempts have been carried out to seek for a rational protocol to produce 7-ACA in a single reactor in order to simplify the reaction process for the sake of lowering production cost. Among the reported protocols, the simultaneous action of DAAO and GA for converting CPC to 7-ACA is studied more extensively and intensively (Aail et al. 1995 and 1996; Bianchi et al. 1998; Luo et al. 2004; Zheng et al. 2007; Tan et al. 2006). However, the main drawback of this method is regarded as that H_2O_2 in the reaction system can decompose the cephalosporin

nucleus into undesirable byproducts and inactivate the catalysts, especially DAAO (Lopez-Gallego et al. 2005; Vicenzi and Hansen 1993). On the other hand, it has been reported that the cephalosporin acylase and the mutants of the N-terminal (Ntn) hydrolases can directly convert CPC to 7-ACA (Matsuda et al. 1987; Nigam et al. 2005; Oh et al. 2003 and 2004; Pollegioni et al. 2005; Wolf et al. 2002). Nevertheless, the successful application of such a single enzyme for 7-ACA production at industrial level has not been found, likely because of the comparatively low enzyme yield. Recently, a new protocol for single-pot conversion of CPC to 7-ACA in absence of H_2O_2 using co-immobilized DAAO and CAT and carrier-immobilized GA has been developed (Lopez-Gallego et al. 2005). It was reported that AKA-7-ACA could be hydrolyzed by GA if the glutaric acid (or H_2O_2) was absent from the reaction mixture due to the sufficient CAT activity in the co-immobilized system (Fig. 1). In this case, it is essential that enough CAT is located near DAAO molecules for in situ elimination of H_2O_2, which formed from the oxidative deamination of CPC to AKA-7-ACA.

Actually, it is well known that DAAO has a relatively poor stability even in the immobilized form (Betancor et al. 2003; Lopez-Gallego et al. 2005; Vicenzi and Hansen 1993). Therefore, if three related enzymes (DAAO, CAT and GA) are simultaneously employed in carrier-immobilized forms (e.g. all enzymes immobilized on a carrier or different carriers) or in free-cell forms (e.g. all enzymes expressed in a host cell or different host cells) to convert CPC to 7-ACA, the residual active catalyst in such a reaction system will be eventually discarded along with the inactivated catalyst because these catalysts are inseparable. As an alternative, we proposed that these enzymes should be prepared in disparate forms. This would permit us to selectively remove the inactivated catalyst and keep the active one for next reactions. Since DAAO is quite unstable, it may be highly desirable to directly utilize DAAO and CAT in cell form for the enzymatic process to avoid the waste of the related catalysts.

In our previous work, DAAO and CAT were co-expressed through a high-density fermentation of the recombinant *Pichia pastoris* (Tan et al. 2007). We have also demonstrated a di-enzymatic protocol for single-pot conversion of CPC to 7-ACA by simultaneous action of DAAO in the permeabilized *P. pastoris* cells and the carrier-immobilized GA (Tan et al. 2006). However, there was an unsettled matter that lots of H_2O_2 was required to feed to the reactor, resulting in negative effects on the employed catalysts. To solve this problem, in this study we directly converted CPC to 7-ACA by a tri-enzymatic system, which included DAAO and CAT present in the permeabilized *P. pastoris* cells and the carrier-immobilized GA. The

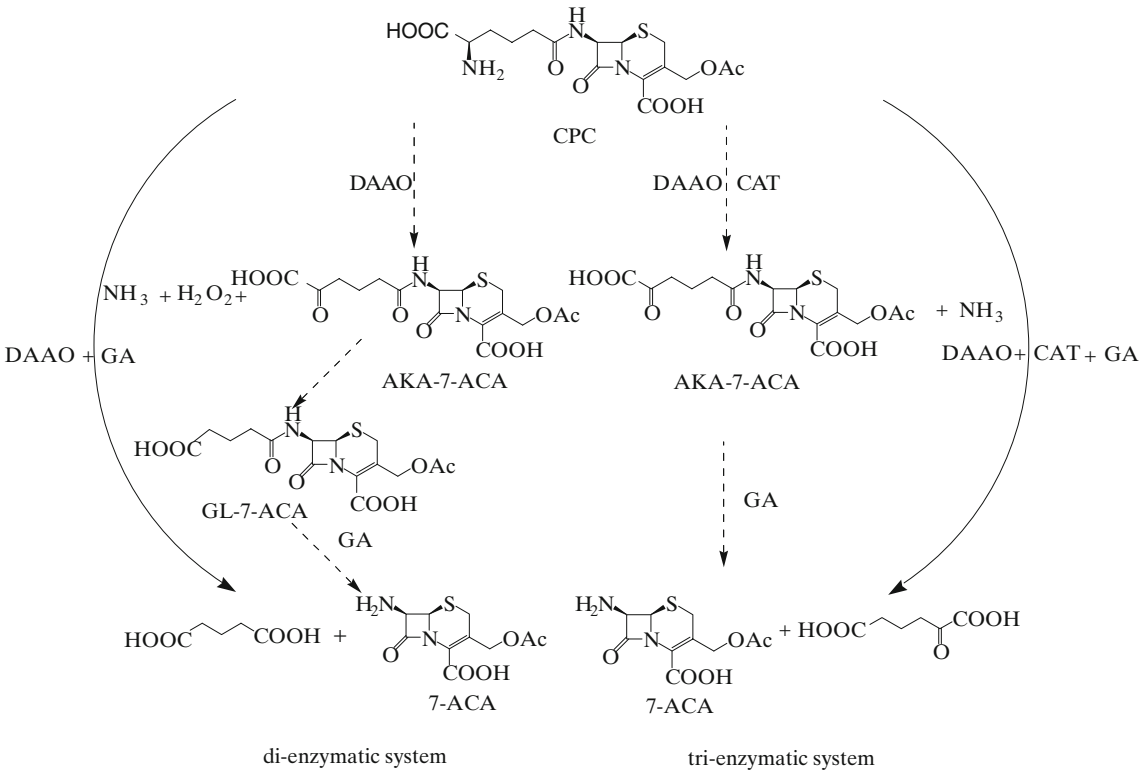

Fig. 1 Conversion of CPC to 7-ACA using the conventional di-enzymatic system and the new tri-enzymatic system

stability of the cellular DAAO and CAT were observed and a fed-batch strategy for the consecutive conversion of CPC to 7-ACA was also investigated.

Materials and methods

Materials

CPC and 7-ACA were kindly supplied by Shijiazhuang Pharmaceutical Group Co. Ltd. (Shijiazhuang, China). D-Alanine and cetyltrimethylammonium bromide (CTAB) were purchased from Fluka Co. GL-7-ACA was synthesized by ourselves as described by Shibuya (Shibuya et al. 1981), and its structure was analyzed by mass spectrum. All other chemicals were of analytical grade.

The recombinant strain *P. pastoris* GS115 ($his^{-}mut^{+}$) with a gene encoding DAAO from *T. variabilis* was kindly donated by Professor Zhongyi Yuan (Shanghai Institutes for Biological Sciences, China). GA immobilized on an epoxy resin modified with poly(methyl methacrylate) was supplied by Hunan Flag Biotech. Co. Ltd. (Changsha, China). The biocatalyst beads were spherical particles with an average diameter of 200 μm, having an activity of 40 U/g measured as the following method.

Ultrasonic disruption of cells

P. pastoris cells (0.62 g, by dry cell weight, DCW) were uniformly suspended in 20 ml of pyrophosphate buffer (50 mM, pH 7.5), containing 25 mM β-mercaptoethanol as DAAO stabilization agent. This cell suspension was incubated in an ice-bath and then disrupted by an ultrasonic cell disruptor (Microson model XL200). Sonication was carried out in short bursts in order to avoid overheating the mixture. The suspension of disrupted cells was used for enzyme assay directly.

Cell permeabilization

P. pastoris cells (0.31 g DCW) were froze at −20°C for 8 h, and then melted at room temperature. The treated cells were washed twice with pyrophosphate buffer (50 mM, pH 7.5) and suspended in 10 ml of the same buffer. On the other hand, equivalent amount of *P. pastoris* cells was suspended at a cell concentration of 31 mg DCW/ml in three kinds of pyrophosphate buffer (50 mM, pH 8.5) containing 30% acetone (v/v), 2.5% (v/v) double-solvent (toluene/ethanol = 1:4, v/v) and 0.4% CTAB (w/v), respectively. The three kinds of suspension were orderly incubated at room temperature with intermittent stirring for 30, 5, 30 min, respectively. The treated cells were collected by centrifugation (6,000×*g*, 10 min) and washed twice with pyrophosphate buffer (50 mM, pH 7.5), and then suspended in 10 ml of the same buffer. The cell suspension was used for enzyme assay.

Heat treatment of the permeabilized cells

The suspension of the CTAB-permeabilized cells was incubated at 65°C for 2 min and immediately cooled to 4°C. The suspension was centrifuged at 6,000×*g* for 10 min to collect cells. Then cells were washed with pyrophosphate buffer (50 mM, pH 7.5) and re-suspended in the same buffer at an appropriate concentration. The cell suspension was used for enzyme assay and subsequent enzymatic conversions either directly or after dilution.

Enzyme assay

The stability of DAAO and CAT within the permeabilized cells was assayed as follows: The cell suspension was incubated at a measurement temperature (4 or 30°C). Aliquots of the cell suspension were withdrawn at regular intervals and the cells were collected by centrifugation for assaying DAAO and CAT activities.

DAAO activity was determined with the permeabilized cells by measuring the formation of keto acid according to the previous methods (Brodelius et al. 1981; Yu et al. 2002). One unit of DAAO activity corresponded to the formation of 1 μmol min^{-1} of pyruvate at 37°C. The activity of immobilized GA was measured following the method described by Monti (Monti et al. 2002). One unit of GA activity was defined as the amount of enzyme which produced 1 μmol min^{-1} of 7-ACA at 37°C. CAT activity was assayed spectroscopically at 240 nm (Aebi 1984). Esterase activity was determined by the method reported by Becka et al. (Becka et al. 2003).

Conversion of CPC to 7-ACA

Three milliliters of the permeabilized cell suspension (20 U of DAAO and approximately 1000 U of CAT) and 0.5 g of carrier-immobilized GA (20 U) were incubated with 50 ml of 1% CPC (w/v) in potassium phosphate buffer (0.1 M, pH 7.5), containing 80 μl antifoam (Dow Corning 2210). The reaction was performed in a mechanically stirred reactor (150 ml) which has been well described in our previous work (Tan et al. 2006). The stirring rate was settled at 120 rpm. The reaction temperature was controlled at 28°C using a circulating water system outside the reactor core. The reaction pH was always maintained at 7.5 using a pH-stat. The pure oxygen gas was injected to the reaction mixture from the bottom of the reactor at a flow rate of 0.2 l min^{-1}. The samples were taken from the reactor each 30 min intervals. After 2–2.5 h, the reaction

mixture was released through the bottom of the reactor and the immobilized GA catalyst was intercepted in the reactor by the wire filter. The cells were filtered outside the reactor and recycled selectively for further conversions. The products present in the supernatant were assayed by high performance liquid chromatography (HPLC).

HPLC assay

The reactive products were measured through HPLC with reverse phase XDB C-18 column (Zorbax, 4.6 × 150 mm). The flow rate of the eluant (25 mM sodium phosphate, pH 3.5, 8% acetonitrile, v/v) was 1 ml min^{-1} and samples were detected at 254 nm. The retention times of different products were as follows: 2.1 min for CPC, 3.2 min for AKA-7-ACA, 8.7 min for GL-7-ACA and 1.7 min for 7-ACA. Other minor peaks were considered as the by-products. The conversion rate was calculated on the basic of peak areas.

All experiments were performed at least in triplicate under the same conditions and the results are presented as the mean value. The experimental errors never exceeded 5%.

Results and discussion

Characterization of enzymes in the permeabilized cells

In this study, no activities of DAAO and CAT were detectable when the whole cells of recombinant *P. pastoris* were used for activity assay. It was implied that the permeability barriers of the cell wall/membrane prevented substrates and products from diffusing through the cells, which restricted the activity exhibition of intercellular enzymes in the whole cells. To remove the permeability barriers of cells for exploiting the intracellular enzyme activities, some researchers have developed various methods including physical and chemical treatments for different microorganisms (Becka et al. 2003; Gough et al. 2001; Gowda et al. 1991; Moreno et al. 2004; Nandita Bachhawat et al. 1996; Upadhya et al. 1999; Vicenzi and Hansen 1993; Zhu et al. 2001). After treatment of these *P. pastoris* cells by different methods, the activities and stability of DAAO and CAT were further analyzed. As shown in Table 1, the frozen-melted cells and the CTAB-permeabilized cells exhibited the lowest and highest apparent enzymatic activities, respectively. However, the highest stability of DAAO occurred at the frozen-melted cells and 132.6% of its initial activity could be retained under the incubation conditions of 4°C, pH 7.5, for 35 days. The lowest stability of DAAO was found in the CTAB-permeabilized cells and 60% of its initial activity was retained under the same incubation conditions (Fig. 2). The half-lives of DAAO and CAT in the frozen-melted cells were 30.5 and 22 days, respectively, compared to 14.5 and 4 days in the CTAB-permeabilized cells at 30°C (Fig. 3). On the other hand, during the early storage period there was an increase in the enzymatic activities in the frozen-melted cells incubated at 4 and 30°C. With regard to these results, we noticed that there was a negative relationship between the stability and the apparent enzymatic activities (Figs. 2 and 3). As to the reasons, we considered that a mild permeabilization treatment caused a slight disruption of the cell wall/membrane to allow only a few substrates and products to diffuse through the cells, offering the treated cells low apparent activity. These treated cells with nearly intact cell wall/membrane could efficiently prevent the intracellular

Table 1 DAAO and CAT activities of the treated *P. pastoris* cells

Cell treatment	DAAO activity (U/g DCW)	CAT activity (U/g DCW)
None	nd	nd
CTAB[a]	293	15984
Frozen-melted[b]	68	528
Double-solvent[c]	220	11754
Acetone[d]	247	12256
Ultrasonic disruption[e]	475	17654

nd not detectable

[a] 0.31 g of cells was incubated with in 10 ml of 0.4% CTAB (w/v) in pyrophosphate buffer (50 mM, pH 8.5) for 30 min

[b] 0.31 g of cells was froze at −20°C for 8 h, and melted at room temperature

[c] 0.31 g of cells was incubated in 10 ml of pyrophosphate buffer (50 mM, pH 8.5) containing 30% acetone (v/v), for 30 min

[d] 0.31 g of cells was incubated in 10 ml of 2.5% toluene:ethanol (1:4, v/v) in pyrophosphate buffer (50 mM, pH 8.5) for 5 min

[e] 0.62 g of cells was suspended in 20 ml of pyrophosphate buffer (50 mM, pH 7.5), containing 25 mM β-mercaptoethanol as DAAO stabilization agent, and then disrupted by a an ultrasonic cell disruptor in a an ice-bath

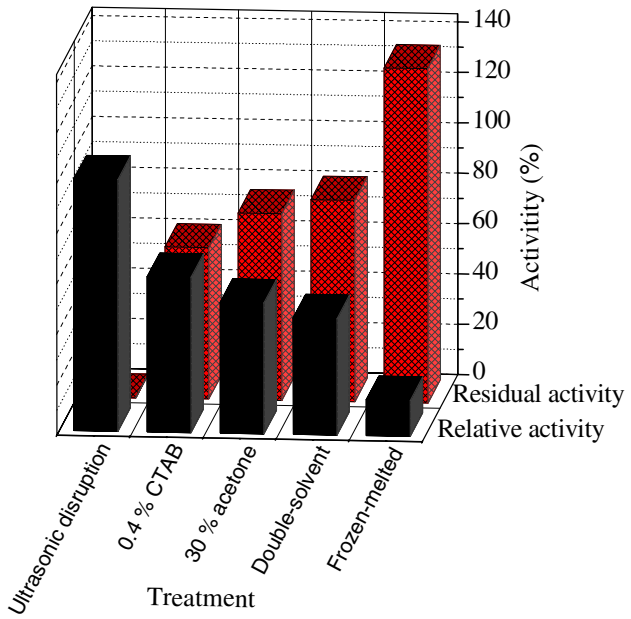

Fig. 2 Effect of the cell treatments on the activity and stability of DAAO assayed with cell suspension. To calculate the relative activity, the activity assayed with the suspension of ultrasonically disrupted cells was taken as 100%. To calculate the residual activity, cell suspension were incubated at 4°C, pH 7.5 for 35 days. The initial DAAO activity assayed with the each cell suspension was taken as 100%

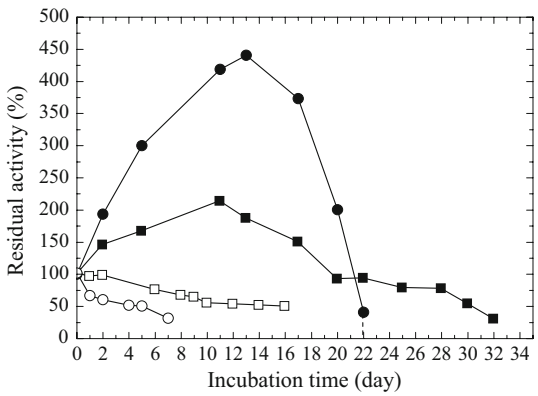

Fig. 3 The stability of the cellular DAAO and CAT under the incubation conditions of 30°C, pH 7.5. The enzymatic activities in the treated cells before incubation were taken as 100%. (■) DAAO and (●) CAT in the frozen-melted cells; (□) DAAO and (○) CAT in the CTAB-permeabilized cells

enzymes from leaking out the cells and protect enzymes against attack from the external environment, presenting a preferable stability behavior accordingly. However, it could be inevitable that a further disruption of the cell/membrane in these cells was triggered by an autolysis process or by an alteration of the membrane structure of peroxisomes, which was regarded as the location of DAAO and CAT in vivo (Upadhya et al. 2000). As a result, this would abate the permeabilized barrier and thus increase the apparent enzymatic activities during the early storage period.

The operation stability of the two cellular enzymes could be improved if the frozen-melted cells were used as the catalysts for bioconversion. In that case, however, we would have to increase the cell concentration to provide the reaction with enough enzymatic activities because the apparent enzymatic activities in the frozen-melted cells were relatively low. This could hamper the matrix diffusion during the reaction and thereby reduce the conversion efficiency. From this point of view, we selected the CTAB-permeabilized cells as the catalysts for the reaction system. Unfortunately, in these permeabilized cells there was a little esterase activity, which acted on the -$OCOCH_3$ side chain to bring undesirable de-acetyl derivatives (byproducts) (Pilone and Pollegioni 2002). After heat treatment (see Materials and methods), esterase activity in these treated cells was entirely eliminated while the activities and stability of DAAO and CAT were not impaired visibly.

Determination of the reaction conditions

The relatively high activities of the DAAO, CAT and GA were obtained in the pH range of 7.5–9.0, 8.0–9.0 and 6.8–8.0, respectively. The superior stability of DAAO and CAT in the CTAB-permeabilized cells was occurred at a low incubation pH value (7.0–7.5). No significant effect of pH values on the stability of the immobilized GA was observed. In addition, CPC and 7-ACA were relatively unstable at high pH values (>8.0), especially for 7-ACA.

The three enzymes displayed the relatively high activities at the temperature range of 30–40°C, but their thermal stability would be weakened with increase in temperature. CPC and 7-ACA suffered a deleterious effect at high temperature and would be decomposed gradually at temperature over 30°C. AKA-7-ACA was considerably unstable at high pH and temperature, and about 44% of its initial concentration was decomposed to different byproducts at pH 8.0, 23°C within 3 h (Vicenzi and Hansen 1993). As far as these results were concerned, the conversion conditions were chosen as pH 7.5, 28°C. However, pH value of reaction mixture was gradually altered with the concentration change of formations, especially the acidic derivatives, such as ketoadipic acid (a by-product of this reaction). Therefore, pH value should be controlled at 7.5 by a pH-stat, where the diluted ammonia solution was monitored to feed to the reactor. Although it has also been reported that DAAO conversion rate with CPC could be increased linearly with the oxygen concentration (Vicenzi and Hansen 1993), we refused to operate the reaction under oxygen pressure for safety issues and avoiding complexity.

Single-pot conversion of CPC to 7-ACA

The cellular DAAO and CAT and the immobilized GA were employed simultaneously for the conversion of CPC to 7-ACA in one reactor. As shown in Fig. 4, CPC was converted to 76.2% 7-ACA, along with 4.8% AKA-7-ACA and 19% unidentified byproducts after 2.5 h. No measurable GL-7-ACA was detected during the reaction process according to the HPLC assay. This fact likely meant that enough CAT and DAAO molecules coexisting in a cell could efficiently hydrolyze the formed H_2O_2 and hence prevented the reaction between H_2O_2 and AKA-7-ACA. Unfortunately, a great deal of byproducts was generated in this reaction. It was suggested that the GA hydrolysis rate with AKA-7-ACA was comparatively low, leading to the decomposition of AKA-7-ACA into various byproducts even under mild reaction conditions. The result was in accord with that reported by Lopez-Gallego: GA preferred GL-7-ACA to AKA-7-ACA as a substrate and the Km of GA for GL-7-ACA was 4-fold lower than that for AKA-7-ACA (Lopez-Gallego et al. 2005).

Single-pot conversion of CPC to 7-ACA using a fed-batch strategy

The activity assay of individual enzymes during the reaction process was performed optionally by sampling these separable catalysts from the reaction mixture. At the end of reaction, DAAO and CAT in the permeabilized cells retained 78% and 83% of the initial activity, respectively (Fig. 5). The activity loss of the two cellular enzymes approximately conformed to a linear trend and the average loss rates were about 0.029 and 1.13 U min^{-1}, respectively. However, the immobilized GA indicated no activity loss.

The loss of DAAO and CAT activities during the reaction could be compensated by continuous addition of the new permeabilized cell suspension to the reactor. The addition rate should be determined by the loss rate of DAAO activity because its activity was a limiting factor in this reaction compared to the enough CAT activity. In this case, we decided to feed the cell suspension to the reaction system at the rate of 6 ml h^{-1} (about 2 U h^{-1} of DAAO) owing to the easy control. As shown in Fig. 6, the equivalent amount of CPC could be converted into 76% 7-ACA, 5.8% AKA-7-ACA and 18.2% byproducts under the same conditions within 2.5 h. These cells could be reused for 6 reaction cycles without any significant decrease in the 7-ACA yield (Fig. 7). However, reuse of these cells for more conversions would lead to a little delay in reaction time for a comparable 7-ACA yield. We inferred that the used permeabilized cells without DAAO activity overcrowded in the reaction mixture, increasing diffusion limitation and thus lowering the conversion rate. Therefore, we preferred to entirely renew the used permeabilized cells every 6 reaction cycles (about 15 h of reaction time). Likewise, the carrier-immobilized GA could be reused for 15 reaction cycles without a significant decrease in

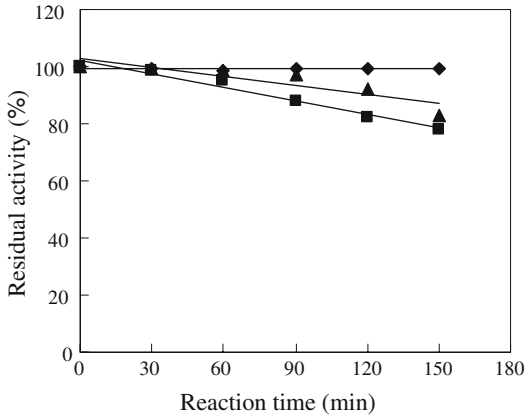

Fig. 5 Assay of each individual enzyme activity during the reaction process. (■) DAAO; (◆) GA; (▲) CAT

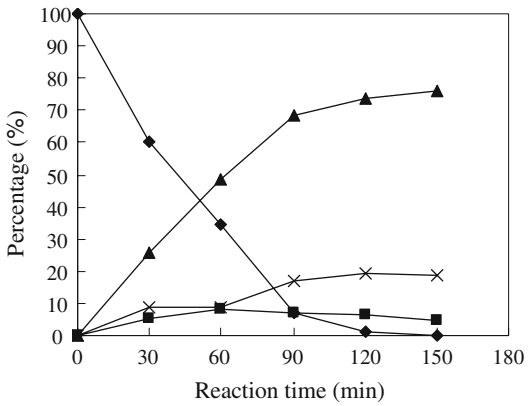

Fig. 4 Conversion of CPC to 7-ACA in one-step. (◆) CPC; (▲) 7-ACA; (×) by-products; (■) AKA-7-ACA

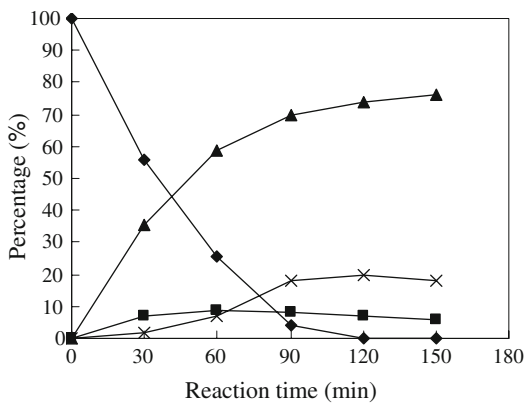

Fig. 6 Fed-batch conversion of CPC to 7-ACA in one-step. (◆) CPC; (▲) 7-ACA; (×) by-products; (■) AKA-7-ACA

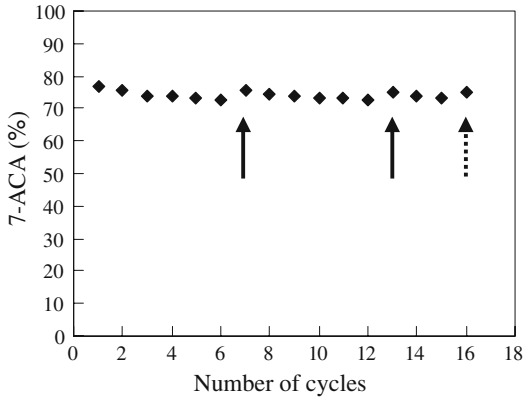

Fig. 7 Recycle of the catalysts for consecutive conversions. The *solid arrows*: the permeabilized cells were renewed at this cycle. The *dotted arrow*: the immobilized GA was renewed at this cycle

conversion efficiency (Fig. 7). However, for the sake of short reaction time, the used GA catalyst was completely replaced by the new one every 15 reaction cycles (37.5 h of reaction time).

Conclusions

This work demonstrates a new protocol that single-pot conversion of CPC to 7-ACA in the absence of H_2O_2 can be realized with a tri-enzymatic system, which includes DAAO and CAT in the permeabilized *P. pastoris* cells and GA immobilized on carrier. From the obtained results, it may be suitable to utilize DAAO and CAT in the permeabilized *P. pastoris* cells for this enzymatic process on the basic of the enzymatic activities and stability. Despite no high yield of 7-ACA obtained in this work, a significant advantage is that we can avoid many complicated processes and consumption of costly support, which are involved in preparing the co-immobilized DAAO and CAT. Meanwhile, the tri-enzymatic system in two disparate forms permits us to selectively separate and recycle the employed catalysts for the consecutive fed-batch conversions, avoiding the waste of the catalysts.

The main drawback occurred at the reaction system is high concentration of by-products. To solve the problem, the preferential solution can be achieved by seeking for the enzyme with high hydrolysis activity toward AKA-7-ACA from natural microorganisms using AKA-7-ACA or its analogs as induced substrates. Additionally, the three enzymes including GA, cephalosporin acylase and penicillin G acylase belong to members of Ntn hydrolase family and the structural homologies at their active-sites are very high (Oh et al. 2004). Therefore, a substantial effort can be devoted to mutate the active-site residuals of these enzymes by directed evolution technology for producing the targeted mutants with new acylase activity toward AKA-7ACA (Lopez-Gallego et al. 2008; Oh et al. 2003 and 2004; Pollegioni et al. 2005; Wolf et al. 2002).

Acknowledgments We thank Professor Zhongyi Yuan for kindly providing the recombinant *P. pastoris* strains. We are also grateful to Dr Tianwen Wang for reading the manuscript. This study was supported by the Key Disciplinary Foundation of Shanghai.

References

Aail P, Bále V, Michalková E (1995) Biotransformation of cephalosporin C to 7-aminocephalosporanic acid with coimmobilized biocatalyst in a batch bioreactor. Bioproc Biosyst Eng 12:249–252

Aail P, Bále V, Michalková E (1996) Biotransformation of cephalosporin C to 7-aminocephalosporanic acid with coimmobilized biocatalyst in a batch bioreact. Bioproc Biosyst Eng 14:105–109

Aebi H (1984) Assay of catalase in vitro conditions. In: Parker L (ed) Methods in enzymology 105. New York, Academic Press, pp 122–127

Bachhawat Nandita, Gowda LR, Bhat SG (1996) Single step method of preparation of detergent-permeabilized *kluyveromyces fragilis* for lactose hydrolysis. Process Biochem 31:21–25

Becka S, Skrob F, Plhackova K, Kujan P, Holler P, Kyslik P (2003) Cross-linked cell aggregates of *Trigonopsis variabilis*: D-amino acid oxidase catalyst for oxidation of cephalosporin C. Biotechnol Let 25:227–233

Betancor L, Hidalgo A, Fernandez-Lorente R, Mateo C, Rodriguez V, Fuentes M, Lopez-Gallego F, Fernandez-Lafuente R, Guisan JM (2003) Use of physicochemical tools to determine the choice of optimal enzyme: stabilization of D-amino oxidase. Biotechnol Prog 19:784–788

Bianchi D, Bortolo R, Golini P, Cesti P (1998) Enzymatic transformation of cephalosporin C to 7-ACA by simultaneous action of immobilized D-amino acid oxidase and glutaryl-7-ACA acylase. Appl Biochem Biotechnol 73:257–268

Brodelius P, Nisson K, Mosbach K (1981) Production of α-keto acids: immobilized cells of *Trigonopsis variabilis* containing D-amino acid oxidase. Appl Biochem Biotechnol 6:293–308

Gough S, Deshpande M, Scher M, Rosazza JPN (2001) Permeabilization of *Pichia pastoris* for glycolate oxidase activity. Biotechnol Lett 23:1535–1537

Gowda LR, Bachhawat N, Bhat SG (1991) Permeabilization of Bakers' yeast by cetyitrimethylammonium bromide for intracellular enzyme catalysis. Enzyme Microb Technol 13:154–157

Lopez-Gallego F, Batencor L, Hidalgo A, Mateo C, Fernandez-Lafuente R, Guisan JM (2005) One-pot conversion of cephalosporin C to 7-aminocephalosporanic acid in the absence of hydrogen peroxide. Adv Synth Catal 347:1804–1810. doi: 10.1002/adsc.200505099

Lopez-Gallego F, Batencor L, Sio CF, Reis CR, Jimenez PN, Guisan JM, Quax WJ, Fernandez-Lafuente R (2008) Evaluation of different glutaryl acylase mutants to improve the hydolysis of cephalosporin C in the absence of hydrogen peroxide. Adv Synth Catal 350:343–348. doi:10.1002/adsc.200700320

Luo H, Yu HM, Li Q, Shen ZY (2004) Cloning and co-expression of D-amino acid oxidase and glutaryl-7-ACA acylase genes in *Escherichia coli*. Enzyme Microb Technol 35:514–518. doi: 10.1016/j.enzmictec.2004.08.036

Matsuda A, atsuyama K, Yamamoto K, Ichikawa S, Komatsu KI (1987) Cloning and characterization of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. J Bacteriol 58:5815–5820

Monti D, Carrea G, Riva S, Baldaro E, Frara G (2002) Characterication of an industrial biocatalyst: immobilized glutaryl-7-ACA acyalse. Biotechnol Bioeng 70:239–244

Moreno JA, Ruiz CA, Catalan J, Galan MA, Chem J (2004) Thermal deactivation and inhibition of D-amino acid oxidase in permeabilized cells of the yeast *Trigonopsis variabilis*. J Chem Technol Biotechnol 79:321–326. doi:10.1002/jctb.954

Nigam VK, Kundu S, Ghosh P (2005) Single-step conversion of cephalosporin C to 7-aminocephalosporanic acid by free and immobilized cells of *Pseudomonas diminuta*. Appl Biochem Biotechnol 126:13–21

Oh B, Kim K, Yoon J, Chung K, Shin Y, Lee D, Kim Y (2003) Deacylation activity of cephalosporin acylase to cephalosporin C is improved by changing the side-chain conformations of active-site residues. Biochem Biophys Res Commun 310:19–27. doi:10.1016/j.bbrc.2003.08.110

Oh B, Kim K, Park J, Yoon J, Han D, Kim Y (2004) Modifying the substrate specificity of penicillin G acylase to cephalosporin acylase by mutating active-site residues. Biochem Biophys Res Commun 319:486–492. doi:10.1016/j.bbrc.2004.05.017

Parmar A, Kumar H, Marwaha SS, Kennedy JF (1998) Recent trends in enzymatic conversion of cephalosporin C to 7-aminocephalosporanic acid (7-ACA). Crit Rev Biotechnol 18:1–12

Pilone M, Pollegioni L (2002) D-amino acid oxidase as an industrial biocatalyst. Biocatal Biotransform 20:145–159. doi:10.1080/10242420290020679

Pollegioni L, Lorenzi S, Rosini E, Marcone GL, Molla G, Verga R, Cabri W, Pilone MS (2005) Evolution of an acylase active on cephalosporin C. Protein Sci 14:3064–3076. doi:10.1110/ps.051671705

Shibuya Y, Matsumoto K, Fujii T (1981) Isolation and properties of 7-*β*-(4-carboxybutanamide)-cephalosporinic acid acylase-producing bacteria. Agric Biol Chem 45:1561–1567

Tan Q, Song QS, Wei DZ (2006) Single-pot conversion of cephalosporin C to 7-aminocephalosporanic acid using cell-bound and support-bound enzymes. Enzyme Microb Technol 39:1166–1172. doi:10.1016/j.enzmictec.2006.02.028

Tan Q, Song QS, Zhang YW, Wei DZ (2007) Characterization and application of D-amino acid oxidase and catalase within permeabilized *Pichia pastoris* cells in bioconversions. Appl Biochem Biotechnol 136:279–290

Upadhya R, Nagajyothi H, Bhat SD (1999) D-Amino acid oxides and catalase of detergent permeabilized *Rhodotorula gracilis* cells and its potential use for the synthesis of α-keto acids. Process Biochem 35:7–13

Upadhya R, Nagajyothi H, Bhat SD (2000) Stabilization of D-amino acid oxidase and catalase in permeabilized *Rhodotorula gracilis* cells and its application for the preparation of α-keto acids. Biotechnol Bioeng 68:430–436

Vicenzi JT, Hansen GJ (1993) Enzymatic oxidation of cephalosporin C using whole cells of the yeast *Trigonopsis variabilis* within a "cross-flow filter-reactor". Enzyme Microb Technol 15:281–285

Wolf KF, Koller KP, Lange G, Liesum A, Sauber K, Schreuder H, Aretz W, Kabsch W (2002) Structure-based prediction of modification in glutarylamidase to allow single-step enzymatic production of 7-aminocephalosporanic acid from cephalosporin C. Protein Sci 11:92–103. doi:10.1101/ps.27502

Yu J, Li DY, Zhang YJ, Yang S, Li RB, Yuan ZY (2002) High expression of *Trigonopsis variabilis* D-amino acid oxidase in *Pichia pastoris*. J Mol Catal B Enzym 18:291–297

Zheng HB, Zhu TB, Chen J, Zhao YH, Jiang WH, Zhao GP, Yang S, Yang YL (2007) Construction of recombinant *Escherichia coli* D11/pMSTO and its use in enzymatic preparation of 7-aminocephalosporanic acid in one pot. J Biotechnol 129:400–405. doi:10.1016/j.jbiotec.2007.01.021

Zhu TB, Shen J, Zhang YF, Yang YL, Jiao RS (2001) Permeabilization of yeast *Trigonopsis variabilis* FA10 cells for enhancing apparent activity of D-amino acid oxidase. Chin J Biotechnol 17:73–77