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One-Pot Conversion of Cephalosporin C to 7-Aminocephalosporanic Acid in the Absence of Hydrogen Peroxide

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Abstract: The main drawback in the production of 7-aminocephalosporanic acid (7-ACA) at the industrial level is the inactivation of the enzymes implicated in the process due to the presence of hydrogen peroxide during the reaction. As an alternative, we have developed the conversion of cephalosporin C to 7-ACA in a single reactor without the presence of hydrogen peroxide during the reaction, achieving more than 80% yield. In order to develop this process, D-amino acid oxidase (DAAO) was co-immobilized with catalase (CAT), which is able to fully eliminate *in situ* the hydrogen peroxide formed by the neighbouring DAAO molecules. Thus, the product of the reaction is only α-ketoadipyl-7-ACA. This system prevents the inactivation of the oxidase by hydrogen peroxide, solving the main problem of the enzymatic process. Moreover, we have found that α-ketoadipyl-7-ACA is recognized as a substrate by glutaryl acylase (GAC) and hydrolyzed as long as glutaric acid is absent from the reaction medium (because it is able to inhibit the hydrolysis). The low stability of α-ketoadipyl-7-ACA justifies the use of a single reactor, in which glutaryl acylase is already present when this substrate is generated. Thus, the whole process may (and must) be performed in a single step, and in the absence of hydrogen peroxide that could affect the stabilities of the involved enzymes.

Keywords: D-amino acid oxidase; 7-aminocephalosporanic acid; catalase; co-immobilized enzymes; glutarayl acylase; hydrogen peroxide

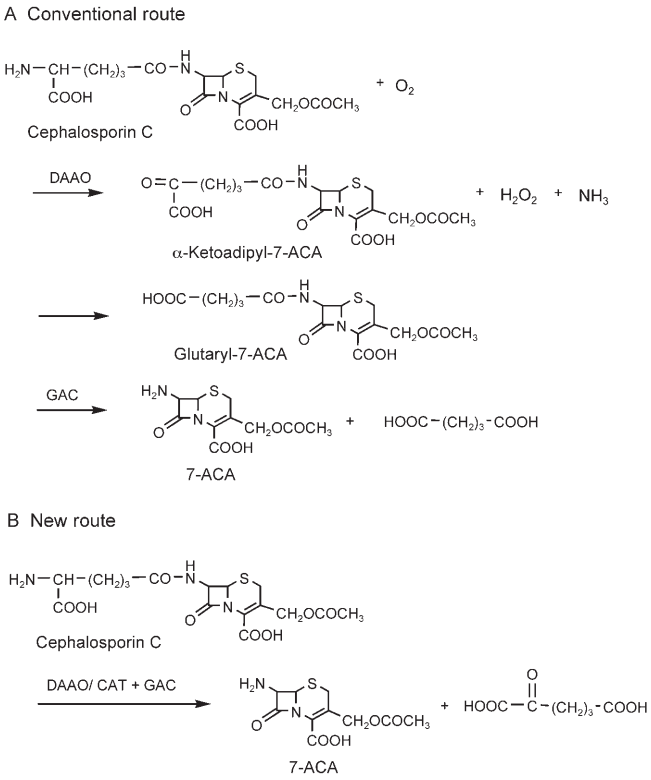
Introduction

7-Aminocephalosporanic acid (7-ACA) is a key intermediate for the production of many semi-synthetic cephalosporin antibiotics. Chemical deacylation of cephalosporin C (CPC),[1] a fermentation product, is the primary method used to produce 7-ACA industrially. This production method is economically feasible using currently established processes; however, there are concerns with environmental and safety issues due to the large quantities of hazardous chemicals used. To overcome these problems, efforts are being made to develop an efficient, entirely enzymatic process for the conversion of CPC to 7-ACA.

The direct conversion of CPC to 7-ACA in one-step by only one enzyme has been previously reported.[2] Nevertheless, the conversion rate of this enzyme is too low for an industrial application. Nowadays, the unique enzymatic route industrially applied for the conversion of CPC to 7-ACA consists in a two-pot process.[3] Firstly, the CPC suffers an oxidative deamination catalyzed by D-amino acid oxidase (DAAO), rendering 7-β-(5-carboxy-5-oxopentanamido)-cephalosporanic acid (α-ketoadipyl-7-ACA) and hydrogen peroxide, this last product is able to carry out the decarboxylation of α-ketoadipyl-7-ACA producing glutaryl-7-aminocephalosporanic acid (GL-7-ACA) spontaneously.[3a] Secondly the GL-7-ACA, formed in the previous step, is hydrolyzed to 7-ACA and glutaric acid by an enzyme named glutaryl acylase (GAC) (Scheme 1).[4]

The principal drawback of this process for the production of 7-ACA is the presence of hydrogen peroxide during the reaction, because hydrogen peroxide can inactivate the enzymes employed in the reaction, especially DAAO.[5]

Many attempts have been carried out to avoid the inactivation of DAAO and GAC by hydrogen peroxide through different immobilization strategies but with only moderate success.[6] The complete elimination of hydrogen peroxide from the reaction medium would be the best solution for this problem. To achieve this goal, we propose catalase (CAT) as a suitable alternative. CAT is an enzyme able to decompose the hydrogen peroxide into molecular oxygen and water.[7] The system



Scheme 1. Different routes for the conversion of CPC to 7-ACA.

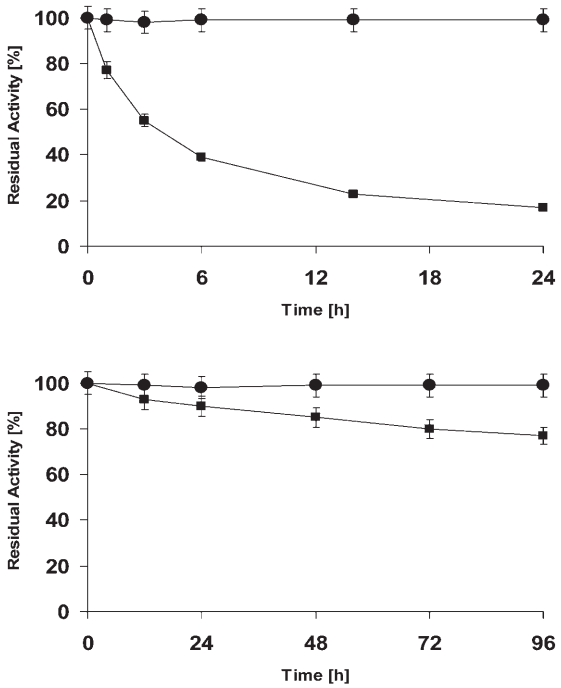


Figure 1. Inactivation of DAAO (*top*) and GAC (*bottom*) by hydrogen peroxide. 4 IU DAAO immobilized on glyoxyl agarose and 12 IU/mL PEI-GAC-glut derivative were separately incubated with and without 10 mM hydrogen peroxide at pH 7 and 25 °C. ●: enzyme without hydrogen peroxide and ■: enzyme with hydrogen peroxide.

DAAO/CAT has been used to produce phenylpyruvic acid, where co-immobilization of both enzymes was necessary to obtain only the desired product.[8] This example also illustrated that in this way, hydrogen peroxide could not inactivate the enzymes present in the reaction system.

In this work, we have designed a one-pot process for converting CPC to 7-ACA *via* a trienzymatic route (DAAO, CAT and GAC) where the oxidative deamination is carried out by DAAO yielding α-ketoadipyl-7-ACA and hydrogen peroxide as products, the hydrogen peroxide is immediately decomposed by CAT co-immobilized near DAAO, and the α-ketoadipyl-7-ACA can then be hydrolyzed to 7-ACA (final product) by GAC (Scheme 1). Therefore, this process could be carried out in one reactor (one-pot) because hydrogen peroxide is not present in the reaction medium.

Results and Discussion

Conversion of CPC to 7-ACA in a Two Reactor Process with a Bienzymatic System

As described in Scheme 1A the classical reaction has to be performed in two different reactors. In the first reactor, DAAO immobilized on glyoxyl agarose carried out the conversion of CPC to GL-7-ACA under very mild reaction conditions (pH 7.5 and 25 °C). This reaction was completed in 200 min with less than 1% CPC remaining at this point. Table 1 shows that the GL-7-ACA yield was 81%. Moreover, approximately 10% α-ketoadipyl-7-ACA was formed during the reaction. This 10% α-ketoadipyl-7-ACA means that not all the hydrogen peroxide was used to oxidatively decarboxylate α-ketoadipyl-7-ACA to GL-7-ACA (Scheme 1A), and therefore, it can inactivate DAAO or any other enzyme present in the reactor. Figure 1 shows the susceptibility of DAAO (top) and GAC (bottom) to hydrogen peroxide, mainly DAAO which, in presence of 10 mM hydrogen peroxide, only retained 20% of its initial activity after 24 h of incubation in presence of hydrogen peroxide.

After that, the reaction products were offered to GAC in a second pot. This hydrolysis reaction was carried out by PEI-GAC-glut derivative, again under very mild conditions (pH 8 and 25 °C). Table 1 shows that the 2nd reaction yielded 78% 7-ACA with only 3% of GL-7-ACA not hydrolyzed in 30 minutes, (very likely due to thermodynamic reasons). Most important was that all the initial α-ketoadipyl-7-ACA formed in the first reaction was not hydrolyzed by GAC.

This is the reason why α-ketoadipyl-7-ACA was not considered to be a substrate of GAC, in many instances

Table 1. Results from the two-pot reaction with a bienzymatic system, single cycle.

Time [min]	Reactors	Conversion [%]		Yield [%]		
		CPC	Glutaryl-7-ACA	α-ketoadipyl-7-ACA	7-ACA	Side products
200	Reactor 1	0	81 ± 4	10.3 ± 0.4	–	4.5 ± 0.2
30	Reactor 2	–	2.8 ± 0.1	9.5 ± 0.5	78.2 ± 4.5	3.6 ± 0.2

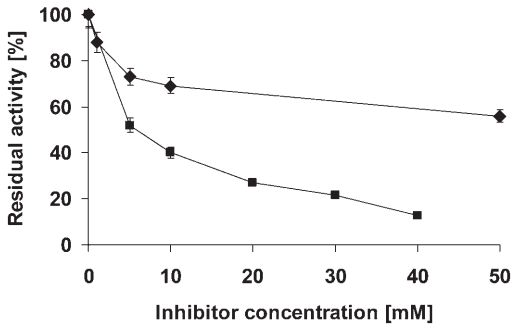


Figure 2. Effect of glutaric acid on the α-ketoadipyl-7-ACA amidase and GL-7-ACA amidase activities. ◆: GL-7-ACA amidase activity and ■: α-ketoadipyl-7-ACA amidase activity. Further details are described in the Experimental Section.

the first products need to be treated with the addition of hydrogen peroxide in a third pot to have 100% glutaryl-7-ACA.[5a,9] This solution would be very positive in terms of 7-ACA yield; however the process would be very more complex and expensive due to the use of an additional reactor.

However, we found that this is due to the accumulation of glutaric acid formed by the more favourable hydrolysis of GL-7-ACA. Figure 2 shows that the glutaric acid induced a higher inhibition in the α-ketoadipyl-7-ACA activity than in the GL-7-ACA activity (Figure 2).

Therefore, the main drawback of this process is the presence of hydrogen peroxide during the reaction, which may inactivate the enzymes employed in the system.

Co-Immobilization of DAAO and CATb to Carry Out the Oxidative Deamination of CPC and the Decomposition of Hydrogen Peroxide *in situ*

Thus, the full elimination of hydrogen peroxide was necessary for a second reason: to increase enzyme stability and to prevent inhibition of the α-ketoadipyl-7-ACA activity. In order to eliminate the hydrogen peroxide during the reaction, the co-immobilization of DAAO and CATb was suggested as a solution. GL-7-ACA was used as an indicator of the hydrogen peroxide formation, in order to evaluate different ratios of co-immobilized DAAO and CATb. Table 2 shows that with a 1:10 (DAAO:CATb) ratio in the co-immobilized derivative,

Table 2. Effect of the DAAO:CATb ratio on the production of GL-7-ACA.

Ratio [mg DAAO:mg CAT]	% Glutaryl-7-ACA
1:0.1	20 ± 1
1:1	0.60 ± 0.03
1:10	0

GL-7-ACA was not produced, which indicates that hydrogen peroxide was not present in the reaction medium. With lower ratios than 1:10 (DAAO: CATb) in the co-immobilized derivative, the full elimination of hydrogen peroxide was not achieved because GL-7-ACA appeared in the reaction medium.

Therefore, the optimal co-immobilized DAAO/CATb derivative was achieved when the amount by weight of CATb was 10-fold higher than the amount by weight of DAAO.

Kinetic Parameters of PEI-GAC-glut with Different Substrates

In order to develop a process without hydrogen peroxide, the oxidative deamination of CPC catalyzed by the co-immobilized DAAO/CATb derivative must produce only α-ketoadipyl-7-ACA. In this way, the kinetic parameters of the GAC derivative (PEI-GAC-glut) with α-ketoadipyl-7-ACA as substrate were analyzed (Table 3).

The GAC derivative carried out the hydrolysis of GL-7-ACA in a more favourable way than the hydrolysis of α-ketoadipyl-7-ACA, because the Km for GL-7-ACA was 4-fold lower than Km for α-ketoadipyl-7-ACA. However, the hydrolysis of α-ketoadipyl-7-ACA was possible, although the hydrolysis rate for this substrate was lower than for GL-7-ACA.

Conversion of CPC to 7-ACA in Two Reactors *via* a Trienzymatic System

The conversion of CPC to 7-ACA without hydrogen peroxide was performed using the co-immobilized DAAO/CATb derivative and, later on, the α-ketoadipyl-7-ACA hydrolysis using PEI-GAC-glut. When the conversion of CPC to 7-ACA was carried out in two reactors, the

Table 3. Kinetics parameters of GAC with GL-7-ACA and α-ketoadipyl-7-ACA.

Kinetic parameters	Glutaryl amidase activity	α-ketoadipyl amidase activity
Km [mM]	1.06 ± 0.05	4.9 ± 0.3
Kcat [min^{-1}]	87.8 ± 0.7	2.8 ± 0.5
Kcat/Km [$min^{-1} \times mM^{-1}$]	82.8 ± 0.4	0.56 ± 0.02

Table 4. Results from the two-pot reaction with a trienzymatic system, single cycle.

Time [min]	Reactors	Conversion [%]		Yield [%]		
		CPC	Glutaryl-7-ACA	α-ketoadipyl-7-ACA	7-ACA	Side products
200	Reactor 1	0	0	54.3 ± 1.1	–	13.3 ± 0.4
133	Reactor 2	–	0	0.20 ± 0.01	48 ± 2	9.19 ± 0.46

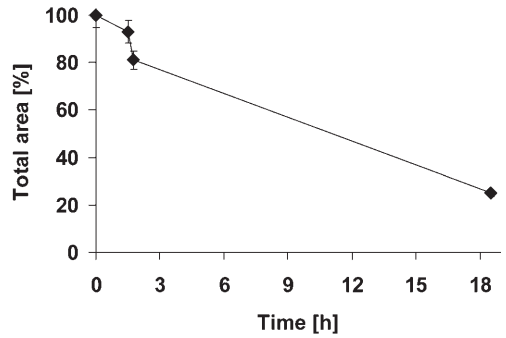


Figure 3. Decomposition of α-ketoadipyl-7-ACA in the reaction conditions. 4 mL of α-ketoadipyl-7-ACA were incubated at pH 7 and 25 °C. The reduction of the peak area corresponding to α-ketoadipyl-7-ACA was monitored by HPLC as described in the Experimental Section.

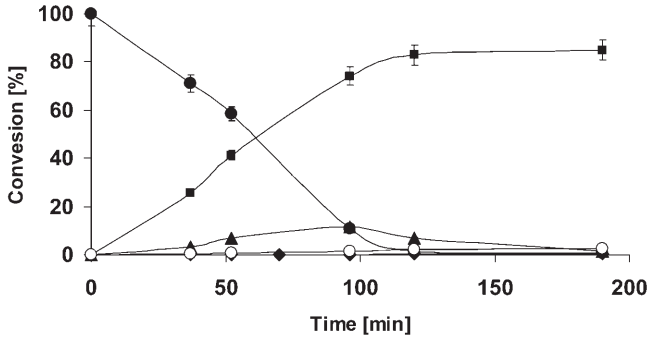


Figure 4. Reaction course of the conversion of cephalosporin C into 7-aminocephalosporanic acid using the trienzymatic, one-pot process. The reaction was carried out as described in the Experimental Section; ■: 7-ACA. ●: CPC. ▲: α-ketoadipyl-7-ACA; ○: side products and ◆: GL-7-ACA.

α-ketoadipyl-7-ACA yield was not high, although GL-7-ACA was not observed (Table 4). This makes that the final 7-ACA yield was much lower (47%) than in the conventional process. This fact could be explained because the α-ketoadipyl-7-ACA was quite unstable and rapidly decomposed into unknown products. Figure 3 shows that 80% of the initial α-ketoadipyl-7-ACA decomposed after 18 h under the reaction conditions (pH 7.5 and 25 °C). In this way, this process did not look industrially interesting.

Direct Conversion of CPC to 7-ACA in only One Reactor (One-Pot Process) *via* a Trienzymatic System

A possible solution to the previous problems is to avoid the accumulation of α-ketoadipyl-7-ACA by hydrolyzing it quickly after it is produced. The development of this system involves only one reactor with two different enzymatic derivatives (DAAO and CATb co-immobilized on glyoxyl agarose and PEI-GAC-glut derivative). In this system, CPC is converted to α-ketoadypyl-7-ACA and hydrogen peroxide by DAAO, and the hydrogen peroxide is decomposed *in situ* by CATb. Moreover, α-ketoadipyl-7-ACA is *in situ* hydrolyzed to 7-ACA by the GAC derivative, avoiding its accumulation (Scheme 1B).

The reaction course of the one-pot system (Figure 4) shows that 80% of 7-ACA was achieved in180 minutes. Moreover, only 2.5% of α-ketoadypyl-7-ACA was not hydrolyzed.

Conclusion

The inactivation of the enzymes, which carry out the conversion CPC to 7-ACA (primarily DAAO) by hydrogen peroxide is the main disadvantage of the conventional process used industrially for the production of the antibiotic core 7-ACA. In this work, we have developed a one-pot system that produces 7-ACA from CPC with three immobilized enzymes (DAAO/CATb and

GAC). The process was performed without hydrogen peroxide in the reaction medium, due to its *in situ* decomposition by CATb co-immobilized with DAAO. This fact is the great advantage of this process in comparison with the conventional two-pot system.

Moreover, another problem in the two-step system was the accumulation of α-ketoadipyl-7-ACA during the 1st reaction. This product could not be hydrolyzed in the 2nd reaction because of the presence of glutaric acid, a potent inhibitor of the hydrolysis of α-ketoadipyl-7-ACA, formed by the hydrolysis of GL-7-ACA (main product of the first reaction). This problem would not occur in the one-pot system, since GL-7-ACA was not produced due to the absence of hydrogen peroxide which could carry out the decarboxylation of α-ketoadipyl to GL-7-ACA.

Hence, the direct conversion of CPC to 7-ACA *via* a trienzymatic system in only one reactor, presented two important advantages with respect to the conventional two-pot enzymatic route. However, this one-pot system may be improved if GAC hydrolysis rate with α-ketoadipyl-7-ACA is improved. The solution to this problem is the search for an enzyme with better properties towards the hydrolysis of α-ketoadipyl-7-ACA than the GAC used in this work. This aim could be achieved with the new techniques of molecular biology as directed evolution. In this direction, new mutants of GAC have been made for carrying out the adipyl-7ADCA hydrolysis (its side chain is very similar to that of α-ketoadipyl-7-ACA) in a very successful way.[10]

Experimental Section

Materials

Cephalosporin C (CPC), and glutaryl-7-aminocephalosporanic acid (GL-7-ACA), were kindly donated by Bioferma Murcia S. A. Glutaryl acylase from *Pseudomonas* sp. (GAC) was obtained from Roche (Basel, Switzerland) and D-amino acid oxidase from *Trigonopsis variabilis* (DAAO) was obtained from Recordati (Milan, Italy). Catalase from bovine liver (CATb) was purchased from Fluka (Buch, Switzerland). Glyoxyl agarose 4-BCL (20 μmoles of glyoxyl groups/g support) was prepared according to previously described methods.[11] Polyethyleneimine 600 KDa-agarose 4BCL support was prepared according to previously described methods.[12] Glutaric acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Kromasil C-8, (5 μm, 250 × 4.6 mm) column was purchased from Analisis vínicos (Tomelloso, Spain). Ammonium acetate (HPLC grade) was purchased from Panreac (Barcelona, Spain), acetonitrile was purchased from Scharlau (Barcelona, Spain). All other reagents were of analytical grade.

Methods

All experiments were performed at least in triplicate and the results are presented as the mean value. Experimental error was never over 5%.

Preparation of Glutaryl-7-ACA Acylase

The commercial preparation of GA was diluted (1/5) in 25 mM potassium phosphate buffer solution pH 7.0 and then dialyzed three times against 100 volumes of 5 mM potassium phosphate buffer, pH 7. The dialyzed enzyme was then centrifuged (12000 rpm for 30 minutes at 4 °C) and the supernatant (containing 16 IU/mL) was used as the enzymatic preparation for further experiments. More than 90% of initial activity was recovered after this process.

Determination of Catalase Activity

Catalase activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2, through the measurement of the change in the absorbance at 240 nm.[13] 2.9 mL of H_2O_2, 0.14% (w/v) in 50 mM sodium phosphate buffer pH 7.0 were incubated with 0.2 mL of enzyme solution. All the measurements were carried out at 25 °C.

One catalase unit was defined as the amount of enzyme that decomposes 1 micromol of H_2O_2 under the previously described conditions.

D-Amino Acid Oxidase Activity Assay

DAAO activity in its soluble form was analyzed spectrophotometrically using cephalosporin C as substrate measuring the increment of absorbance at 445 nm promoted by coupling the oxidative deamination of the substrate with the reaction between the hydrogen peroxide and *o*-phenylenediamine catalyzed by peroxidase.[13] The reaction mixture consisted of 1.5 mL of 25 mg/mL cephalosporin C solution in 100 mM potassium phosphate at pH 7.5, 0.5 mL of 1.85 mM *o*-phenylenediamine in distilled water and 0.1 mL of a 1 mg/mL peroxidase solution in 50 mM potassium phosphate at pH 7.5. The reagents were preincubated at 25 °C. The reaction was initiated by adding a maximum of 0.1 units of DAAO.

One DAAO unit is defined as the amount of enzyme able to oxidize one micromole of cephalosporin C per minute in the previously described conditions.

Glutaryl Acylase Activity Determination

The activity of both immobilized and soluble enzyme was measured as follows: the initial reaction rates were measured with a pH-STAT using an automatic titrator (Crison micro TT 2050) to determine the amount of glutaric acid formed. The assays were carried out by adding 0.1 mL of soluble enzyme or suspension of immobilized enzyme to 10 mL of a 10 mM solution of GL-7-ACA 0.1 M potassium phosphate buffer pH 7.5, and titrating the reaction mixture with 25 mM NaOH at 25 °C.

One unit of glutaryl 7-ACA acylase activity was defined as the amount of enzyme that is necessary to produce 1 micromol of glutaric acid per minute in the previously described conditions.

Product Analysis by HPLC

The products of the reactions were determined through HPLC with a Kromasil C8 (5 μm, 250 × 4.6 mm) column, mobile phase 20 mM ammonium acetate pH 5.2; acetonitrile (95:5 v/v). The retention times of the different products were: 4.1 minutes for CPC, 4.9 minutes for 7-ACA, 6.7 minutes for α-ketoadipyl-7-ACA and 12.3 minutes for GL-7-ACA. Other minor peaks were considered as side products.

Immobilization of DAAO on Glyoxyl Agarose

10 g of glyoxyl agarose 4BCL (20 μmoles of glyoxyl groups/g support) were added to 20 mL of a 1 mg/mL DAAO solution in 25 mM sodium carbonate at pH 10.00. The suspension was gently stirred at 4 °C. Periodically, samples of the supernatants were withdrawn and analyzed for enzyme activity and protein concentration. After 15 hours of gently stirring at 4 °C, 1 mg of solid $NaBH_4$ was added per mL of suspension and 30 min later the reduced derivative was washed with 25 mM potassium phosphate buffer at pH 7.0. Finally, the derivative was stored at 4 °C until further use.

Co-Immobilization of DAAO and CATb on Glyoxyl Agarose

100 mL of a CATb solution at different concentrations (1 mg/mL, 0.1 mg/mL or 0.01 mg/mL) were added to 10 g of unreduced, immobilized DAAO (2 mg DAAO/g support) on glyoxyl agarose to permit the co-immobilization of CATb. The suspension was gently stirred at 25 °C. Periodically, samples of the supernatants were withdrawn and analyzed for enzyme activity and protein concentration. After 3 h of gentle shaking at 25 °C, 1 mg of solid $NaBH_4$ was added per mL of suspension and 30 min later the reduced derivative was washed with 25 mM potassium phosphate buffer at pH 7.0. The derivative was stored at 4 °C until further use.

Covalent Immobilization of GAC Adsorbed on PEI through Glutaraldehyde Treatment of the Adsorbed Protein

10 g of PEI-agarose were suspended in 60 mL of GAC solution prepared as previously described. The suspension was gently shaken at 25 °C. Periodically, samples of the supernatants were withdrawn and analyzed for enzyme activity and protein concentration. When the immobilization was completed, the suspension was filtered and suspended in 40 mL of a 0.5% glutaraldehyde solution in 25 mM potassium phosphate buffer at pH 7. The suspension was left under mild stirring at 25 °C during 1 h. This step permits the activation of primary amino groups of the support as well as the enzyme with one molecule of glutaraldehyde.[12] Then the suspension was filtered, washed with 25 mM potassium phosphate buffer at pH 7, resuspended in the same buffer and further incubated at 25 °C for 20 h to have an intense support-enzyme cross-linking.[14] The final derivative, termed PEI-GAC-glut, was stored at 4 °C until further use.

Kinetic Constants of GAC for GL-7-ACA and α-Ketoadipyl-7-ACA

Km, Kcat and Km/Kcat were determined for the GL-7-ACA and α-ketoadipyl-7-ACA activities of the PEI-GAC-glut derivative. The GL-7-ACA activity was determined with an automatic titrator as previously described, with different concentrations of GL-7-ACA (0.5–5 mM). The α-ketoadipyl-7-ACA activity was determined by incubating 0.1 g of PEI-GAC glut with 1.5 mL of α-ketoadipy-7-ACA solution at different concentrations and 25 °C (1.75–13.8 mM) prepared in 0.1 M potassium phosphate buffer at pH 7. Supernatant samples were withdrawn at different times and analyzed by HPLC as described above. The Michaelis constant was determined for each substrate from Lineweaver–Burk plots.

Inhibition of the GL-7-ACA and α-Ketoadipyl Activities of the PEI-GAC-glut Derivative by Glutaric Acid

The inhibition by glutaric acid on the GL-7-ACA activity was determined measuring the hydrolysis of GL-7-ACA with an automatic titrator as previously described in the presence of different glutaric acid concentrations (0–50 mM).

The inhibition of glutaric acid on the α-ketoadipyl-7-ACA activity was determined by measuring the formation of 7-ACA via HPLC as described above, in a suspension of 0.1 g of derivative with 1.6 mL of 13.8 mM α-ketoadipyl-7-ACA and glutaric acid at different concentrations (0–40 mM) at pH 7.5 and 25 °C.

Conversion of CPC to 7-ACA in Two Reactors (Two-Pot Process)

1st Reactor. Conversion of CPC to GL-7-ACA or α-Ketoadipyl-7-ACA: 0.2 g of immobilized DAAO or co-immobilized DAAO-CATb on glyoxyl agarose were added to 4 mL of 40 mM CPC in potassium phosphate buffer at pH 7.5 and 25 °C to convert the CPC to GL-7-ACA or α-ketoadipyl-7-ACA, respectively. Samples were withdrawn from the supernatant at different times and analyzed by HPLC as described above. When all CPC was completely converted, the suspension was filtered and the supernatant was recovered for further experiments

2nd Reactor. Conversion of GL-7-ACA or α-Ketoadipyl-7-ACA to 7-ACA: 4 mL of GL-7-ACA or α-ketoadipyl-7-ACA obtained as described above were added to 1.5 g of PEI-GAC-glut derivative (60 mg GAC/g support) and the suspension pH was increased to 8.0. The suspension was gently shaken and the pH was maintained at 8.0 by continuous addition of 4 M NaOH. Periodically, samples were withdrawn and analyzed *via* HPLC as described above.

Direct Conversion of CPC to 7-ACA in One Reactor (One-Pot Process)

0.2 g of DAO-CATb (2 mg DAAO:20 mg CATb/g support) co-immobilized on glyoxyl agarose plus 1.5 g of PEI-GAC-glut derivative (60 mg GAC/g support) were added to 4 mL of 40 mM CPC in potassium phosphate buffer at pH 8 and 25 °C. The suspension was gently stirred and the pH was maintained at 8.0 by adding 4 M NaOH during all process. Periodically, samples were withdrawn and analyzed *via* HPLC as described above.

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