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Stabilization of D-Amino Acid Oxidase from Yeast *Trigonopsis variabilis* used for Production of Glutaryl–7–Aminocephalosporanic Acid from Cephalosporin C

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ABSTRACT

The studies to improve the production of glutaryl-7-ACA from cephalosporin C are described in this paper.

During the conversion of cephalosporin C to keto-adipyl-7-amino-cephalosporonic acid by D-amino acid oxidase (D-AAO), with the simultaneous production of equimolar amount of hydrogen peroxide, an incomplete nonenzymatic conversion of the keto form into the glutaryl form occurs, where cephalosporin C as well as D-AAO are partly destroyed in the presence of hydrogen peroxide.

D-AAO was immobilized to different carriers in order to achieve better enzyme stability. The activity of immobilized D-AAO on manganese oxide remained above 100% during the first 9 h of a semicontinuous conversion of cephalosporin C. The presence of catalase co-immobilized with D-AAO and coupled to CNBr-activated Sepharose 4B improved the operation stability of D-AAO.

An additional approach for the continuous transformation of cephalosporin C used whole cells of *Trigonopsis variabilis*, containing D-AAO, immobilized to magnetic iron oxide particles.

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Index Entries: Immobilized D-amino acid oxidase; production of glutaryl-7-ACA; production of 7-ACA; stabilization of D-amino acid oxidase; stabilization of cephalosporin C.

Abbreviations: PMSF: phenylmethylsulfonyl fluoride; 2,4-DNPH: 2,4-dinitrophenylhydrazine; D-AAO: D-amino acid oxidase; PEG: polyethylene glycol; CPG: controlled pore glass.

INTRODUCTION

The yeast *Trigonopsis variabilis* has been found (*1*) to produce rare D-amino acid oxidase, that has the capacity to transform cephalosporin C into keto-adipyl-7-aminocephalosporanic acid.

Keto acids are used for chronic uremia therapy (*2*), and many of the researchers in this area (*3–7*) have used microbial sources of amino acid oxidase to obtain keto acids from amino acids. Our goal was to include D-AAO in tirst step of cephalosporin C conversion into 7-aminocephalosporanic acid (7-ACA) via keto-adipyl-7-ACA and glutaryl-7-ACA (Fig. 1). This paper describes ways to stabilize the system that takes part in the conversion of cephalosporin C into glutaryl-7-ACA.

MATERIALS AND METHODS

Materials

Iron oxide, 1 μm, magnetite (BDH Chemicals Ltd., Poole, England). 3-Aminopropyltriethoxysilane (EGA-Chemie, Steinheim, FRG). Mangan (IV) oxide, $NaBH_3CN$, LiChrosorb RP-18 (25×0.4 cm, 5 μm), PEG 35 000 (E. Merck, Darmstadt, FRG). Glutaraldehyde (Fluka Chemie, Buchs, Switzerland). Cephalosporin C and glutaryl-7-ACA (Eli Lilly Laboratory, Indianapolis, IN). Beef liver catalase, α-keto acid standards and phenylmetylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). Dextran T10, and Sepharose 4B (Pharmacia, Uppsala, Sweden). 2,4-Dinitrophenylhydrazine (KEBO Lab AB, Stockholm, Sweden). DE-52 cellulose (Whatman Ltd., Springfield Mill, Maidstone, England). Cellulose HBS research grade (Serva, FRG). Affi-gel (Bio-Rad, Richmond, CA). Controlled pore glass, CPG 1350 Å pore size (Pierce Chemical Co., Rockford, IL).

Source of Enzyme

Partially purified D-AAO was obtained from *Trigonopsis variabilis* after isoelectric precipitation at pH 5.3 (*8*).

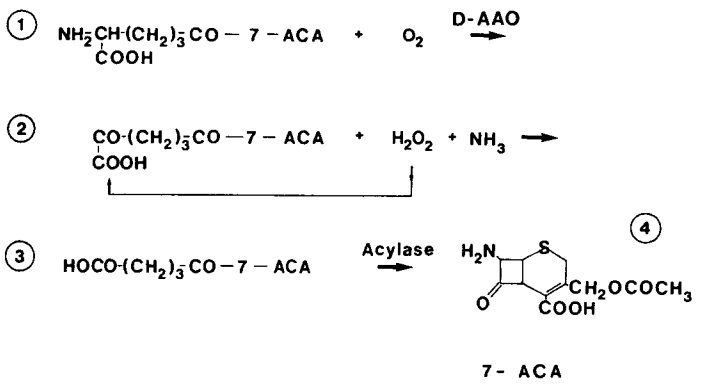

Fig. 1. Reaction scheme for conversion of cephalosporin C (1) into glutaryl-7-aminocephalosporanic acid (3) via keto-adipyl-7-aminocephalosporanic acid (2) by D-amino acid oxidase. The step glutaryl-7-ACA (3) to 7-ACA (4) is catalyzed by glutaryl acylase.

Immobilization of Enzyme

Activation of Sepharose

Sepharose 4B was activated with CNBr according to Cuatrecasas (*9*). The coupling buffer was 50 m*M* phosphate, pH 8. Sepharose 4B was activated as well with tresylchloride (*10*).

Alkylamination of Oxide Particles

The manganese oxide particles were crushed and sieved to 1–4 μm (microscopic evaluation). One gram of dry particles were then mixed with 3-aminopropyltriethoxysilane (10%, 25 mL, dissolved in water) and adjusted to pH of the range 3.9–4.1 with 6*M* HCl. The suspension was incubated at 90°C for 1 h with constant mixing, and was washed thoroughly with water before drying at 120°C for 2 h.

Derivatization with Glutaraldehyde

One gram of alkylamine-metal oxide particles (or alkylamine derivatized CPG) was incubated with glutaraldehyde (2.5%, 20 mL in 50 m*M* phosphate buffer), pH 7.0, sonicated and kept under vacuum for 2 h at room temperature with intermittent mixing. After removing the glutaraldehyde solution, the particles were then washed with 50 m*M* phosphate buffer, pH 7.0, rinsed repeatedly with 1*M* NaCl (in the same buffer), and again with 50 m*M* phosphate buffer, pH 7.0.

Coupling

One gram of the wet activated carrier (*see above*) was mixed with the enzyme preparation (2 mg protein in 15 mL 50 m*M* phosphate buffer, pH 7.0) $NaBH_3CN$ (156 mg) and $Ni(CH_3COO)_2$ (28 mg according to Miller and

Robyt) (*11*)). The coupling was carried out in the cold room for 2 h by rotating the mixture end-over-end. The particles with the immobilized enzyme were then washed with coupling buffer, 0.5*M* NaCl (in coupling buffer), 0.1*M* acetate buffer pH 5.5 and again with coupling buffer.

Aqueous Two-Phase Systems

Stock solutions of the polyethylene glycol and dextran were mixed with buffer. Enzyme (40 μL enzyme/mL) and cephalosporin C (at final concentration 10 m*M*) were added and the enzymatic reaction was allowed to proceed for 2 h at 23°C rotating end-over-end. The concentration of substrate and products in the upper and lower phases were determined as described below.

Assay Methods

The standard assay for D-amino acid oxidase activity toward cephalosporin C was performed as follows: 100–200 mg of the immobilized enzyme or cell paste were added to 3 mL of 10 m*M* cephalosporin C (in 50 m*M* phosphate buffer, pH 7.5) and rotated end-over-end for 40 min at room temperature. The products of biotransformation in the supernatant were determined both by HPLC and by a colorimetric method using 2.4-dinitrophenylhydrazine (DNPH). A LiChrosorb RP-18 column with particle size 5 μm was used. The mobile phase was 3% methanol in phosphate buffer (50 m*M*, pH 7.0) at a flow rate of 1 mL/min and detection wavelength 254 nm.

The colorimetric determination of the α-keto-adipyl-7-ACA with DNPH was compared against standard curves for α-keto isocaproic and α-keto isovaleric acid. The enzymatic activity was also assayed by converting the keto acid to glutaryl-7-ACA and measuring the amount of product formed by using a HPLC system (Fig. 2).

Cell Permeabilization and Immobilization

Permeabilization of yeast cells was achieved by freezing and thawing the cells four times. Then, part of the cells were crosslinked using glutaraldehyde (0.25% in 50 m*M* phosphate pH 7.0) for 30 min at room temperature and with end-over-end rotation. The cells were collected by centrifugation (3000*g*, 5 min) and washed with 50 m*M* phosphate, pH 7 and deionized water. A suspension of equal parts of cells and water was mixed with a thick suspension of magnetic particles (one part of particles with one part of water) for 20 min at room temperature. The cells were recovered with a permanent magnet.

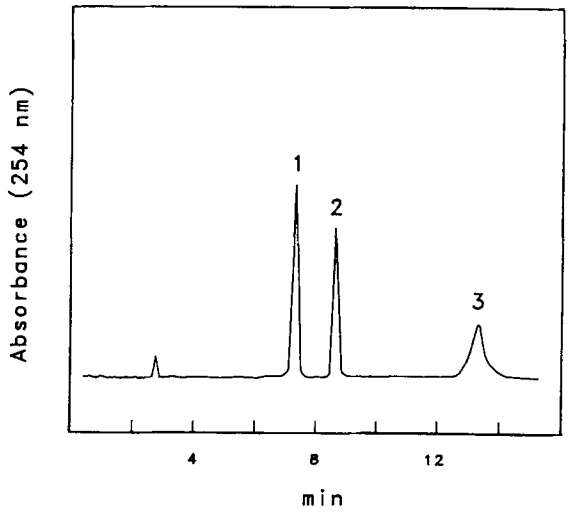

Fig. 2. HPLC chromatogram of products from cephalosporin C conversion. 1—cephalosporin C; 2-glutaryl-7-ACA; 3-keto-adipyl-7-ACA.

RESULTS AND DISCUSSION

We observed that at 50°C, the activity of the free D-amino acid oxidase remained 100% for 30 min in 25 m*M* phosphate buffer at pH 7.5. This encouraged us to go further and to use the enzyme for semicontinuous biotransformation of cephalosporin C into glutaryl-7-ACA using the immobilized enzyme or cell preparations. The three steps involved in the transformation of cephalosporin C into glutaryl-7-ACA are described in Fig. 1.

The high yield of the transformation of cephalosporin C into glutaryl-7-ACA is dependent on the concentration of cephalosporin C and on the supplied oxygen (Fig. 1).

We tried to stabilize the D-AAO and cephalosporin C and to increase the oxygen supply to the system. One of the main reasons why catalase or agents with a catalase-like activity are usually added during the enzymatic deamination of amino acids is to protect the keto-product from hydrogen peroxide oxidation (*3*). Glutaryl-7-ACA is a substrate in the third step of the reaction catalyzed by acylase to yield 7-ACA (*see* Fig. 1), that is of great industrial interest as a basic moiety for the preparation of semisynthetic cephalosporins (*8*).

In spite of using D-amino acid oxidase without catalase, we found that not all hydrogen peroxide formed during the reaction was used for the production of glutaryl-7-ACA. The amount of unconverted keto product was around 40%. We isolated keto-adipyl-7-ACA from a HPLC separa-

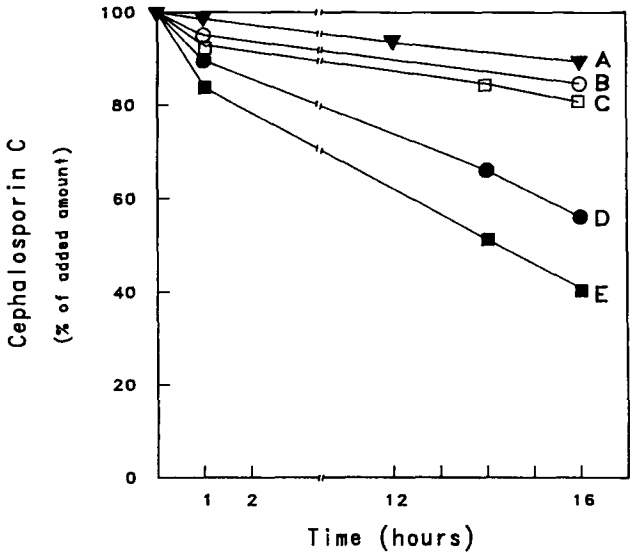

Fig. 3. Protecting effect of MnO_2 on cephalosporin C from by hydrogen peroxide damage. ▼—▼ A—10 m*M* cephalosporin C in 50 m*M* phosphate buffer, pH 7.5; ○—○ B—A supplemented with 5 m*M* hydrogen peroxide with MnO_2 (20mg/mL), pH 7.5; □—□ C—like B only at pH 8; ●—● D—A supplemented with 5 m*M* hydrogen peroxide, pH 7.5; and ■—■ E—like D only at pH 8.

tion run (Fig. 2, the peak with the retention time of about 12.5 min) and converted one portion into glutaryl-7-ACA using 50 m*M* hydrogen peroxide. Another portion of the isolated substance was used for keto group evaluation in a colorimetric test with 2,4-dinitrophenylhyrazine.

To follow the stability of cephalosporin C supplemented with 5 m*M* hydrogen peroxide, the following experiment was performed (Fig. 3): after 1 h at pH 7.5 and at pH 8.0, cephalosporin C was reduced by about 9 and 15%, respectively. After 16 h, this loss increased to 44 and 60%, respectively. After addition of MnO_2 (20 mg/mL of 10 m*M* cephalosporin C at pH 7.5), the stability was nearly the same as in the sample without hydrogen peroxide. At pH 8.0 under similar conditions, we noted a 10% decrease of cephalosporin C. We also used iron oxide particles in an attempt to protect cephalosporin C from hydrogen peroxide, but did not notice the same effect as with MnO_2 (results now shown). It is well documented that metal oxides are good hydrogen peroxide destroying agents to yield oxygen. Brodelius et al. (*3*) have shown that the presence of manganese oxides increases the production of α-keto-methiol-butyric acid from D-methionine by whole cells of *Trigonopsis variabilis* and protects the keto product from further degradation. Manganese oxide was successfully used as a carrier by Duvnjak and Lilly (*12*), who entrapped MnO_2 together with glucose oxidase by crosslinking them with 2.5% glutaraldehyde. When we followed their procedure, our enzyme lost most of the activity. A preliminary test with the free enzyme and MnO_2 present was performed.

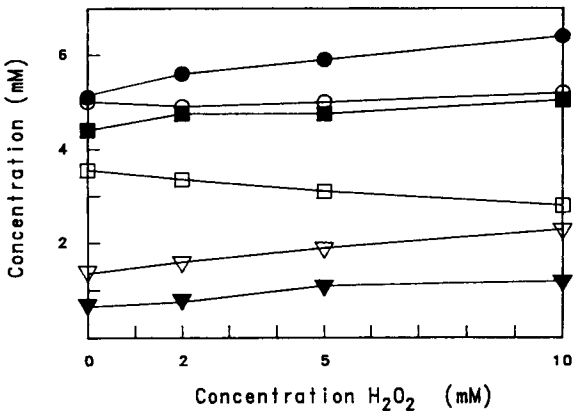

Fig. 4. Conversion of 10 m*M* cephalosporin C into keto-adipyl-7-ACA and glutaryl-7-ACA by D-AAO in an aqueous two-phase system (10% PEG 35,000/8% dextran T 10,100 m*M* K-phosphate, pH 7.8). ▼—▼ the level of the glutaryl-7-ACA with MnO_2; ▽—▽ the level of glutaryl-7-ACA without MnO_2; ■—■ the level of keto-adipyl-7-ACA with MnO_2; □—□ the level of keto-adipyl-7-ACA without MnO_2; ●—● the level of the total product concentration with MnO_2; and ○—○ the level of the total product concentration without MnO_2.

In this case, the D-AAO activity decreased about 60% compared to the one without manganese oxide particles. To protect the enzyme from inactivation by manganese oxide as well as to immobilize the enzyme in a simple way, we examined a few aqueous two-phase systems (polyethylene glycol/dextran). We found that in such systems, the enzyme was indeed protected from denaturation by the metal oxide. The system containing PEG 35,000 and dextran T 10 (10%/8% in 100 m*M* K-phosphate, pH 7.8) was used for testing the influence of externally added hydrogen peroxide and MnO_2 on the enzymatic activity of D-AAO. The manganese oxide particles were found to partition completely to the dextran phase, that was also the phase preferred by the enzyme. Figure 4 shows that in the absence of MnO_2 and in the presence of 10 m*M* H_2O_2 2.9 μmol/mL keto-adipyl-7-ACA and 2.3 μmol/mL glutaryl-7-ACA are formed (total 5.2 μmol/mL of product). After addition of 20 mg/mL of MnO_2 to the two-phase system, the amount of keto-adipyl-7-ACA increased to 5.1 μmol/mL and glutaryl-7-ACA decreased to 1.3 μmol/mL (total amount of product was 6.4 μmol/mL). The increase in the amount of product was probably on account of the increased oxygen concentration in the system containing MnO_2.

For the immobilization, different carriers and coupling methods were screened, the methods presented here were those of our final choice.

Glutaraldehyde activated alkylamino-MnO_2, as well as alkylamino-CPG (1350 Å) were used to immobilize the D-AAO. An increase of the activity of the MnO_2 immobilized enzyme was observed (130% recovered activity). The activity of the free enzyme used for the immobilization was determined in the presence of excess of catalase in the enzymatic test.

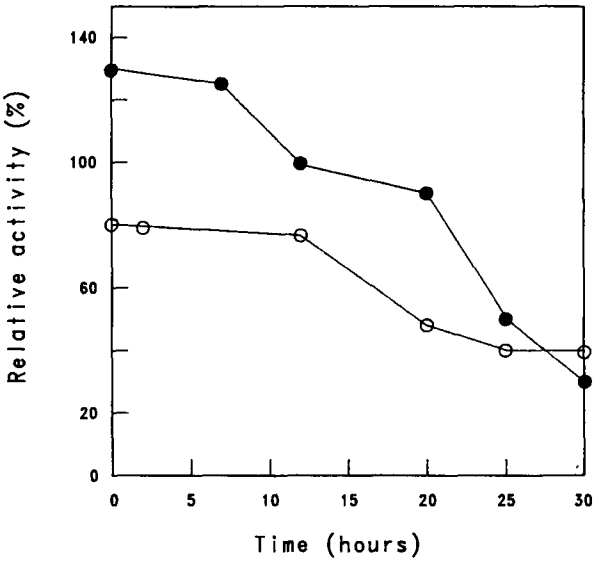

Fig. 5. Operation stability of D-AAO immobilized to glutaraldehyde-activated alkylamine-MnO_2 and CPG 1350 Å. ●—● MnO_2; ○—○ CPG. Enzyme activity before immobilization was 100%.

The activity increase of the immobilized enzyme was probably related to the very close contact between the enzyme and the metal oxide core, that has a function similar to that of catalase. The released oxygen in such close contact can probably be used immediately in the oxidase reaction. The activities of these preparations during semicontinuous operation are shown in Fig. 5. The half lives of the D-AAO activity coupled to glutaraldehyde activated alkylamino-CPG or on MnO_2 were about 25 h in both cases. The relative enzymatic activity after 20 h was 36% higher for the MnO_2-immobilized D-AAO than for the CPG-immobilized enzyme.

Besides these two inorganic carriers, we used Sepharose 4B activated with CNBr (*9*). 80% of the enzymatic activity of the D-AAO-Sepharose 4B complex was recovered after immobilization. In Fig. 6, different combinations of immobilization and coimmobilization of D-AAO and catalase are shown. The half life of the D-AAO immobilized to Sepharose 4B was found to be 6 h (Fig. 6, curve A). Crosslinking of the same preparation with glutaraldehyde shifted the half life of the enzyme from 6 h to 10 h (Fig. 6, curve B). Coimmobilization of catalase with D-AAO improved the half life from 10 to 20 h (curve C). This is probably caused by the increased oxygen supply, but could also be on account of the disappearance of free radicals, killers of flavine coenzymes (*13*), produced from hydrogen peroxide in the absence of catalase. By changing the sequence in which the enzyme is immobilized on the solid carrier, the operation stability was improved (Fig. 6, curve D). There was, however, no remarkable increase in stability if superoxide dismutase was coimmobilized with D-AAO and

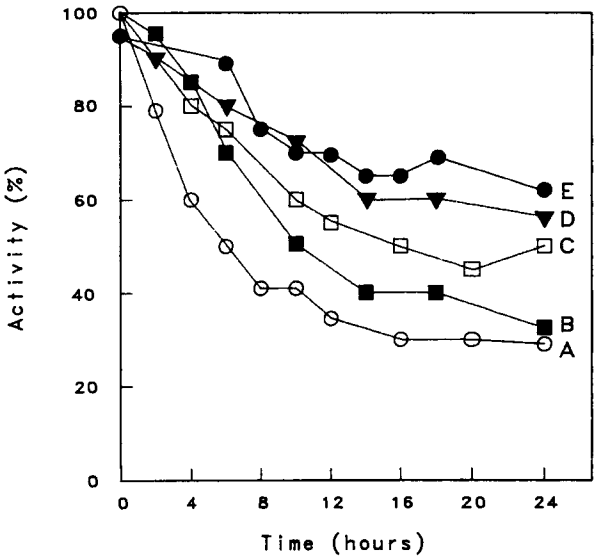

Fig. 6. Operation stability of D-AAO immobilized in different ways to CNBr-activated Sepharose 4B. Amount of D-AAo was 23 mg/g wet Sepharose. ○—○ A—only D-AAO coupled directly to CNBr-activated Sepharose 4B; ■—■ B—like preparation A but also crosslinked with 0.05% glutaraldehyde; □—□ C—like preparation A but also coimmobilized with catalase by crosslinking with glutaraldehyde (0.05%); ▼—▼ D—catalase coupled to CNBr-activated Sepharose 4B and coimmobilized with D-AAO by crosslinking with glutaraldehyde (0.05%); and ●—● E—catalase coupled like in D, followed by coimmobilization with a mixture of D-AAO and superoxide dismutase crosslinked by glutaraldehyde (0.05%).

catalase (curve E). In some cases, we tried to restore the lost activity by incubating the immobilized preparations with FAD or FMN (for 48 h), but without restoring it (*13*).

Tresylchloride was also used for activation of Sepharose 4B, but the enzyme coupled to such a carrier showed no activity.

Moreover, we used cellulose fibers activated with CNBr (*8*) and Affi-gel 10 as well. The recovery of the D-AAO activity in both cases was around 40%.

Sepharose 4B, with a six carbon spacer arm activated with carbodiimide or derivatized with glutaraldehyde, was also used. D-AAO coupled to the carbodiimide-activated spacer lost almost all enzymatic activity. D-AAO immobilized to glutaraldehyde-derivatized Sepharose 4B showed the same activity and stability as the preparation in Fig. 6, curve B.

We got about 80% recovery of activity and a relatively thermostable preparation of D-AAO immobilized to DE-52 cellulose. However, since this immobilization was based on medium strong ionic interactions, we could not raise the concentration of cephalosporin C above 2.5 m*M*, which is too low for efficient systems.

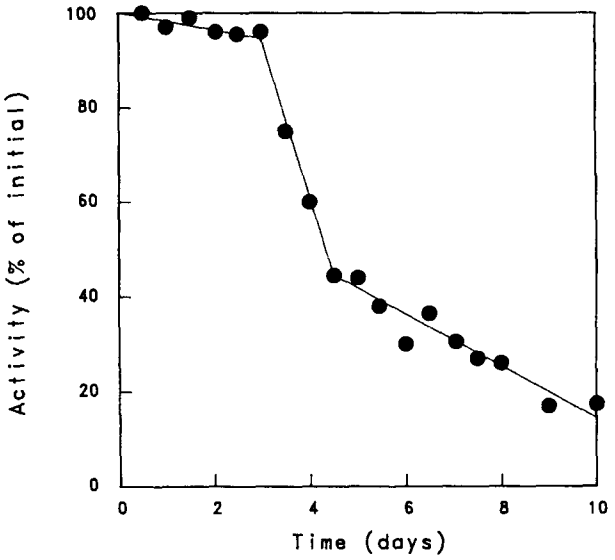

Fig. 7. The continuous conversion of 10 m*M* cephalosporin C (in 5 m*M* Tris-HCl, pH 7.5, containing 0.1 m*M* PMFS) by *Trigonopsis variabilis* cells immobilized to iron oxide particles.

To avoid hydrogen peroxide production, we have used para-benzoquinone and additionally also methyl viologen, potassium ferricyanide and trifenyltetrazolium chloride. None of these could replace oxygen or partly support the function of oxygen. In the presence of oxgygen, *p*-benzoquinone even acted as an inhibitor. Artificial electron acceptors are well known in enzymology, but their use has been of minor importance for preparative purposes. In spite of this fact, Alberti and Klibanov (*14*) and Adlecreutz and Mattiasson (*15*) succeeded in using para-benzoquinone.

Besides using immobilized enzymes and "free" enzymes contained in aqueous two-phase systems (unpublished data), we also used whole cells for the continuous transformation of cephalosporin C into keto-adipyl-7-aminocephalosporanic acid. The cells were permeabilized (Materials and Methods), crosslinked with 0.25% glutaraldehyde, and directly adsorbed to iron oxide particles. It was easy to handle such immobilized cells because of their magnetic properties. Magnetically immobilized cells have been described by Flygare and Larsson (*16*), who first modified the surface of the particles to achieve a good contact between cell surface and particles; this was not necessary in our system.

The D-AAO activity of the magnetically immobilized *T. variabilis* cells carried out in batch reactions started to decrease after the third day of continuous operation, and after 10 d, only 15% of the starting activity remained (Fig. 7).

CONCLUSIONS

The attempts to establish optimal conditions for the D-amino acid oxidase and cephalosporin C were made because we wanted to use D-AAO coimmobilized together with glutaryl-7-ACA acylase for producing 7-ACA. The results obtained show that only 60% of keto-adipyl-7-ACA is converted nonenzymatically to the glutaryl-7-ACA form by the simultaneously formed hydrogen peroxide in the reaction mixture. Hydrogen peroxide is harmful for cephalosporin C as well as for the enzyme–coenzyme. Therefore, hydrogen peroxide should be added after the enzymatic transformation is completed. Externally added hydrogen peroxide leads to higher yields of glutaryl-7-ACA. The use of manganese oxide in combination with hydrogen peroxide is shown to be beneficial, as the oxide both increases the oxygen concentration by breaking down the hydrogen peroxide, and protects the enzyme and cephalosporin C. D-Amino acid oxidase can be used in coimmobilization with catalase. From a practical point of view, the results with whole cells are very encouraging. Production of 7-ACA in the combined system of two enzymes on one carrier has been shown to be impractical.

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