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Crosslinked Penicillin Acylase Aggregates for Synthesis of β-Lactam Antibiotics in Organic Medium

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Abstract

Crosslinked enzyme aggregates (CLEAs) of a partially purified penicillin acylase from a recombinant *Escherichia coli* strain have been produced as a novel type of biocatalysts well endowed to perform in organic media. Different protein precipitants were studied and glutaraldehyde was used as the crosslinking agent. Precipitation curves were obtained for all precipitants to determine the concentrations at which all the protein precipitated out of the solution. The effect of the glutaraldehyde-to-protein ratio was studied with respect to process recovery and the specific activity and stability of the biocatalyst. Recovery of penicillin acylase activity was moderately high, about 50%; major losses of enzyme activity were produced at the precipitation step. Specific activities of all CLEAs were very high, which is one of the advantages of using nonsupported biocatalysts. Ammonium sulfate and *tert*-butyl alcohol were the best precipitants at a glutaraldehyde-protein mass ratio of 2 and were selected to perform the kinetically controlled synthesis of ampicillin in 60% (v/v) ethylene glycol medium. At comparable conversion yields, volumetric and specific antibiotic productivity were much higher for CLEAs than for carrier-bound penicillin acylases.

Index Entries: Crosslinked enzyme aggregates; enzyme immobilization; penicillin acylase; ampicillin; β-lactam antibiotics; organic cosolvents.

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Introduction

The use of enzyme biocatalysts to perform reactions of organic synthesis is gaining widespread use *(1)*. Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is a very versatile biocatalyst for organic synthesis and has been used for the synthesis of antibiotics and other amide-bond-containing compounds *(2–4)*. Reactions of organic synthesis often require being conducted in nonaqueous media, so considerable attention has been focused on studying the performance of enzyme biocatalysts in organic media *(5–7)* and, more recently, in ionic liquids *(8–10)*. Biocatalysts adequate for performing in such media are thus required. Among the many strategies and procedures developed *(11–14)*, crosslinked enzyme crystals *(15)*, and, more recently, crosslinked enzyme aggregates (CLEAs) *(16–18)* appear to be particularly promising biocatalysts for organic synthesis. These nonsupported recoverable biocatalysts are favorably compared with carrier-bound enzymes, as recently highlighted *(19)*. CLEAs have the additional advantage of not requiring purified enzymes, and the procedure is rather straightforward and amenable for scale-up. The procedure is an extension of the well-known system of enzyme aggregation by molecular crosslinking with bifunctional reagents. However, in this case, aggregation is produced over already precipitated protein molecules, making the biocatalyst robust enough to cope with the harsh conditions of synthesis. CLEAs have been proposed as a suitable form of biocatalyst for penicillin acylase *(17)* and other enzymes as well *(20,21)*. The combination of CLEAs with polymeric coaggregates *(22)* and with encapsulation into hydrophilic gels *(23,24)* has also been proposed as a suitable form of biocatalyst for organic synthesis with penicillin acylase.

In this article, we present the results obtained in the production of CLEAs from a partially purified penicillin acylase from recombinant *Escherichia coli*. Several precipitants were assessed and conditions at each step of the production process were determined considering recovery of enzyme activity and specific activity and stability of the biocatalyst. The selected biocatalysts were tested in the kinetically controlled synthesis of ampicillin used as a model reaction to compare their performance with carrier-bound penicillin acylase biocatalysts.

Materials and Methods

Chemicals and Reagents

Penicillin acylase from *E. coli* with 230 ± 30 IU/mL and 22 ± 2 mg/mL of protein was kindly provided by Antibióticos S.A. (León, Spain). The enzyme was centrifuged and dialyzed prior to use. This partially purified preparation, with 240 IU/mL and 21 mg of protein/mL, was used throughout this work and remained fully stable for more than 1 yr stored at 4°C. Immobilized penicillin acylase (PGA450) from *E. coli* with 320 ± 20 IU/g was from Roche (Mannheim, Germany). Agarose 10 BCL was from Iberagar

(Coina, Portugal), penicillin G potassium salt (PGK) and 6-aminopenicillanic acid (6-APA) were kindly provided by Natsus S.A. (Lima, Perú), (*R*)-(–)-2-phenylglycine methylester hydrochloride (PGME) and ampicillin were from Sigma (St. Louis, MO), and (*R*)-(–)-2-phenylglycine (PG) was from Aldrich (Milwaukee, WI). Polyethylene glycol (PEG) of 600 and 10,000 Daltons and ammonium sulfate were from Merck (Darmstadt, Germany), and *tert*-butyl alcohol and glutaraldehyde solution (25% [v/v]) were from Fluka (Buchs, Switzerland). Ethylene glycol, glycidol, and all other reagents were of analytical grade and were from either Sigma-Aldrich or Merck.

Analysis

The hydrolytic activity of penicillin acylase was determined from initial rate data analyzing 6-APA formation as reported by Shewale et al. *(25)*. Alternatively, it was determined using a pH-stat (Mettler Toledo, DL50) to titrate the H^+ produced by the hydrolysis of 10 m*M* penicillin G in 0.1 *M* sodium phosphate buffer, pH 7.8.

One international unit of activity (IU) was defined as the amount of penicillin acylase that catalyzes the hydrolysis of 1 µmol of PGK/min from 134 m*M* PGK solution in 0.1 *M* phosphate buffer, pH 7.8, at 30°C and 250 rpm. Substrates and products of synthesis were identified and analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV UV-Vis detector and a CBM-101 Shimadzu HPLC/PC integrator. The column used was a µ-Bondapack C_{18} (300 × 3.9 mm) from Waters (Milford, MA). Samples were eluted isocratically with a sonicated mixture of 70% (v/v) 20 m*M* phosphate buffer, pH 6.0, and 30% (v/v) methanol at a flow rate of 1 mL/min and analyzed in the UV detector at 214 nm. Elution times were 2.8, 3.7, 8.9, and 12.5 min for 6-APA, PG, Amp, and PGME, respectively. The concentration of substrates and products was calculated from calibration curves using stock solutions.

Protein was determined according to Bradford *(26)*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Electrophoresis Power Supply EPS 600) was performed using molecular mass standards of 14,400 (α-lactoalbumin), 20,100 (trypsin inhibitor), 30,000 (carbonic anhydrase), 67,000 (albumin), and 94,000 Daltons (phosphorylase *b*).

Production of Biocatalysts of Penicillin Acylase

The process for the production of CLEAs consists of four operations. The first is precipitation, in which the enzyme is contacted with the precipitating agent; the second is crosslinking, in which the protein precipitate is reacted with a bifunctional reagent, glutaraldehyde in this case; the third is reduction, to stabilize the Schiff base formed by reaction of the aldehyde groups of the reagent and the amino groups in the protein (mainly ε-amino group from lysine), forming a stable secondary amine. The final operation

is a thorough washing of the biocatalyst to remove any adsorbed protein and reagents. For each 10 mL of partially purified penicillin acylase extract, the required amount of precipitating agent is added slowly under agitation and held for 45 min (precipitation was in all cases complete after 30 min). Four precipitants were selected: PEG 600 and 10,000 Daltons, ammonium sulfate, and *tert*-butyl alcohol. For each one, a precipitation profile was done to determine the concentration required to precipitate all the protein out of the solution. Then, the required volume of glutaraldehyde (25% [v/v]) was added dropwise and allowed to react for 1 h under agitation; the amount of glutaraldehyde varied according to the glutaraldehyde-protein mass ratio employed, from 0.5 to 2. A solution of 100 m*M* bicarbonate solution, pH 10.1, containing 100 m*M* phenylacetic acid and 25% (v/v) glycerol was added to double the volume of the reaction. $NaBH_4$ was added to a concentration of 1 mg/mL and allowed to react for 30 min under agitation. All these operations were performed in a water-ice bath, with the temperature in the reactor never exceeding 3°C. Precipitation and cross-linking were done at a controlled pH of 7.0. The suspension containing the CLEAs was then centrifuged at 3°C for 10 min at 12,000*g* and the supernatant discarded. The CLEAs were resuspended in 200 m*M* phosphate buffer, pH 7.0, and again centrifuged; this operation was repeated four times and then the biocatalyst was stored wet at 4°C. No enzyme inactivation or leakage has been detected during prolonged storage.

Glyoxyl-agarose-immobilized penicillin (GAP) acylase was prepared from the partially purified enzyme extract according to the procedure described by Alvaro et al. *(27)*. Glyoxyl-agarose gel beads were produced as reported by Guisán *(28)*. The immobilization procedure was modified by using phenylacetic acid instead of penicillin G sulfoxide as protecting agent, and the time of immobilization was extended to 20 h, determined as the optimum for biocatalyst stability *(29)*. The glyoxyl-agarose-immobilized penicillin acylase biocatalyst (GAP) was stored wet at 4°C. No enzyme inactivation or leakage has been detected during prolonged storage.

Commercial penicillin acylase, PGA-450, was used directly after incubation for half an hour at reaction conditions.

Stability of CLEAs of Penicillin Acylase

The stability of CLEAs produced by precipitation with PEG (600 and 10,000 Daltons), ammonium sulfate, and *tert*-butyl alcohol at different glutaraldehyde-protein ratios was determined by incubating the biocatalysts under nonreactive conditions in 60% (v/v) ethylene glycol medium, pH 7.0, at 50°C. Experiments were conducted in 80-mL Pyrex glass reactors with a working volume of 50 mL and equipped with a paddle impeller to keep CLEAs in suspension. Samples were taken at intervals to determine the residual activity of the biocatalyst. The concentration of CLEAs was the same as in the experiments of synthesis.

Synthesis of Ampicillin With CLEAs and Carrier-Bound Penicillin Acylase

Syntheses of ampicillin with CLEAs produced by precipitation with ammonium sulfate (AS-CLEA) and *tert*-butyl alcohol (TB-CLEA) were conducted under kinetic control at pH 7.0, 0°C, and 60% (v/v) ethylene glycol medium using PGME as acyl donor and 6-APA as the limiting substrate at a PGME-6-APA molar ratio of 3. The amount of biocatalyst in all experiments was equivalent to 125 IU/mmol of 6-APA. These conditions were selected for being close to the optimum previously determined for the synthesis of cephalexin and ampicillin with GAP and PGA-450 *(30–32)*, so that results obtained with CLEAs can be properly compared *(30–32)*. Syntheses were performed batchwise with temperature and pH control in 80-mL Pyrex glass reactors with a working volume of 50 mL and equipped with a paddle impeller to keep CLEAs in suspension. Samples were taken at intervals through a filter (or centrifuged) to keep CLEAs out and were properly diluted prior to being assayed by HPLC. Molar conversion yield (Y) was defined as the maximum molar conversion of 6-APA into ampicillin. Volumetric productivity (mM/h) was determined as the amount of ampicillin produced per unit time and unit reaction volume at maximum yield. Specific productivity (mM/[h·g]) was determined as the amount of ampicillin produced per unit time, unit reaction volume, and unit mass of biocatalyst at maximum yield. Experiments were done in duplicate and samples assayed in triplicate with variations below 5% among them.

Results and Discussion

Production and Characterization of Biocatalysts of Penicillin Acylase

CLEAs were produced from the partially purified penicillin acylase preparation using four different precipitating agents: PEG 600 and 10,000 Daltons, ammonium sulfate, and *tert*-butyl alcohol; glutaraldehyde was used as the crosslinking agent in all cases. Precipitation profiles for penicillin acylase and total protein are presented in Fig. 1 for the different precipitants. Activity and total protein were measured on the redissolved precipitate and supernatant. The supernatant underwent SDS-PAGE to monitor the disappearance of penicillin acylase and contaminant proteins during precipitation; penicillin acylase subunits had molecular masses of 30,000 and 67,000 Daltons. Penicillin acylase and total protein followed similar patterns of precipitation. The enzyme was completely precipitated at 60% (v/v) PEG 600, 20% (v/v) PEG 10,000, ammonium sulfate at 50% saturation, and 50% (v/v) *tert*-butyl alcohol. Therefore, such precipitant concentrations were used to produce the corresponding CLEAs.

Enzyme activity and protein balances were done at each step of the production process. Table 1 summarizes the results for the case of PEG 600. A global recovery of 50% was obtained for the enzyme activity, with major losses produced at the precipitation and washing steps. In the first case,

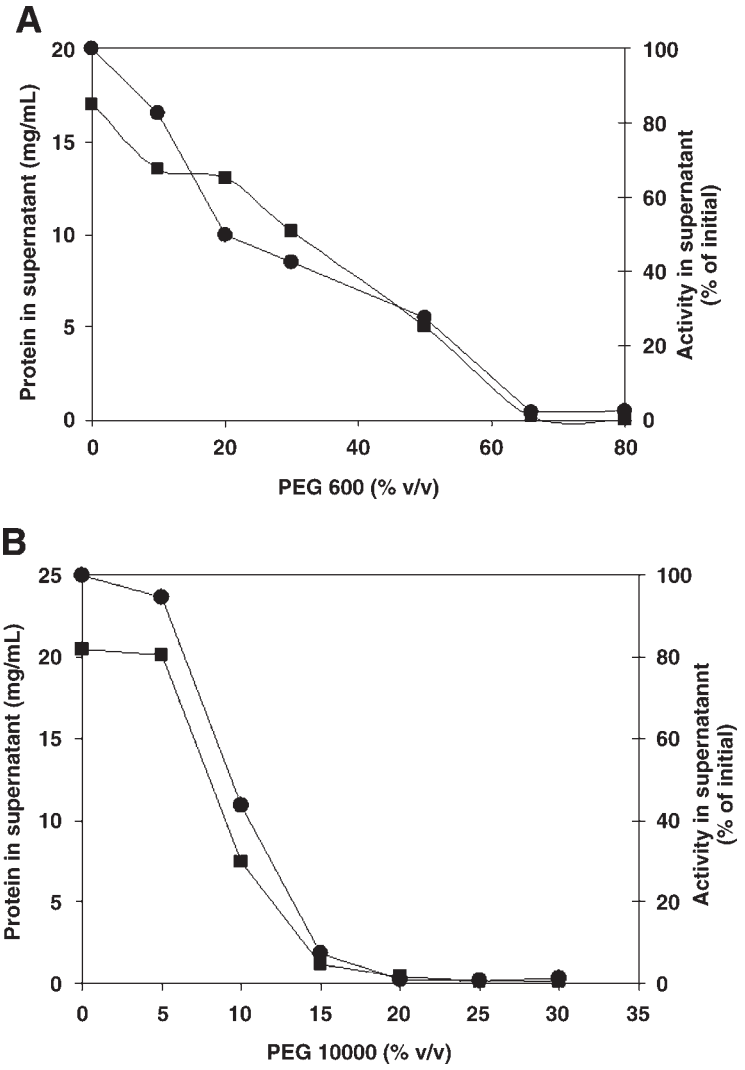

Fig. 1. Precipitation profiles of partially purified penicillin acylase from recombinant *E. coli*: **(A)** PEG 600; **(B)** PEG 10,000; (■) protein; (●) penicillin acylase activity.

losses were owing to enzyme inactivation, because almost no activity was recovered in the supernatant after precipitation; diffusional restrictions may also be partly responsible for such loss. In the washing step, losses were owing to noncrosslinked protein desorption from the CLEA; in this case activity was obtained in the washings that corresponded to a substantial portion of the activity lost at this step. Global protein recovery was 80% and major losses were produced at the washing step, which is consistent with activity losses. Results with other precipitating agents differed only at the precipitation stage and not very significantly. The only exception was PEG 10,000, with which higher losses were produced at the

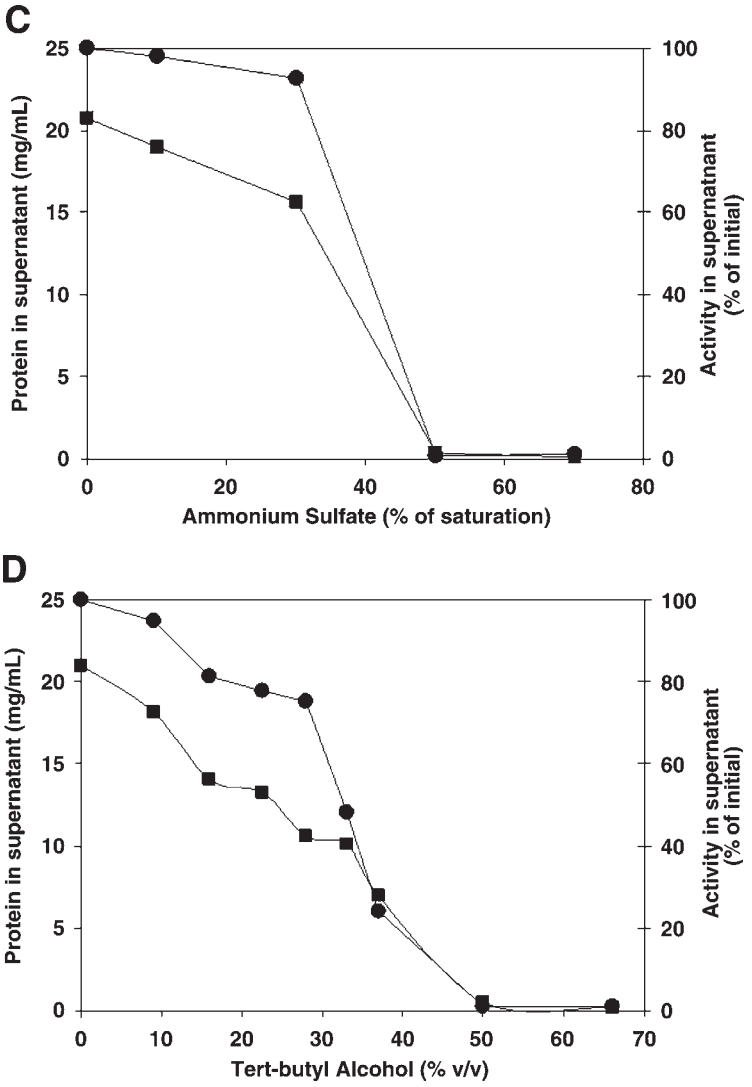

Fig. 1. *(continued)* Precipitation profiles of partially purified penicillin acylase from recombinant *E. coli*: **(C)** ammonium sulfate; **(D)** *tert*-butyl alcohol; (■) protein; (●) penicillin acylase activity.

other steps. Table 2 presents the findings for recovery at the precipitation stage and global recovery for all precipitants. Specific activities of the CLEAs obtained are also presented in Table 2; as seen, values were significantly higher for AS-CLEA. PEG 10,000 was not considered for further studies because of the low specific activity of the CLEA and the low global recovery of the process; furthermore, the mechanical properties of the biocatalyst were poor and made it difficult to handle.

The effect of the glutaraldehyde-to-protein mass ratio was then studied for PEG 600 and ammonium sulfate CLEAs in the range 2–0.5.

Table 1
Penicillin Acylase and Protein Recovery
at Each Step of Production of CLEAs
Using PEG 600 as Precipitant and Glutaraldehyde-to-Protein Mass Ratio of 2

	Recovery at step (%)		Global recovery (%)	
Step	Penicillin acylase	Protein	Penicillin acylase	Protein
1. Precipitation	74	96	74	96
2. Crosslinking	97	93	72	89
3. Reduction	93	97	67	86
4. Washing	73	92	49	79

Table 2
Penicillin Acylase Recovery at Precipitation Step
and Global Recovery in Production of CLEAs,
and Specific Activity of Biocatalysts
Using Different Precipitants and Glutaraldehyde-to-Protein Mass Ratio of 2

Precipitant	Recovery at precipitation (%)	Global recovery (%)	Specific activity (IU/g)
PEG 600	74	49	590
PEG 10,000	80	39	515
$(NH_4)_2SO_4$	77	54	998
tert-Butyl alcohol	69	48	618

The results, which are summarized in Table 3, indicate that more compact CLEAs were produced at higher concentrations of crosslinking agent, impairing activity most probably owing to mass transfer limitations. Specific activities were always higher for ammonium sulfate than for PEG 600 CLEAs and increased substantially at the lower glutaraldehyde-protein ratio. However, the stability of the biocatalysts obtained at the different ratios was assessed before choosing the most appropriate value. Inactivation was therefore conducted under nonreactive conditions at pH 7.0 and 50°C in 60% (v/v) ethylene glycol. The concentration of ethylene glycol was chosen to simulate the conditions at which ampicillin synthesis was tried and the high temperature to increase inactivation rates so that differences among CLEAs could be better appreciated. Figure 2 presents the results for CLEAs obtained with PEG 600 at different glutaraldehyde-protein ratios and those obtained with ammonium sulfate and *tert*-butyl alcohol at a glutaraldehyde-protein ratio of 2. Table 4 reports half-life values. CLEAs produced with *tert*-butyl alcohol as precipitant were the most stable, and the less stable were those produced with PEG 600 as precipitant, and the stability was significantly higher at the glutaraldehyde-protein ratio of 2.

Table 3
Global Recovery of Penicillin Acylase in Production of CLEAs and Specific Activity of Biocatalysts Using PEG 600 and Ammonium Sulfate at Different Glutaraldehyde-to-Protein Mass Ratios

	Global recovery (%)		Specific activity (IU/g)	
G/P[a]	PEG 600-CLEA	AS-CLEA	PEG 600-CLEA	AS-CLEA
2	49	54	590	998
1	50	56	820	1020
0.5	68	65	960	1180

[a]G/P, glutaraldehyde-to-protein mass ratio.

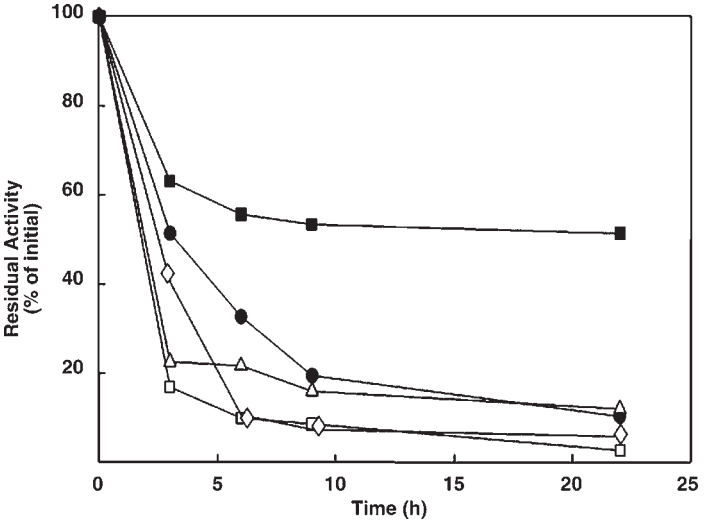

Fig. 2. Inactivation of penicillin acylase CLEAs at 50°C, pH 7.0, and 60% (v/v) ethylene glycol: (◇) PEG 600-CLEA, (●) AS-CLEA, (■) TB-CLEA, all at glutaraldehyde-to-protein mass ratio of 2; (□) PEG 600-CLEA at glutaraldehyde-to-protein mass ratio of 1; (△) PEG 600-CLEA at glutaraldehyde-to-protein mass ratio of 0.5.

Considering the recovery of enzyme activity, the specific activity, and the stability of the different CLEAs produced, those obtained with ammonium sulfate and *tert*-butyl alcohol as precipitants at a glutaraldehyde-protein ratio of 2 were selected for performing the kinetically controlled synthesis of ampicillin. Both CLEAs have remained fully active at 4°C for more than 6 mo.

GAP was produced with a specific activity of 220 ± 20 IU/g. PGA-450 had a specific activity of 300 ± 20 IU/g. Both biocatalysts have remained fully stable under storage at 4°C for 1 yr.

Table 4
Half-Life of CLEAs at pH 7.0 and 50°C
in 60% (v/v) Ethylene Glycol Obtained With Different
Precipitating Agents and Glutaraldehyde-to-Protein Mass Ratios

Precipitant	Glutaraldehyde-to-protein ratio	Half-life (min)
PEG 600	2	150
	1	115
	0.5	120
$(NH_4)_2SO_4$	2	190
tert-Butyl alcohol	2	510

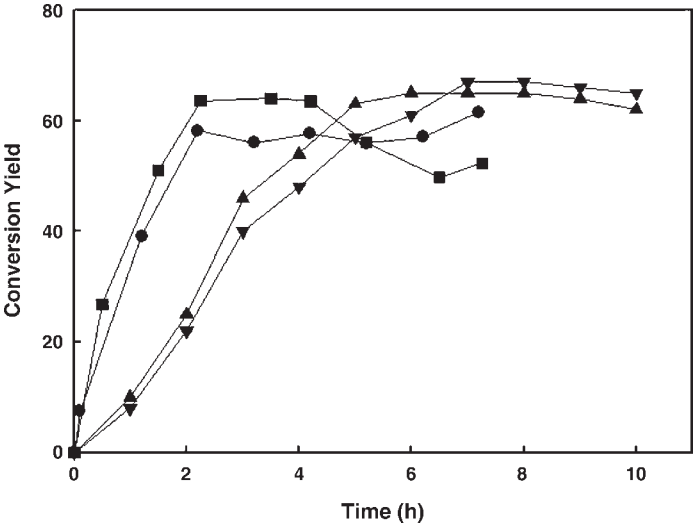

Fig. 3. Synthesis of ampicillin at 0°C, pH 7.0, 60% (v/v) ethylene glycol, 30 m*M* 6-APA, 90 m*M* PGME, and 125 IU/mmol of 6-APA: (■) CLEA-SA; (●) CLEA-TB; (▼) GAP; (▲) PGA-450.

Synthesis of Ampicillin With CLEAs and Carrier-Bound Penicillin Acylase

Syntheses of ampicillin with AS-CLEA and TB-CLEA and also with GAP and PGA-450 were conducted under kinetic control at pH 7.0, 0°C, and 60% (v/v) ethylene glycol medium using PGME as acyl donor and 6-APA as the limiting substrate at a PGME-6-APA molar ratio of 3. The amount of biocatalyst in all experiments was equivalent to 125 IU/mmol of 6-APA; Fig. 3 presents the results. Table 5 reports the conversion yields, volumetric productivities, and specific productivities obtained. Conversion yields obtained with CLEAs were very close to those obtained with carrier-bound penicillin acylases. Conversion yields for ampicillin rarely exceed 65% *(30,33–36);* it is a well-known fact that 6-APA is not a very good

Table 5
Molar Conversion Yield, Productivity, and Specific Productivity for Kinetically Controlled Synthesis of Ampicillin With AS-CLEA and TB-CLEA, GAP and PGA-450 at pH 7.0, 0°C, and 60% (v/v) Ethylene Glycol at FGME-to-6-APA Molar Ratio of 3 and 125 IU/mmol of 6-APA

Biocatalyst	Conversion yield (%)	Productivity (m*M*/h)	Specific productivity (m*M*/[h·g])	Reference
AS-CLEA	65	8.5	45.2	This work
TB-CLEA	62	7.8	25.7	This work
GAP	67	2.9	3.4	This work
GAP	57	3.0	3.0	*37*
PGA-450	65	3.3	5.3	This work
PGA-450	58	2.3	3.9	*30*
Agar-PA	60	4.7	7.8	*36*

nucleophile for penicillin acylase if compared, e.g., with 7ADCA *(32)*. Working with a glyoxyl agarose immobilized penicillin acylase, a maximum conversion yield of 57% at 4°C and pH 6.5 in aqueous medium was recently reported *(37)*. As seen in Table 5, ampicillin volumetric productivities were significantly higher for CLEAs than for carrier-bound penicillin acylases. Differences were even higher (about one order of magnitude) in favor of CLEAs when compared in terms of ampicillin specific productivity, because of the higher specific activity of CLEAs, which is one of the advantages of using nonsupported immobilized enzymes.

Operational stability of AS-CLEA and TB-CLEA was determined by measuring residual activity after synthesis. After 15 h of operation, residual activities of AS-CLEA and TB-CLEA were 72 and 81%, respectively. Assuming first-order kinetics of inactivation, a projected half-life of 32 and 49 h can be estimated for AS-CLEA and TB-CLEA, respectively. These values are 10 and 6 times higher than those obtained under nonreactive conditions. These differences are mainly owing to the different temperatures in both cases; however, protection by substrates and/or products of the reaction cannot be ruled out, as already proven for the hydrolysis of penicillin G with penicillin acylase *(38)*.

Results indicate that penicillin acylase CLEAs are very powerful biocatalysts for the synthesis of β-lactam antibiotics in organic media that compare quite favorably with good carrier-bound penicillin acylases. Specific productivities are much higher at comparable yields and operational stabilities are acceptable. The performance of penicillin acylase CLEAs in the synthesis of β-lactam antibiotics is now being tested at high substrate concentrations. This will permit evaluation of the potential of these novel type of biocatalysts under aqueous solution precipitate *(39,40)* and solid-state *(41,42)* modes of operation in which very promising results have been

obtained already. Further improvement in productivity is still to be expected when working at substrate concentrations close to saturation and beyond, as already proven for the synthesis of cephalexin with carrier-bound penicillin acylase *(43)*. Recovery of CLEAs from the heterogeneous medium after reaction is a challenge for this kind of system.

Conclusion

CLEAs of penicillin acylase from recombinant *E. coli* have been produced using different precipitants and ratios of crosslinking agent to protein mass. Precipitants were used at concentrations at which all the protein was precipitated out of the original enzyme solution. Activity and protein balances were made on each step of the production process, with major losses produced at the precipitation and washing steps. However, global recovery of enzyme activity was moderately high, on the order of 50%.

Ammonium sulfate and PEG 600 CLEAs were the best precipitants in terms of recovery and specific activity and were selected to evaluate the effect of glutaraldehyde-protein ratio in the crosslinking step. Even though specific activity of the biocatalysts increased at decreasing glutaraldehyde-protein ratio, stability decreased, so a ratio of 2 was chosen as the best condition for CLEA production.

Considering the recovery of enzyme activity, the specific activity, and the stability of the different CLEAs produced, those obtained with ammonium sulfate and *tert*-butyl alcohol as precipitants at a glutaraldehyde-protein ratio of 2 were selected for performing the kinetically controlled synthesis of ampicillin. Syntheses with selected CLEAs were conducted at conditions previously optimized for in-house and commercial carrier-bound penicillin acylases to compare their performance on an equal ratio of enzyme activity to limiting substrate. Performance was much better for CLEAs in terms of productivity and specific productivity of ampicillin at comparable conversion yields, even though conditions for synthesis have been not optimized for CLEAs.

CLEAs are very promising novel type of biocatalysts for the synthesis of β-lactam antibiotics in organic media and are being tested at high substrate concentrations to the limit of nucleophile solubility and beyond.

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