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Enzymatic Transformation of Cephalosporin C to 7-ACA by Simultaneous Action of Immobilized D-Amino Acid Oxidase and Glutaryl-7-ACA Acylase

DANIELE BIANCHI,* ROSSELLA BORTOLO, PAOLO GOLINI, AND PIETRO CESTI

EniChem S.p.A. — Centro Ricerche Novara, Istituto G. Donegani, Via Fauser 4, I-28100 Novara, Italy

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ABSTRACT

The enzymatic transformation of cephalosporin C (CEPH C) to 7-amino-cephalosporanic acid (7-ACA) using D-amino acid oxidase (DAO) and glutaryl-7-ACA acylase (Gl-7-ACA) is reported. The enzymes have been immobilized separately on different carriers, in order to maximize the catalytic activity and the stability. The reaction has been carried out in single-step-like conditions, using the two enzymes simultaneously. The effect of catalase contamination, present in the DAO preparations, was balanced by addition of extra hydrogen peroxide. In optimum conditions, the conversion of CEPH C to 7-ACA was higher than 90%, with byproduct formation lower than 4%. The mixture of immobilized enzymes was reused in repeated reaction cycles, showing an appreciable operational stability.

Index Entries: Enzymatic transformation; cephalosporin C; 7-amino-cephalosporanic acid; D-amino acid oxidase; glutaryl-7-ACA acylase.

INTRODUCTION

7-Amino-cephalosporanic acid (7-ACA) is a key intermediate for the production of a wide variety of semisynthetic cephalosporin antibiotics

*Author to whom all correspondence and reprint requests should be addressed.

(6). The traditional methods for its production involve the chemical deacylation of the corresponding 7-acylaminocephalosporanic acids (e.g., cephalosporin C [CEPH C]), which are produced directly by fermentation. The chemical deacylation reactions, such as nitrosyl chloride method *(7)*, or iminoether method *(8)*, are carried out in organic solvents, using toxic reagents and laborious procedures. In recent years, a new method for the enzymatic hydrolysis of the side chain of CEPH C has been proposed as an alternative to the conventional procedures *(9)*. The enzymatic transformation can be performed by a two-step process, including the oxidative deamination of CEPH C to glutaryl-7-ACA (Gl-7-ACA), catalyzed by a D-amino acid oxidase (DAO), and the subsequent hydrolysis to 7-ACA, catalyzed by a Gl-7-ACA acylase (Fig. 1). Despite extensive attempts only few reports about the one-step direct conversion of CEPH C to 7-ACA by using a single enzyme (CEPH C acylase) were reported, all of them characterized by poor yield and low catalytic efficiency *(10)*. The enzymatic route is attractive from an environmental point of view, since it is conducted in water rather than in organic solvents, which avoid the isolation of CEPH C from its aqueous fermentation broth. As in the case of hydrolysis of penicillin G to 6-aminopenicillanic acid, it is recognized that the development of an enzymatic process for the production of 7-ACA to replace the existing chemical technology will have considerable cost-saving advantages *(11)*.

With the goal of developing an industrial catalytic system, the authors' previous works reported two procedures for the immobilization of DAO from *Trigonopsis variabilis (1)* and Gl-7-ACA acylase from recombinant *Escherichia coli (2)*, by covalent coupling to porous supports. The immobilized enzymes showed a remarkable stability against thermal and pH inactivation.

The present work reports the simultaneous use of the two enzymes, immobilized on different supports in order to maximize the catalytic performances, in the one-step-like hydrolysis of CEPH C.

MATERIALS AND METHODS

Materials

Amberlite XAD7 (Röhm and Haas, Philadelphia, PA) and Duolite A365 were supplied from Röhm and Haas. 1,2-Diaminoethane, D-alanine, and glutaraldehyde were purchased from Fluka (Buchs, Switzerland). 7-ACA and cephalosporin C sodium salt dihydrate (purity 94.8%) were from Farmitalia Carlo Erba (Milan, Italy). Partially purified DAO from *T. variabilis* and G1-7-ACA acylase from *E. coli* were prepared as previously described *(1,2)*.

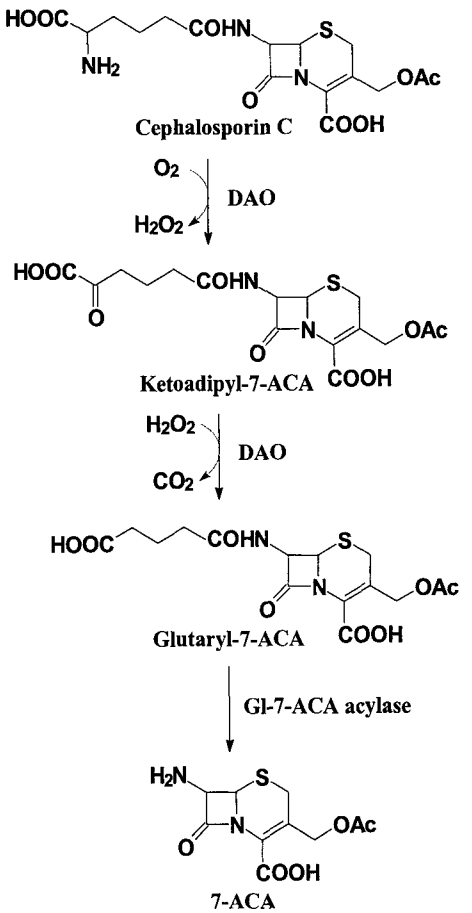

Fig. 1. Bienzymatic transformation of CEPH C to 7-ACA.

D-amino acid Oxidase Activity Assay

The determination of the immobilized DAO activity was performed on CEPH C as substrate, under the following conditions: A weighed amount of enzyme bound to Duolite A365 (0.1 g, wet wt) was added to 2 mL of the substrate solution (25 m*M* in phosphate buffer, 100 m*M*, pH 7.5) at 28°C. Oxygen was bubbled in the reaction vessel at a flow rate of 3 L/h. The catalytic activity was evaluated by monitoring the amount of ketoadipyl-7-ACA and Gl-7-ACA produced at the initial stage of the reaction (conversion less than 10%). The progress of the oxidation reaction was followed by HPLC, using 25 m*M*, pH 4.4, potassium phosphate/acetonitrile as eluent on a LiChrospher 100 RP-18 column (250 × 4 mm). 1 U corresponds to the amount of enzyme that oxidizes 1 μmol of CEPH C per minute, in phosphate buffer, pH 7.5, saturated with oxygen at 28°C.

In order to test the residual enzymatic activity present in the washing solutions of the resin after the immobilization procedure, a more sensitive assay was used: the modified Trinder reaction *(3)*. In this case, activity was evaluated by using D-alanine as substrate in 100 m*M* phosphate buffer, pH 7.5, saturated with oxygen at 37°C.

Glutaryl-7-ACA Acylase Activity Assay

The activities of both soluble and immobilized enzymes were determined in a stirred reactor using a solution of Gl-7-ACA (1% w/v) in 100 m*M* phosphate buffer, pH 7.5, (20 mL) at 28°C. The progress of the hydrolytic reaction was monitored by HPLC, utilizing 25 m*M* potassium phosphate, pH 4.4/methyl alcohol 1/1 as eluent on a LiChrospher 100 RP-18 column (250 × 4 mm). Between 3 and 14 U of soluble or immobilized enzyme were used in each experiment. 1 U corresponds to the amount of enzyme that cleaves 1 μmol of Gl-7-ACA to 7-ACA per minute, at pH 7.5 and 28°C.

DAO Immobilization

Preparations of partially purified D-amino acid oxidase from *T. variabilis* (0.19 U/mg protein) were used *(1)*. Duolite A365 (10 g, dry wt) was washed with methyl alcohol and water, then suspended in a 10% solution of glutaraldehyde (325 mL) in phosphate buffer 100 m*M*, pH 7.5. After shaking (250 rpm) 30 min at 4°C, the resin was washed with cold buffer. 60 mL of a protein solution (220 U) was mixed with the activated resin suspended in 140 mL buffer. The coupling reaction was carried out over a period of 4 h, at 4°C, on a rotary shaker (250 rpm). The resin was then washed with cold buffer. The immobilization yield was calculated from the oxidase activity and protein content remaining in the washing solutions, compared to the starting solution. DAO was immobilized on modified Amberlite XAD7 beads by using the same procedure.

Preparation of Amberlite XAD7 Modified with 1,2-Diaminoethane

23 g of Amberlite XAD7 dry beads, previously washed with water (400 mL) and methyl alcohol (400 mL), were suspended in 180 mL 1,2-diaminoethane. The suspension was mechanically stirred (150 rpm) at 110°C. After 8 h, the mixture was cooled and the polymer filtered off. The beads were washed with distilled water until neutral pH, and with methyl alcohol (500 mL) to desorb the residual 1,2-diaminoethane. After drying under reduced pressure, 24 g modified polymer were recovered. The degree of modification has been assayed both by total nitrogen determination (Kjeldal method) *(4)* and by primary amino group titration (argentometric titration of chloride, after transforming into hydrochloride) *(5)*.

After the modification procedure, 1.20 mmol of total nitrogen and 0.66 mmol of primary amino groups were detected per gram of dry support.

Glutaryl-7-ACA Acylase Immobilization

Preparations of partially purified Gl-7-ACA acylase from *E. coli* (1.2 U/mg protein) were used (*2*). An aqueous solution of glutaraldehyde (10% w/v, 40 mL) was slowly added to 10 g (dry wt) of modified Amberlite XAD7, suspended in 50 m*M* sodium phosphate buffer, pH 7.5 (150 mL) at 4°C. After 30 min under mechanical stirring (150 rpm), the suspension was filtered and the solid washed with buffer (200 mL). The activated resin was resuspended in 50 m*M* sodium phosphate buffer, pH 7.5 (100 mL), and a solution of Gl-7-ACA acylase (250 U) was added. The suspension was stirred (150 rpm) for 2 h at 4°C, and for an additional 24 h at 20°C. The immobilized Gl-7-ACA acylase was recovered by filtration, washed, and stored in 0.1 *M* sodium phosphate buffer, pH 7.5, at 4°C. Gl-7-ACA acylase was immobilized on Duolite A365 beads by using the same procedure.

Operational Stability of Immobilized DAO

100 U of DAO from *T. variabilis* immobilized on Duolite A365 (4.7 g dry wt), or on modified Amberlite XAD7 (10 g of weight), were added to a solution of CEPH C (1 g, 2.42 mmol) in potassium phosphate buffer 100 m*M*, pH 7.5 (100 mL). The reactions were carried out at 28°C in a reactor (200-mL vol), equipped with a glass filter on the bottom, through which a stream of O_2 (30 L/h) was bubbled in the suspension. After complete conversion of the substrate, the supported enzymes were recovered by filtration and reused in repeated reaction cycles. The operational stability was calculated by comparing the times at which 50% conversion was reached.

Influence of pH on DAO and Gl-7-ACA Acylase Activity

In order to determine the optimum pH, DAO, and Gl-7-ACA acylase activities were assayed, according to the procedure described above, between pH 6.0 and 9.5. Activity measurements were performed in glycine/Tris/H_3PO_4 120 m*M* buffer solution, brought to the given pH by adding sodium hydroxide. The stability as a function of pH was determined by measuring the residual activity at each pH after storage for 24 h at 28°C.

Hydrolysis of CEPH C by Simultaneous Action of DAO and Gl-7-ACA Acylase

59 U of DAO immobilized on Duolite A365 (2.8 g dry wt) and 56 U of GI-7-ACA acylase immobilized on modified Amberlite XAD7 (5.6 g dry wt)

were added to a solution of CEPH C (2.25 g, 5.44 mmol) in potassium phosphate buffer 100 m*M*, pH 7.5 (225 mL). The reaction was carried out at 28°C in a mechanically stirred reactor (500-mL volume), equipped with a glass filter on the bottom, through which a stream of O_2 (50 L/h) was bubbled in the suspension. After 3.5 h, the reaction mixture typically had the following percentage composition: CEPH C (0%), ketoadipyl-7-ACA (20%), Gl-7-ACA (6%), 7-ACA (72%), and not identified β-lactam products (2%).

Effect of Hydrogen Peroxide Addition

The transformation was carried out using the same procedure described above. During the reaction, a solution of hydrogen peroxide (H_2O_2; 3% w/w, 2.6 mL, 2.2 mmol) was added continuously for 3 h. After 3.5 h the reaction mixture typically had the following percentage composition: CEPH C (0), ketoadipyl-7-ACA (0), Gl-7-ACA (5), 7-ACA (91), and not identified β-lactam products (4).

After complete conversion of the substrate, the supported enzymes were recovered by filtration and reused in repeated reaction cycles.

7-ACA was isolated from the reaction mixture by precipitation at 5°C, after adjusting the pH of the solution at the isoelectric point of the product (pH 3.5). The precipitation yield was 87%, corresponding to an overall 7-ACA yield of 79%.

RESULTS AND DISCUSSION

In attempts to find a simple and effective method for the immobilization of the enzymes involved in CEPH C transformation, the authors tested a series of synthetic carriers suitable for protein binding by physical adsorption, such as Amberlite XAD 2, XAD 4, and XAD 7, or by covalent linkage, such as Duolite A365, aminoalkylated Amberlite XAD 7, aminopropyl-controlled-pore glass, aminopropyl-controlled-pore silica gel, and oxirane-acrylic beads (Eupergit C).

The best results in the immobilization of DAO from *T. variabilis* were obtained using glutaraldehyde-activated Duolite A365, a macroreticular polystyrene resin functionalized with primary amino groups *(1)*. The selected carrier for immobilization of Gl-7-ACA from acylase *E. coli* was the polymethacrylic esteradsorbent resin Amberlite XAD 7, modified with aliphatic diamines *(2,12)*. Since Amberlite XAD 7 has no ion-exchange capacity, the resin was modified by aminoalkylation with 1,2-diaminoethane [R-COOMe→R-CONH$(CH_2)_2NH_2$]. As in the case of Duolite A 365, the primary amino groups are suitable for activation with glutaraldehyde, through Schiff's base formation, followed by covalent coupling with the free amino groups of the enzyme.

Table 1
Immobilization of DAO and Gl-7-ACA Acylase on Different Carriers

Enzyme	Carrier	Particle size (mm)	Total added enzyme (U g^{-1})	Immobilization yield (%)[a]	Expressed activity[b] (U g^{-1})	Expressed activity[b] (%)
DAO	Duolite A365	0.8	22	97	12	56
DAO	Duolite A365	0.1	22	97	21	98
DAO	Amberlite XAD7	0.3–0.7	22	91	10	50
Acylase	Duolite A365	0.8	25	12	0.7	23
Acylase	Amberlite XAD7	0.3–0.7	25	98	11	45

[a] Based on the residual activity of the immobilization solution.
[b] Based on the activity of the bound enzyme.

The immobilized DAO and Gl-7-ACA acylase showed good expressed activity, and were successfully used for CEPH C deamination and Gl-7-ACA hydrolysis, respectively. As suggested in a previous study, the simplest way to carry out the transformation of CEPH C into 7-ACA is the combination of the two steps through the simultaneous action of the two enzymes co-immobilized on the same carrier *(13)*. With the aim of developing a new co-immobilization procedure, the authors initially tested the catalytic features of DAO supported on modified Amberlite XAD 7, and Gl-7-ACA acylase supported on Duolite A 365.

As reported in Table 1, DAO was efficiently bound to Amberlite XAD 7, but, as shown in Fig. 2, its operational stability, measured at 28°C and pH 7.5, was lower than that displayed by the enzyme immobilized on Duolite A 365. Conversely, Duolite A 365 showed a low efficiency in binding Gl-7-ACA acylase, both in terms of expressed activity and amount of loaded enzyme. The data of total proteins bound was higher than that of acylase immobilization yield, indicating the preferential binding of the contaminant proteins present in the partially purified enzymatic preparation. This is probably caused by the higher molecular hinderance of Gl-7-ACA acylase (mol wt 134 kDa) with respect to DAO (mol wt 86 kDa).

On the basis of these results, it appears that co-immobilization on the same carrier is disadvantageous in terms of catalytic performances, at least for one of the involved enzymes. A more beneficial alternative would be the use of a mixture of separately immobilized enzymes, each one exploiting its maximum catalytic potential. In this particular case, the simultaneous use of a mixture of carriers in the same reactor is possible, since Duolite A 365 and Amberlite XAD 7 are both synthetic organic resins with similar chemical and mechanical properties.

In order to make an homogeneous mixture, and to minimize diffusion limitations, the resins were mechanically ground, selecting the fraction

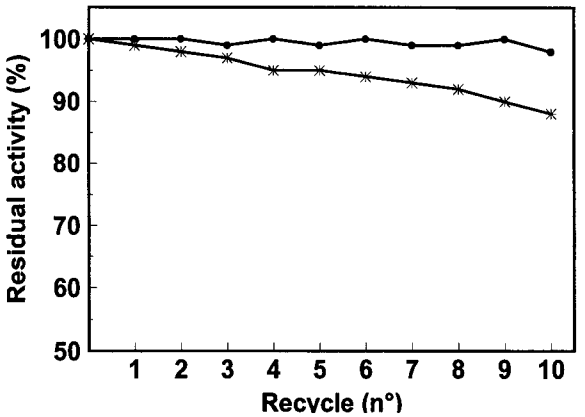

Fig. 2. Operational stability of immobilized DAO. The stability was evaluated after repeated batch reactions with CEPH C as a substrate at pH 7.5, 28°C. D-amino acid oxidase from *T. variabilis* immobilized on Duolite A365 (●) and Amberlite XAD-7 modified with 1,2-diaminoethane (*).

with average particle size near 0.1 mm. In this way, a sharp rise of the DAO activity was observed (Table 1), probably caused by the improved oxygen diffusion, but immobilized Gl-7-ACA acylase was almost unaffected by the reduction of the carrier particle size.

The effect of pH on the activity of immobilized DAO and Gl-7-ACA acylase was studied at 28°C, using separately immobilized enzymes, and is reported in Fig. 3. Both the enzymes showed a maximum activity between pH 8.0 and 8.5.

The residual activity of the immobilized enzymes after storage for 24 h at a given pH is shown in Fig. 4. In the pH range of 6.5–8.0 both DAO and Gl-7-ACA acylase showed a quite similar behavior, being equally stable, but a sharp decrease of activity was observed at higher pH values.

Although the highest activity of the immobilized enzymes is around pH 8.0, the bienzymatic transformation was carried out at pH 7.5, in order to minimize the nonenzymatic deacetylation of CEPH C in position 3, which becomes relevant at more alkaline pH values.

The total amount of catalyst used in the bienzymatic transformation was maintained lower than 50 g/L, to prevent stirring and diffusion limitation. The immobilized enzymes were maintained in suspension by mechanical stirring with the aid of the oxygen flow, bubbled in through the bottom of the reactor.

The maximum concentration of CEPH C was limited by the competitive product inhibition of glutaric acid on Gl-7-ACA acylase (K_i = 0.16 ± 0.03 mmol/dm, calculated for the soluble enzyme) (*2*). In a representative experiment (total immobilized catalyst = 37 g/L), a transformation of

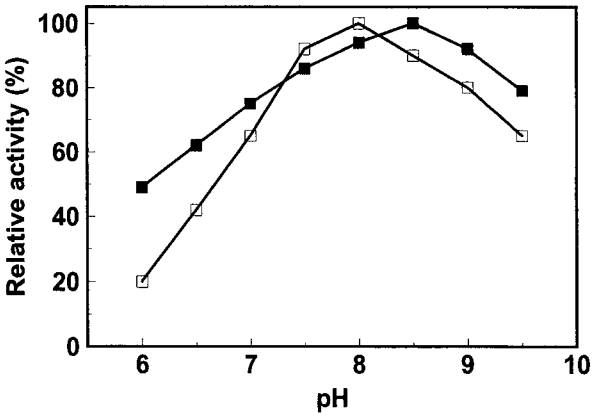

Fig. 3. Effect of pH on DAO and Gl-7-ACA activity. The reaction were carried out at 28°C. Data are expressed as percentage of highest activity value. DAO from *T. variabilis* immobilized on Duolite A365 (■); Gl-7-ACA acylase from *E. coli* immobilized on Amberlite XAD-7 modified with 1,2-diaminoethane (□).

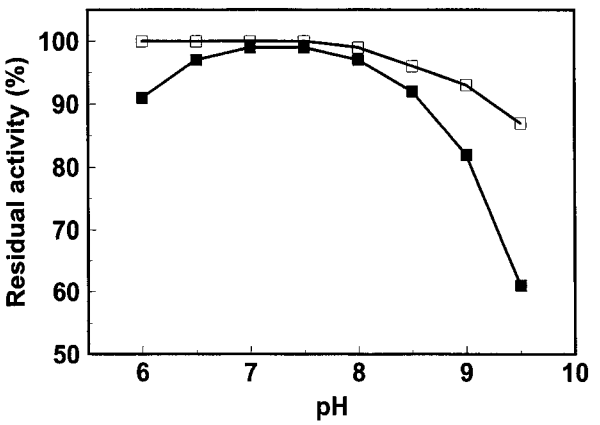

Fig. 4. Effect of pH on DAO and Gl-7-ACA stability. Samples were incubated at 28°C at the indicated pHs for 24 h. Data are expressed as percentage of initial enzyme activity. DAO from *T. variabilis* immobilized on Duolite A365 (■); Gl-7-ACA acylase from *E. coli* immobilized on Amberlite XAD-7 modified with 1,2-diaminoethane (□).

practical interest (90% conversion in 3 h) can be achieved operating only with a CEPH C concentration not exceeding 1%.

On the basis of these results, the selected conditions for the bienzymatic system were: 260 U/L of DAO (on Duolite A365), 250 U/L of Gl-7-ACA acylase (on Amberlite XAD 7), and 25 m*M* CEPH C (10 g/L). Since the oxidative deamination rate was strongly affected by the oxygen concentration, the authors chose to use pure oxygen (200 $L/h^{-1}/L^{-1}$ reaction medium), instead of air.

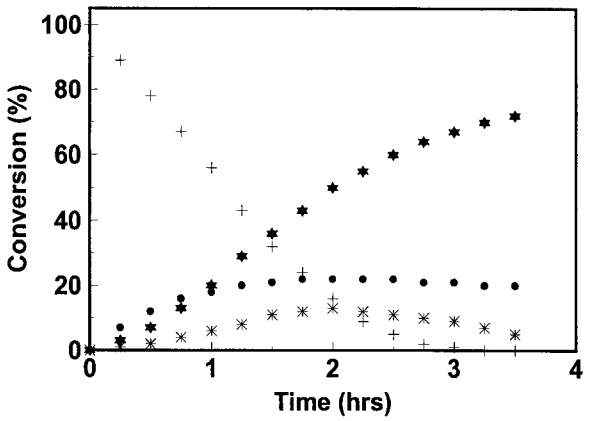

Fig. 5. Bienzymatic transformation of CEPH C. Reaction conditions: DAO on Duolite A365 (260 U/L), Gl-7-ACA acylase on Amberlite XAD 7 (250 U/L), CEPH C (10 g/L−1), O_2 (200 $L/h^{-1}/L^{-1}$), pH 7.5, 28°C. CEPH C (+), Gl-7-ACA (*), Ketoadipyl-7-ACA (●), 7-ACA (★).

As shown in Fig. 1, the oxidation of CEPH C to Gl-7-ACA involves two consecutive reactions: The first one is the oxidation of CEPH C to ketoadipyl-7-ACA, accompanied by the formation of one equivalent of H_2O_2. These two products rapidly undergo a nonenzymatic oxidative decarboxylation to form Gl-7-ACA. If catalase is present as a contaminant of the enzyme preparation, the produced H_2O_2 is partially decomposed into water and oxygen, determining an accumulation of the undesired ketoadipyl derivative. The concentration of ketoadipyl-7-ACA should be kept as low as possible, since glutaryl-7-ACA acylase is specific only for the hydrolysis of the glutaryl derivative. In order to minimize this side reaction, catalase-free enzymatic preparation should be used.

Unfortunately, because of the large amount of catalase present in *T. variabilis* cells, the complete decontamination of DAO preparations is very difficult, even after specific deactivating treatments and chromatographic purification *(14)*, but the *E. coli* strain used for Gl-7-ACA acylase production is essentially catalase free *(15,16)*.

As shown in Fig. 5, the consequence of a small catalase contamination on the simultaneous bienzymatic transformation of CEPH C, carried out as described, is the production of a significant amount of ketoadipyl-7-ACA (up to 20% of the starting CEPH C). A recent paper suggests, as a possible solution to this problem, the addition of a catalase inhibitor (sodium azide) or of extra H_2O_2. In the reported reaction conditions (33°C, pH 7.9), using DAO and Gl-7-ACA acylase co-immobilized on controlled pore glass, both these methods lead to low 7-ACA yields and enhanced byproducts formation *(13)*. Conversely, the authors found that, operating at 28°C, with a

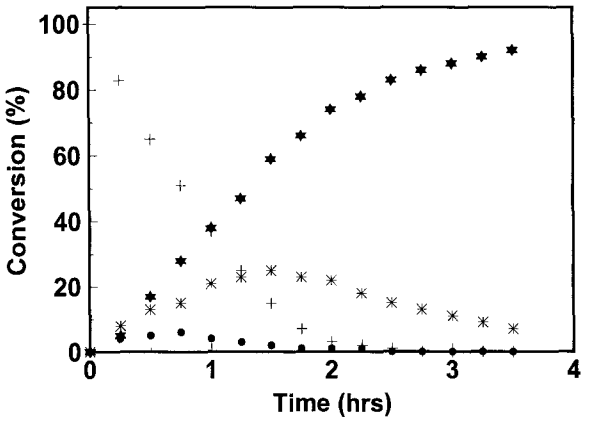

Fig. 6. Bienzimatic transformation of CEPH C: effect of extra H_2O_2 The reaction was carried out as reported in Fig. 5. Extra H_2O_2, corresponding to 40% of the starting CEPH C, was added continuously for 3 h. CEPH C (+), Gl-7-ACA (*), ketoadipyl-7-ACA (●), 7-ACA (★).

more efficient catalytic system, the nonenzymatic decomposition processes, such as the hydrolysis of the β-lactam ring or the sulfur oxidation by H_2O_2 can be drastically reduced.

In Fig. 6 is reported the transformation profile obtained operating as described in Fig. 5, but supplying the reaction mixture with diluted H_2O_2 in order to balance the fraction decomposed by the catalase. In order to avoid accumulation, the total amount of H_2O_2 corresponding to 40% of the starting CEPH C, was added continuously in 3 h. In this way, the intermediate ketoadipyl-7-ACA was completely transformed into Gl-7-ACA, and the final conversion to 7-ACA was improved up to 91%, with byproduct formation lower than 4%. The stability of immobilized enzymes was not affected by the presence of extra H_2O_2, since they could be recovered by filtration and reused for five consecutive reaction cycles without any significant loss of activity.

From these results, it can be concluded that the proposed immobilized bienzymatic system is effective in the one-step-like hydrolysis of CEPH C to 7-ACA in multigram scale. The reaction and the reactor design are currently under investigation, to scale-up the process for industrial applications.

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