DIRECT EVALUATION OF PHENYLACETYL-CoA: 6-AMINOPENICILLANIC ACID ACYLTRANSFERASE OF *PENICILLIUM CHRYSOGENUM* BY BIOASSAY

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The enzyme phenylacetyl-CoA: 6-Aminopenicillanic acid acyltransferase of *Penicillium chrysogenum* was evaluated by direct bioassay against *Micrococcus luteus* ATCC 9341. The enzyme required dithiothreitol, was inactivated by 0.2 mM Hg^{2+} (100%), Zn^{2+} (80%), Cu^{2+} (60%), 1 mM *N*-ethylmaleimide (80%), and showed maximal catalytic activity at pH 8.4 and 20°C. The V_{50} values for phenylacetyl-CoA and 6-aminopenicillanic acid were 0.55 mM and 1 μM, respectively. When octanoyl-CoA was employed as substrate similar results were obtained. In both cases the product generated showed strong antibacterial activity which was quickly lost when incubation was carried out with β-lactamase. Reactions performed in the presence of *Escherichia coli* penicillin acylase did not generated active products when phenylacetyl-CoA was the substrate; they did with octanoyl-CoA. Time-course experiments revealed that the highest enzyme levels are found in 36 hours mycelium and remained almost constant from 48 to 96 hours; thereafter the level of the enzyme slowly decreased.

The biosynthetic pathway of penicillins, cephalosporins and cephamycins[1~3] is partially a common metabolic pathway which starts with non-ribosomal condensation of three amino acids (L-α-aminoadipic acid, L-cysteine and D-valine) (ACV)[4~5]. At a later step this molecule is cyclized to isopenicillin N (IPN) by the isopenicillin N synthetase (IPNS)[6~9]. In the specific pathway of penicillins the α-aminoadipic acid moiety present in the IPN molecule is exchanged with other side-chain precursors (phenylacetic acid, phenoxyacetic acid, octanoic acid) generating different penicillins (benzyl-, phenoxymethyl-, octanoyl-)[10]. These transfer reactions are brought about in two different steps[11]. In the first, activation of the side-chain precursor to its CoA derivatives occurs[12,13], whereas in the second, a different enzyme (transferase) catalyzes either *N*-acylation of the penicillin nucleus (6-aminopenicillanic acid, 6-APA) or direct transacylation between phenylacetyl-CoA and the α-aminoadipyl moiety present in the isopenicillin N molecule (without formation of free 6-APA)[14~20]. Until now all the assays for this enzyme have been carried out by measuring either the incorporation of [^{14}C]-phenylacetyl-CoA or other radioactive side-chain precursors into the penicillin molecule[11,18] or by the appearance of [^{3}H]penicillin after incubating [^{3}H]6-APA with an acyl-CoA derivative[19].

Our contribution reports the best conditions for evaluating this enzyme directly by bioassay (without employing radioactive precursors) and describes its optimal catalytic conditions.

Materials and Methods

Materials

Phenylacetyl-CoA (lithium salt) and octanoyl-CoA (free acid) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Benzylpenicillin (potassium salt), 6-aminopenicillanic acid, octanoylpenicillin, 7-aminocephalosporanic acid (7-ACA), 7-aminodeacetoxycephalosporanic acid (7-ADCA) and cephalosporin C (sodium salt) were from Antibióticos S.A., León, Spain.

Table 1. Partial purification of phenylacetyl-CoA/octanoyl-CoA: 6-APA acyltransferase.

Treatment	Total protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Purification (fold)
Crude extract	1,382	8,562	6.19	1
Protamine sulfate precipitate	756	8,303	10.98	1.77
Ammonium sulfate precipitate	164	6,424	39.17	6.32
Sephadex G-25 eluate	85	4,167	49.02	7.91
DEAE-cellulose eluate				
Fraction 14	4.1	825	201	32.49*
Fraction 15	5.1	1,530	300	48.46*
Fraction 16	2.4	553	232	37.47

* All these fractions were mixed and a single fraction (40-fold purified acyltransferase) were obtained.

All other products used were of analytical grade.

Methods

Microorganisms

The strain of *Penicillium chrysogenum* AS-P-78 used in all experiments was from Antibióticos S.A. This fungus produces about 8,000 U/ml of benzylpenicillin when incubated in a complex production medium[21].

Micrococcus luteus ATCC 9341 was used for the routine determinations of the different penicillins by bioassay[22].

Alcaligenes faecalis ATCC 8750 was employed to evaluate the bioactivity of cephalosporin C derivatives. Strains were kept lyophilized or in liquid N_2 (gas phase).

Media and Culture Conditions

Inoculum of *P. chrysogenum* was developed in a complex medium as previously described[23].

Preparation of Mycelial Extracts

Mycelium of *P. chrysogenum* cultured in complex broth was collected at 30 hours of growth except for the acyltransferase time-course experiments. Mycelium was centrifuged (12,000×*g*, 30 minutes), washed with sterile saline solution and resuspended in the buffer solution of GATENBECK[18] slightly modified (0.05 M Tris, 0.1 M NaCl and 0.02 M KH_2PO_4), pH 8.4. Cells (1.5 g of wet mycelium/10 ml of buffer) were disrupted with glass beads using a Braun MSK mechanical disintegrator[9]. Ultracentrifuged (100,000×*g*, 1 hour, 4°C) crude extracts (130 ml with 10.6 mg of protein/ml) were mixed in an ice bath with 15 ml of a concentrated solution of protamine sulfate (20 g/liter) in the above described buffer (TCP buffer). The extract was kept on ice for 20 minutes and then centrifuged at 25,000×*g* for 30 minutes at 2°C. The pellet was discarded and the supernatant fluid was precipitated with ammonium sulfate. The fraction which precipitated between 30~80% was collected, dissolved in 8 ml of 0.05 M Tris, 0.02 M KH_2PO_4, pH 8.4 (TP buffer) and centrifuged again at 15,000×*g* for 30 minutes at 2°C in order to remove the residual pellet. Excess of ammonium sulfate was eliminated by passing the enzyme solution (4×2 ml) through a Sephadex G-25 PD-10 column (Pharmacia) equilibrated with TD buffer. Aliquots of one ml were collected and assayed for acyltransferase activity. Fractions 3~5, containing maximal activity, were mixed and the total volume (8 ml) was applied to a DEAE-cellulose column (2.5×15 cm) (Pharmacia). Elution was carried out with NaCl gradient (0~0.8 M) as described by KOGEKAR and DESHPANDE[11] and fractions of 5 ml were collected (flow rate 25 ml/hour). The phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase activity eluted between fractions 14~16. The contents of the tubes with maximal enzymatic activity were pooled and lyophilized. By this treatment the enzyme was purified about 40-fold (Table 1).

Enzyme Assay

In a total volume of 100 μl the reaction mixture contained the following: TCP buffer 25 μl, 10 mM phenylacetyl-CoA (octanoyl-CoA) 10 μl, 0.3 mM 6-APA 10 μl, 15 mM dithiothreitol (DTT) 5 μl, en-

zyme 50 μl (0.05 mg of protein). Incubations were carried out at 20°C for 30 minutes (10 minutes for kinetic studies) and stopped by addition of 100 μl of methanol.

One unit of enzymatic activity is defined as the amount of enzyme required to synthesize 1 μg of benzylpenicillin or octanoylpenicillin at 20°C over 30 minutes. Specific activity is expressed as units/mg protein. Protein was measured by the method of LOWRY *et al.*[24].

Characterization of Reaction Products

In order to isolate a sufficient quantity of the reaction products for their identification, a large reaction (240 ml) was carried out. After incubating each substrate (phenylacetyl-CoA or octanoyl-CoA) in the above described conditions for 2 hours, the reaction mixture was extracted with isobutyl-acetate and the antibiotic-rich fraction was transferred to 50 mM phosphate buffer pH 7.0[22]. By this procedure about 300 μg of benzylpenicillin and octanoylpenicillin were obtained. Purification of the penicillins was carried out by HPLC, using a Perkin-Elmer Chromatograph Series 3B with a LC-75 Spectrophotometric Detector and a Sigma 10B Chromatography Data Station. A μBondapack C-18 column of 30 cm (Waters Associates Inc.) was used. The mobile phase for benzylpenicillin was composed of 83% of 0.1 M sodium acetate/acetic acid, pH 4.5 and 17% of acetonitrile (benzylpenicillin retention time 12.3 minutes). The mobile phase used for octanoylpenicillin was 0.025 M KH_2PO_4 - methanol (50 : 50). Retention time for octanoylpenicillin was 9.6 minutes.

Results

6-APA: Acyltransferase

The formation of benzylpenicillin *via* direct acylation of 6-APA by the phenylacetic acid moiety from its CoA derivative, was shown by the accumulation of an antibiotic substance with the biological characteristics of benzylpenicillin (Fig. 1). When octanoyl-CoA was employed as substrate, an antibiotic molecule (octanoylpenicillin) was also synthesized. Both reaction products were completely inactivated by β-lactamase from *Bacillus cereus* (Difco), but when incubated with *Escherichia coli* penicillin acylase, bioactivity was only lost in the product generated when phenylacetyl-CoA was

Fig. 1. Bioassay of the benzyl (or octanoyl) penicillin formed by acylation of 6-APA.

(a) 1: Reaction control (1 mM phenylacetyl-CoA). 2: Incubated with β-lactamase from *B. cereus*. 3: Incubated with *E. coli* penicillin acylase (4 I.U./ml). 4: Control without phenylacetyl-CoA. 5: Control without 6-APA.

(b) 1: Reaction control (1 mM octanoyl-CoA). 2: Incubated with *E. coli* penicillin acylase (4 I.U./ml). 3: Cyclization of ACV (0.2 mM). 4: Idem 3 but incubated with 10 mM phenylacetyl-CoA. 5: Reaction control incubated with β-lactamase.

(c) 1: Reaction control (30 μM 6-APA, 1 mM phenylacetyl-CoA). 2: Idem 1 with 0.25 mM phenylacetyl-CoA. 3: Idem 1 with 3 μM 6-APA. 4: Idem 1 with 6 μM 6-APA. 5: Idem 1 with 0.01 μM 6-APA.

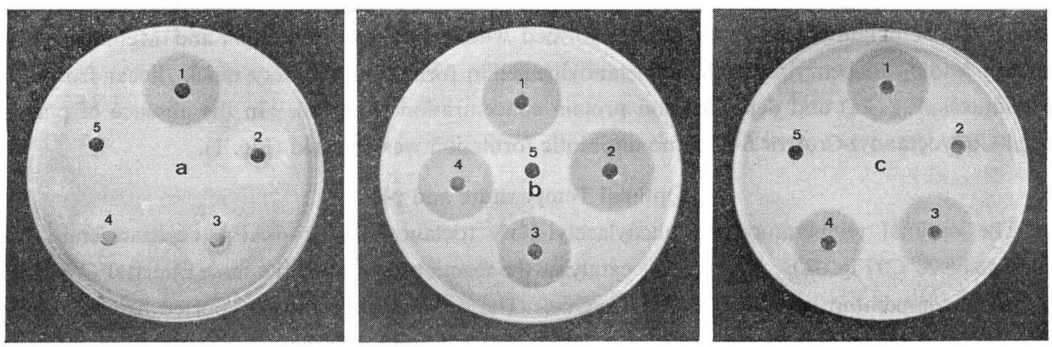


Fig. 2. Effect of time of incubation (a) and increasing concentration of proteins (b) on the formation of benzylpenicillin (●) or octanoylpenicillin (○).

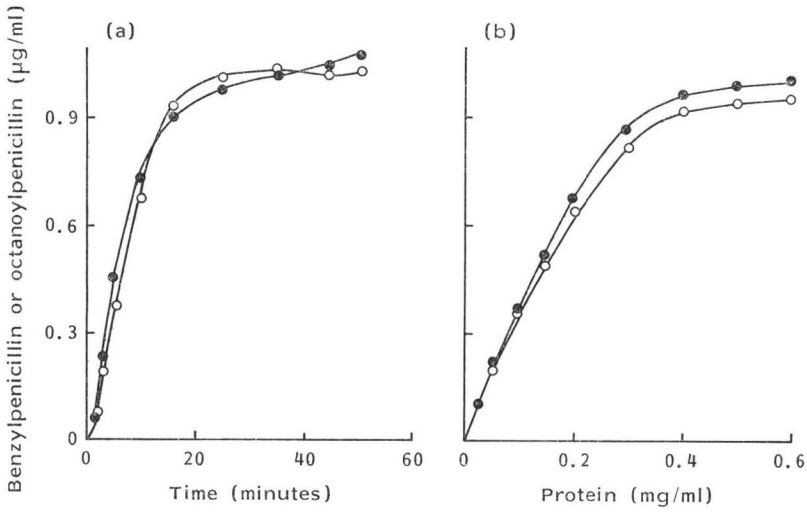
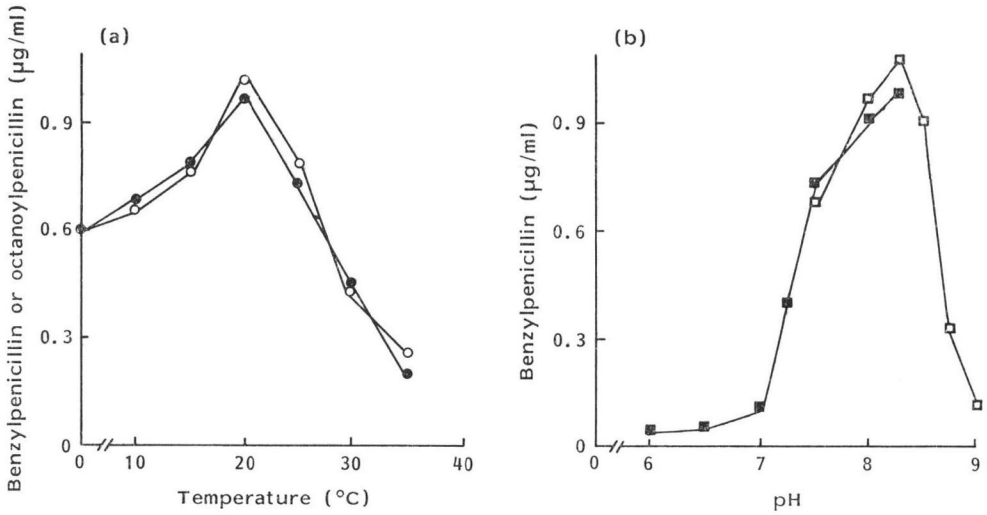


Fig. 3. Effect of temperature (a) (●, benzylpenicillin; ○, octanoylpenicillin) and pH (b) (■, 0.05 M phosphate buffer; □, TP buffer) on the phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase activities.



used as substrate (Figs. 1a and 1b). Identification of the antibiotic formed with both substrates (phenylacetyl-CoA or octanoyl-CoA) was also performed with HPLC (see Materials and Methods). The reaction leading to benzylpenicillin or octanoylpenicillin formation was very quick (linear from 0 to 15 minutes) (Fig. 2a) and dependent on protein concentration (Fig. 2b). In the absence of phenylacetyl-CoA/octanoyl-CoA or 6-APA no antibiotic formation was detected (Fig. 1).

Optimal Temperature and pH

The optimal temperature for phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase was found to be 20°C (Fig. 3a). The enzyme catalyzes the reactions at almost the same rate at 0°C perhaps due to the temperature sensitivity of the enzyme. The optimal temperature reported here is notably different from others previously described: 28°C[19], 32°C[16] and 37°C[15]. The optimal catalytic ac-

Fig. 4. Effect of DTT on the phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase activities.
●, Benzylpenicillin; ○, octanoylpenicillin.

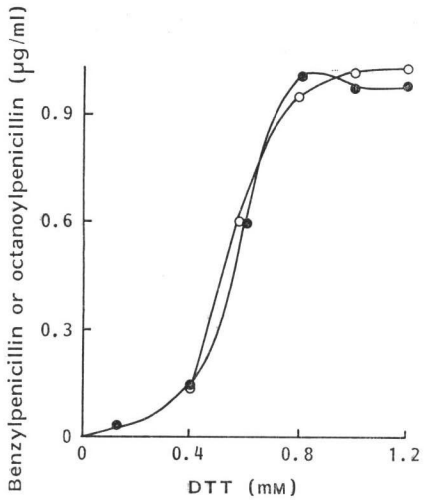


Table 2. Effect of different cations on phenylacetyl-CoA/(octanoyl-CoA): 6-APA acyltransferase activities.

Addition	Inhibition (%)	
	Phenylacetyl-CoA	Octanoyl-CoA
Hg^{2+} (0.2 mM)	100	100
Zn^{2+} (0.2 mM)	80	81
Cu^{2+} (0.2 mM)	60	58
N-Ethylmaleimide (1 mM)	85	80

Other cations (K^+, Na^+, Li^+, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} and Fe^{2+}) did not modify the enzyme activity.

tivity was found at pH 8.4 (Fig. 3b). This value is similar to that reported by GATENBECK and BRUNSBERG[16] (pH 8.5), but different from the values described by others: pH 7.8[11], 7.4[15] and 7.0[19].

In order to avoid the possible effect of ionic strength variation, the molarity of the buffer used in the reaction mixture was modified (from 0.02 M to 0.2 M). No variations in the catalytic activity were detected either using phenylacetyl-CoA or octanoyl-CoA (data not shown).

Effect of DTT and Ions

The presence of DTT or other thiol reagents (mercaptoethanol or reduced glutathione) is absolutely necessary for the activity of this enzyme. Fig. 4 shows the catalytic activity of the phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase enzyme in the presence of different DTT concentrations. These results are in good agreement with those of KOGEKAR and DESHPANDE[11] and suggest the presence of the SH groups on the enzyme active site. The inhibition of the activity by *N*-ethylmaleimide and some divalent cations (Cu^{2+}, Zn^{2+} and Hg^{2+}) (Table 2) agrees with this hypothesis. Other ions tested (K^+, Na^+, Li^+, Ca^{2+}, Mg^{2+}, Mn^{2+}) did not modify enzyme activity.

Substrate Kinetics

The effect of substrate concentration was studied for both substrates (6-APA and phenylacetyl-/octanoyl-CoA) in the presence of saturating concentrations of the other one. Under these conditions the calculated $S_{V0.5}$ (substrate concentration at which $V=1/2\ V_{max}$) for 6-APA was 1 μM, while for phenylacetyl-CoA and octanoyl-CoA they were 0.55 and 0.60 mM, respectively (Fig. 5). These results indicate either that the affinity of the enzyme is much higher for 6-APA than for the side chain precursors employed, or that the enzyme preparations contain enzyme(s) which hydrolyze the acyl-CoA derivatives. Incubations of phenylacetyl-CoA/octanoyl-CoA and enzyme at different times, followed by the addition of 6-APA showed that the total benzylpenicillin/octanoylpenicillin generated was equivalent in all cases (100%). This result clearly indicates that the acyl derivative was not hydrolyzed during the reaction process.

Effect of Other Molecules

When the nucleus of cephalosporins, 7-aminocephalosporanic acid (7-ACA) or the close structural molecule 7-aminodeacetoxycephalosporanic acid (7-ADCA), were used as substrates replacing 6-APA,

Fig. 5.

(a) Kinetic of phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase activity *versus* substrate (○, octanoylpenicillin, $S_{V0.5}$=0.60 mM) phenylacetyl-CoA (PA-CoA); (●, benzylpenicillin, $S_{V0.5}$= 0.55 mM) octanoyl-CoA (Oc-CoA).

(b) Idem *versus* 6-APA (●, benzylpenicillin, $S_{V0.5}$=1 μM).

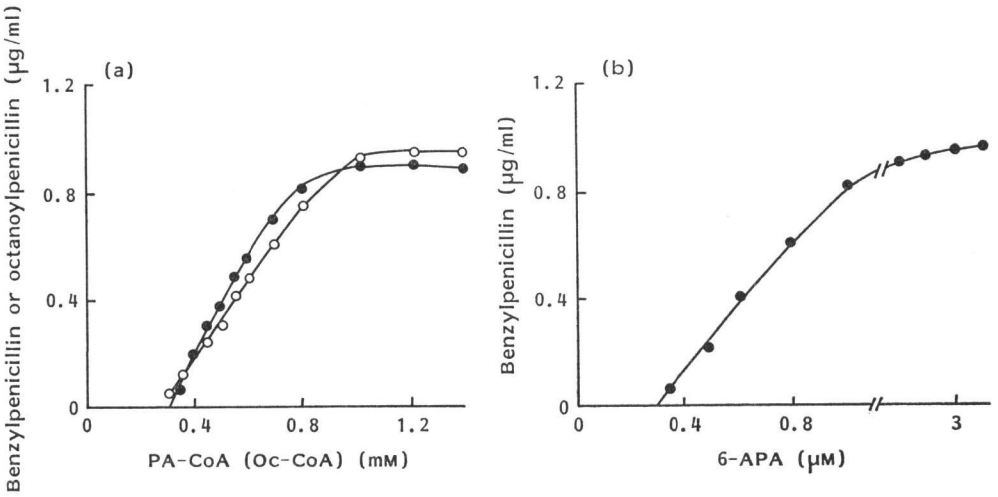
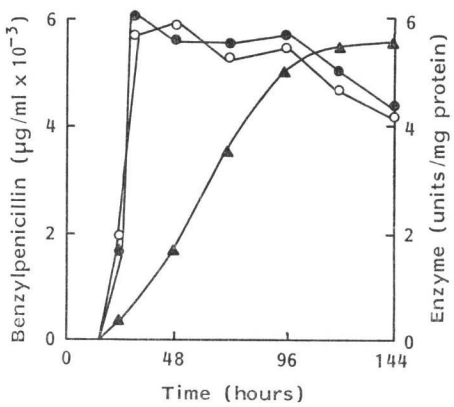


Table 3. Effect of different molecules on isopenicillin N synthetase (IPNS) activity.

Addition	IPNS activity (%)	
	Crude enzyme	40-fold purified enzyme acyltransferase
Reaction control[a]	100	100
+6-APA (30 μM)	98	97
+phenylacetyl-CoA (1 mM)	210	101
+octanoyl-CoA (1 mM)	230	95

[a] The IPNS was assayed in the acyltransferase assay system containing 1 mM Na-ascorbate, 0.1 mM $FeSO_4$, 0.2 mM ACV, acyltransferase (0.05 mg protein from the crude or from the 40-fold purified fraction), 0.05 mg of 20-fold purified IPNS[6] (without acyltransferase activity) and, if required, 6-APA or phenylacetyl-CoA/octanoyl-CoA.

Fig. 6. Time-course of phenylacetyl-CoA: 6-APA acyltransferase (●), octanoyl-CoA: 6-APA acyltransferase (○) activities and benzylpenicillin formation (▲).



they are not accepted by the acyltransferase enzyme. Furthermore, if 6-APA and 7-ACA/7-ADCA were pooled together, the efficiency of the reaction did not decrease (102 and 98% when 6-APA/7-ACA and 6-APA/7-ADCA were incubated in presence of phenylacetyl-CoA; and 106 and 101% when 6-APA/7-ACA and 6-APA/7-ADCA were incubated in the presence of octanoyl-CoA). Such results imply that these molecules do not reset with the enzyme and consequently they can not be acylated. Moreover, when ACV was added to the reaction mixture, without 6-APA, but in presence of 1 mM ascorbate and 0.1 mM Fe^{2+} (which do not affect the acyltransferase enzyme) the cyclization of ACV to isopenicillin N takes place; however the biological activity of the generated product did not in-

crease even when high amounts (10 mM) of the side chain precursors were present (Fig. 1b). Also the addition of 6-APA to the reaction (without phenylacetyl-CoA) did not modify the cyclization reaction (Table 3).

Time Course of 6-APA: Phenylacetyl-CoA Transferase

Phenylacetyl-CoA (octanoyl-CoA): 6-APA acyltransferase activity during the course of penicillin fermentation was determined in extracts of *P. chrysogenum* AS-P-78. The enzyme which was not detected during the first 20 hours of fermentation, was formed rapidly after a decrease in growth rate, reaching maximal levels at 36 hours and remained constant until 96 hours. From this time to the end of fermentation (144 hours), the level of 6-APA: Phenylacetyl-CoA transferase slowly decreased. When octanoyl-CoA was assayed as substrate, the results were identical (Fig. 6). The time course formation of this enzyme follows a similar pattern as other enzymes involved in benzylpenicillin biosynthesis. For example homocitrate synthetase[23], the first enzyme of the biosynthetic pathway and isopenicillin N synthetase[9] showed maximal activity at 48 and 24 hours of fermentation respectively.

Discussion

Phenylacetyl-CoA: 6-APA acyltransferase of *P. chrysogenum* AS-P-78 is an enzyme that converts 6-APA into benzylpenicillin in the presence of phenylacetyl-CoA. This enzyme shows an optimal catalytic activity at pH 8.4 and 20°C. The best assay conditions are in contrast with the optimal pH of penicillin fermentations (pH 6.0~6.5). However, isopenicillin N synthetase, a very important enzyme for the biosynthesis of penicillins and cephalosporins, also shows an optimal pH of about 8.0[8,9,25]. The optimal temperature (20°C), although differing from other data previously described[11,15,18], is somewhat different from the temperature range used for penicillin production (23~27°C). The enzyme requires DTT for its catalytic activity and this fact seems to indicate that it has SH group(s) in its active site. The presence of DTT in the reaction mixture, probably protects the enzyme against inactivation by oxygen. The strong inhibition caused by Hg^{2+}, Zn^{2+} and *N*-ethylmaleimide provides good support for such hypothesis. The similar optimal pH, temperature, DTT dependence, ionic inhibition and kinetic parameters observed when 6-APA was acylated by other side-chain precursor (octanoyl-CoA), suggest that this reaction is carried out by the same enzyme molecule. The affinity of the enzyme for the two different side-chains (0.55 and 0.6 mM, Fig. 5a) contrasts with the high affinity observed for 6-APA (Figs. 1c and 5b). This suggests that the lower specifity for the side-chain, could explain why *P. chrysogenum* is able to synthesize different penicillins (octanoyl-, DF, F, benzyl-, phenoxymethyl) from a common nucleus (6-APA). The fact that 7-ACA and 7-ADCA can not be joined to the enzyme clearly indicates that this system is specific for 6-APA and consequently for hydrophobic penicillin synthesis but not for cephalosporin-producing strains. The absence of this enzyme activity in *Acremonium chrysogenum* cell-free extracts (data not shown) agrees with the above concept.

The fact that when phenylacetyl-CoA/octanoyl-CoA was added to the cyclization reaction (Table 3) the bioactivity of the product did not increase (Fig. 1b) implies that, under our conditions, the isopenicillin N generated after ACV cyclization, is not used as a substrate of 6-APA phenylacetyl-CoA transferase. This effect could be due to the lack of special catalytic requirements or to the disorganization of the "*in vivo*" enzymatic functional system. It is also possible that the formation of 6-APA requires the participation of a different enzyme, isopenicillin amidolyase[26]. The absence of this enzyme from the partially purified extract could quite justifiably account for the fact that isopenicillin N is not converted into benzylpenicillin. The increase in bioactivity observed when phenylacetyl-CoA/octanoyl-CoA was present in the reaction mixture, when a crude extract was used as the enzyme source (Table 3), could be due to some of the above reasons.

Further experiments are needed to clarify whether these proteins (cyclase, acyltransferase and probably others) are coupled in a single enzymatic system which leads to benzylpenicillin formation or whether they are different enzymes with different cellular locations[27]. Better knowledge of the en-

zymes involved in the penicillin biosynthetic pathway is necessary to advance the field of genetic manipulation of penicillin-producing microorganisms[28].

Acknowledgments

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