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REVIEW

Penicillin Acylases

An Update

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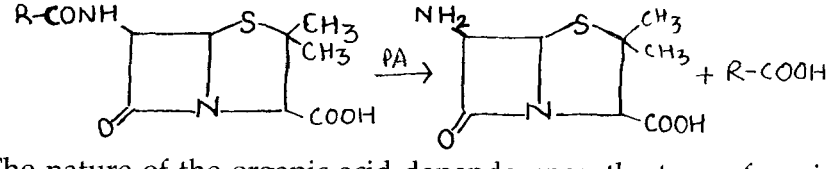
Index Entries: Penicillin acylase, purification schemes for; physicochemical properties, of penicillin acylase; active site studies, of penicillin acylase; regulation of biosynthesis, of penicillin acylase; genetics of biosynthesis, of penicillin acylase.

INTRODUCTION

Penicillin acylase [penicillin amidohydrolase; EC 3.5.1.11] catalyses the hydrolysis of penicillins into 6-aminopenicillanic acid and an organic acid (*1*) according to the equation:



The nature of the organic acid depends upon the type of penicillin hydrolyzed. Phenylacetic acid is produced when benzylpenicillin* is hydrolyzed, whereas POAA is the product of hydrolysis of Pen V. On the basis of their substrate specificity, acylases have been classified into three types. Most of these enzymes also catalyse the synthesis of penicillin from an organic acid and 6-APA (*2*). The latter compound forms the nu-

*Abbreviations used: 6-APA, 6-aminopenicillanic acid; Pen G, benzylpenicillin; Pen V, phenoxymethylpenicillin; PA, penicillin acylase/s; PAA, phenylacetic acid; POAA, phenoxyacetic acid.

cleus of a large number of semisynthetic penicillins that have additional therapeutic advantages (*3*). The possibility of using PA for the large scale production of 6-APA, which in turn would be utilized for the manufacture of semisynthetic penicillins by the antibiotic industry, was first recognized as early as 1950 (*4,5*). Since then, exhaustive screening of the microbial kingdom has been carried out in search of a strain that would catalyze the reaction more efficiently (*6–9*). Attention has been focused more on the commercial exploitation of the enzyme and relevant aspects such as screening procedures, distribution of the enzyme in the microbial kingdom, and use of the enzyme in various forms for the production of 6-APA, which have been discussed in greater detail elsewhere (*2,10–14*). For some unknown reason, information on the basic enzyme chemistry of PA has been very scanty. This is evident from the fact that of the several hundred papers published on this subject in the past three decades, only a few report purification and physicochemical characterization of the enzyme. However, needless to say, such basic information would be of immense importance for a better understanding of the physiological role of the enzyme and might be useful as well in developing its applications. In this review the discussion is restricted to the purification, assay methods, and physicochemical as well as the kinetic properties of PA. A short summary of the regulation and genetics of biosynthesis of the enzyme is also included.

PURIFICATION SCHEMES

Penicillin acylases have been isolated from a wide variety of microorganisms ranging from different groups of bacteria to basidiomycetes. Consequently, various protocols have been applied (Table 1). Except for the *Bacillus megaterium* enzyme (*15*), other acylases are intracellular and extraction and purification has been achieved using conventional methods (*15–24*). These procedures include several chromatographic steps, thereby affecting the recovery considerably. Only recently, the purification procedures involving specific interactions between the enzyme and substrate/analog have been attempted (*25–27*). Although the yields obtained using these procedures are higher, the degree of purity is very low, indicating a lack of specificity in the affinity columns used and clearly suggesting that other factors might be involved in the interaction of the enzyme with the ligand. Recently, involvement of hydrophobic interactions in the retention of the *Escherichia coli* (NCIM 2400) acylase on so-called affinity columns has been reported by Mahajan and Borkar (*28*).

ASSAYS

Methods for detection of the hydrolytic activity of PA basically involve estimation of the unhydrolyzed substrate penicillins (*29*) or deter-

mination of the reaction products, i.e., 6-APA or the side chain acid (*29–46*). Alkalimetry has been used as a method of choice for estimation of the side-chain carboxylic acid (*19,30–33*), although occasionally gas chromatographic (*34*) or colorimetric (*35,36*) methods have been used. Estimation of 6-APA has been the most common means of detecting PA activity, and this has been achieved using several methods such as hydroxylamine assay (*37*), bioassay (*37,38*), spectrophotometry (*39,40*), indicator method (*41*), and colorimetry (*42–46*). Synthetic substrates and amino acid derivatives have also been used in order to develop a simple colorimetric procedure (*17,19,46–48*). Szewczuk used phenylacetyl-4-aminobenzoic acid as a substrate (*48*). After hydrolysis of the substrate at pH 7.8, the resulting aminobenzoic acid was quantitated by diazotization reaction. This method may be used as a spot test for a quick screening of PA-producing organisms as well as for the immobilised enzyme reactions. Another colorimetric method based on estimation of 6-APA using the ninhydrin reaction has been reported by Baker (*49*). Since the reaction of 6-APA with ninhydrin reagent is highly pH dependent and cannot be used for estimating PA activity when ampicillin is used as a substrate, Baker has developed another method (*50*) in which the unhydrolyzed ampicillin is reacted with the biuret reagent.

Veronese et al. (*23*) have employed a fluoroscopic method for estimation of nanomolar quantities of 6-APA using fluorescamine. Recently, the reaction of fluorescamine with 6-APA has been well characterized by Baker (*51*). Although this method is very rapid and sensitive, the interference by penicilloic acid has not been studied. Use of high pressure liquid chromatography has been shown to alleviate this difficulty (*52–54*). In the method reported by Dhawale et al., Pen G is hydrolyzed using immobilized PA and the reaction mixture is subjected to HPLC on a strong anion exchange resin column, SAX (*53*). The extraordinarily high resolving ability of this technique makes it possible to estimate any one of the components in the reaction mixture without interferences. The drawbacks of this method are the requirement of sophisticated instrumentation and its limited application to the immobilized enzyme systems.

It is clear from the foregoing discussion that the choice of the assay method for detecting PA activity would mainly depend on the type of study undertaken. The colorimetric assays should prove advantageous for a rapid screening of activity. Quantitative estimations of the reaction products in the nanomolar range needed for the kinetic characterization of the enzyme would, however, demand a sophisticated and sensitive assay.

GENERAL PROPERTIES

The general properties of PA purified from different sources have been summarized in Table 2. Molecular weights of most of the acylases

TABLE 1
Purification Schemes for Penicillin Acylases

Organism[a]	Localization	Purification steps	Fold purification	Percent recovery
F. semitectum BC 805 (*20*)	Intracellular	Extraction with 0.2*M* NaCl for 16 h Alkali precipitation; 3x DEAE-cellulose treatment; G-25 column; acetone precipitation; Amberlite IRC-50 column	300	5
B. megaterium (*15*)	Extracellular	Celite adsorption; salt precipitation; CM-cellulose column; Celite adsorption and salt precipitation	96	33
F. semitectum (*21*)	Intracellular	Extraction with 0.2*M* NaCl; alkali and acetone precipitation; ultracentrifugation; G-100 and hydroxylapatite column chromatography	21	42
E. coli ATCC 11105 (*17*)	Intracellular	Homogenization; acid and salt precipitation; SE-Sephadex C-50 and DEAE-Sephadex A-50 columns; salt precipitation and crystallization	100	25
E. aeroideae (*22*)	Intracellular	Homogenization; lysozyme treatment; Streptomycin sulfate and salt precipitation; Cellulose-123, G-25, and G-100 columns	98	14

K. citrophila (*16*)	Intracellular	Sonication; streptomycine sulfate, and salt precipitation; DEAE-cellulose and hydroxylapatite columns, and isoelectric focusing	123	61
B. plumbea (*19*)	Intracellular	Homogenization; G-25, DEAE-cellulose, Biogel P-200 and hydroxylapatite columns	220	40
P. rettgeri (*18*)	Intracellular	Homogenization, DEAE-Cellulose, SP-Sephadex, DEAE-Sephadex and G-150 columns	580	9
E. coli SV (*23*)	Intracellular	Extraction by freeze-thawing, DEAE- and CM-cellulose columns; salt precipitation and hydroxylapatite chromatography	80	25
B. megaterium (*27*)	Extracellular	Celite adsoprtion, salt precipitation and PenV–Sepharose column	446	63
E. coli (*25*)	Extracellular	Extraction and chromatography on *p*-aminophenylacetyl-alinyl-methacrylate gel	3	—[b]
E. coli (*26*)	Intracellular	Extraction, and chromatography on PAA—methacrylate gels with or without carbon spacer arms	15	90
E. coli NCIM 2400 (*28*)	Intracellular	Extraction, hydrophobic-interaction chromatography and DEAE-cellulose treatment	48	75

[a]Numbers in parentheses indicate references.
[b]—, value not published.

TABLE 2
Physicochemical Properties of Pencillin Acylases

Organism[a]	pI	Molecular weight	Optimum pH	Optimum Temp., °C	Best substrate[b]	Inhibitors[c]	Remarks
F. semitectum (*20, 21*)	—	65,000	7.8–8.0	50	Pen V (4.75 m*M*)	—	Almost homogeneous; has two Zn^{2+} atoms; can be separated from aminoacylase activity
B. megaterium (*15*)	—	120,000	8.0–9.0	45	Pen G (4.5 m*M*)	PAA (45 m*M*) 6-APA (26 m*M*)	Homogeneous upon ultracentrifugation; metal ions do not stimulate activity; low inhibition by Co^{2+}, Ni^{2+}, and Mn^{2+}
E. coli ATCC 11105 (*17*)	6.3–6.5 and 6.5–6.8	70,000	8.1	—	Pen G (20 m*M*)	PAA (200 m*M*) 6-APA (15 m*M*)	At least two charge forms Partial dissociation in 1% SDS into a smaller polypeptide of 20,500
E. aroideae (22)	—	62,000	5.5	—	Pen V	—	Two active forms upon nondenaturing electrophoresis; glycyltripeptides hydrolyzed but *N*-acetyl amino acids not cleaved. No synthetic activity reported
K. citrophilia (*16*)	8.2	63,000	7.3	55	Cephaloridine Cephalexin (1.4 m*M*)	—	Single band on nondenaturing electrophoresis; preferentially cleaves cephalosporins; synthetic activity max. at

B. plumbea (*19*)	—	88,000	7.5	52	Pen V (1.4 m*M*)	—	No inhibition by metal chelators or hydrolysis products; di- or tripeptides and derivatives of amino acids not cleaved
P. rettgeri (*18*)	—	65,000	—	—	Pen G (2 m*M*)	PAA (4 m*M*) 6-APA (1 m*M*)	Nonhomogeneous on electrophoresis; inhibition by Zn^{2+}, Co^{2+}; ampicillin synthesis max. at pH 6.6
E. coli (23)	—	—	7.8	37	Pen G (8.5 μ*M*)	PAA (50 m*M*)	Enzyme stable to guanidine hydrochloride, ethanol, and acetone
B. megaterium (*27*)	—	—	7.0	40	Pen G (4.5 m*M*)	PAA (0.45 m*M*) 6-APA (0.26 m*M*)	Purified enzyme nonhomogeneous on electrophoresis; Pen V acts as a competitive inhibitor (K_i 9.4 m*M*)
E. coli NCIM 2400 (*28*)	6.3–6.5	67,000	7.5	40	Pen G	PAA 6-APA	No subunits detected in SDS-gel electrophoresis or ultracentrifugation of purified enzyme

[a]Reference numbers in parentheses.
[b]K_m values in parentheses.
[c]K_i values in parentheses.

range between 62,000 and 88,000 and these values have been obtained using gel permiation or thin layer chromatography or SDS-gel electrophoresis. The enzymes from *Bacillus megaterium* and *B. sphaericus* appear to be larger in size with apparent molecular weights of 120,000 (*15*) and 140,000 (*55*). However, it is not clear whether this difference in the molecular weights of these enzyme molecules has any correlation with their extracellular nature.

Under nondenaturing electrophoretic conditions, the enzymes of *E. coli* ATCC 11105 (*17*) and *Erwinia aroideae* (*22*) exhibit multiple active bands as detected by activity staining. Kutzbach and Rauenbusch (*17*) have separated two main types of active forms of the *E. coli* ATCC 11105 enzyme using preparative isoelectric focusing, with p*I* values between 6.3 and 6.4 and 6.7 and 6.8. The enzyme from *E. coli* NCIM 2400 also reveals two forms upon isoelectric focusing having p*I* values between 6.3 and 6.5 (*28*), but the *Kluyvera citrophila* enzyme behaves as a single species with p*I* 8.12 (*16*). The *E. coli* ATCC 11105 enzyme has been found to be partially dissociated into two forms with molecular weights of 70,000 and 20,500 by SDS treatment (*17*). The smaller protein is proposed to be a subunit of the enzyme. However, the enzymatic activity of the individual or reconstituted "subunits" has not been reported. The enzyme from other *E. coli* strains does not exhibit any heterogeneity in SDS-gel electrophoresis or high velocity sedimentation analysis (*23,28*). Thus the information regarding the subunit nature of PA is contradictory, and it is difficult to ascertain whether or not the enzyme exhibits charge and/or size heterogeneity. Further studies, such as the N-terminal analysis and the kinetics of the individual "subunits" and the reconstituted enzyme might throw some light on this problem.

The enzyme from *Fusarium semitectum* has two atoms of Zn^{2+} per molecule (*20,21*) that seem to be essential for the catalytic function. Interestingly, the purified enzyme from the basidiomycete *Bovista plumbea* is not inhibited by EDTA or 8-hydroxyquinoline (*19*), indicating that the metal ion is not an absolute requirement for the fungal acylase activity. The bacterial enzymes are not known to contain any metal ions.

Most acylases are rapidly inactivated at temperatures above 60°C and pH above 8.5. The hydrolytic reaction proceeds optimally between 37 and 55°C. The optimum pH of the hydrolytic reaction lies between pH 7.0 and 8.0 for most acylases [except in *Erwinia aroideae*, where the optimum pH is 5.6 (*22*)]. Stability studies indicate that lyophilization of the *E. coli* acylase causes denaturation (*17,28*), and organic solvents such as acetone rapidly inactivate the enzyme (*17*).

KINETIC PROPERTIES

Studies on the kinetic properties of PA have been carried out under widely differing conditions and hence it is very difficult to compare and

correlate the results obtained. Substrate specificity of PA from different sources has been investigated extensively. In fact, this has been accepted as an important criterion for classifying different types of the enzyme. Thus, the enzyme cleaving Pen V, either from fungi (*19–21*) or from bacteria (*22,55,57,58*) is classified as type I. The type II enzymes are characterized by their high specificity for Pen G as a substrate and are found in bacteria (*14*). A third group of enzymes (e.g., those from *Pseudomonos melanogenum*) is said to be specific for hydrolyzing ampicillin (*59,60*), and the *Kluyvera citrophila* enzyme is more active on cephaloridine (*16*). Apart from Pen G, cephalosporins (such as cephalotin and cephalosporidine) are also hydrolyzed by the *Proteus rettgeri* enzyme (*18*). The substrate specificity of the *E. coli* enzyme has been thoroughly investigated mainly using amino acid esters as substrates (*11,33,61,62*). Introduction of a hydroxyl group in the para position of the benzyl ring of phenylacetamide results in a decrease in the rate of the hydrolysis (*61*). Furthermore, this enzyme appears to exhibit stereospecificity since the L-isomers of phenylacetyl amino acids are cleaved at a much faster rate than the D-isomers (*33*). The nature of the side chain of the amino acids in the phenylacetyl compounds and the thiazolyl ring of penicillins also influence the rate of hydrolysis of these compounds (*33*). Margolin et al. (*62*) have found that the structure of the leaving group in the Pen G molecule also affects the action of the enzyme. The extracellular PA of *B. megaterium* utilizes Pen G as the best substrate although other penicillins and amides of PAA are also hydrolyzed at a much slower rate (*15*). The intracellular enzyme produced by *B. sphaericus* hydrolyzes Pen V (*55*). The type I acylase of *F. semitectum* was initially thought to be active on Pen V as well as some amino acid derivatives (*20*). However, further attempts on the purification of the enzyme by Baumann et al. (*21*) have allowed these workers to separate the aminoacyl hydrolyzing activity from the PA activity.

The purified enzyme from *B. plumbea* is also highly specific for Pen V and its *p*-hydroxy derivatives (*19*). Therefore, the type I acylases appear to exhibit a higher degree of specificity than the type II acylases. It must be noted at this point that the type I enzyme of *Erwinia aroideae* exhibits its activity in a pH-dependent manner. At pH 5.6, only Pen V is hydrolyzed, whereas at pH 8.0, only cloxacillin and methycillin are hydrolyzed, but not Pen V (*22*). This enzyme does not exhibit any synthetic activity towards Pen G or Pen V over a wide range of pH 4.0–8.0. Studies on the *E. coli* enzyme have shown that the substrate specificity of the synthetic reaction is similar to that of the hydrolytic reaction and is optimal between pH 5.0 and 7.0 (*11*). Similar values have been reported for *K. citrophila* (*16*) and *P. rettgeri* (*18*) acylases.

In general, the type II acylases are inhibited by the products of hydrolysis of Pen G. Phenylacetic acid acts as a competitive inhibitor and 6-APA inhibits the type II enzyme noncompetitively. The enzyme from *E. coli* is also inhibited by higher substrate concentrations. Balasingham

et al. (*43*) have shown that while the K_s and K_i values of the *E. coli* NCIB 8743 enzyme for Pen G and PAA, respectively, are not affected by pH, the K_i for 6-APA varies markedly with pH. The *B. megaterium* enzyme is not inhibited by the substrate (*15,63*), whereas the type I acylase of *B. plumbea* is inhibited neither by the substrate Pen V nor by the products (*19*). The intracellular Pen V acylase of *B. sphaericus* is inhibited by both the products of hydrolysis; however, in this case, 6-APA acts as a competitive inhibitor and POAA as a noncompetitive inhibitor (*55*).

Based on the preliminary kinetic data, different models have been proposed to explain the mechanism of action of PA (*63–65*).

The thermodynamic parameters of the reactions of the *E. coli* acylase have been evaluated by Svedas et al. (*66,67*). They have observed that the values of the standard Gibbs energy change for the hydrolysis of several β-lactam antibiotics are not affected by the change in the structure of the nucleus of the molecule, but are more sensitive to changes in the structure of the acyl moiety. These workers have also suggested the use of esters of PAA in the synthesis of β-lactam antibiotics in order to provide more favorable thermodynamic conditions.

Recently, Konecny (*68–70*) has proposed the hypothesis that the enzymatic hydrolysis of Pen G proceeds via an acyl enzyme intermediate. Thus, first the acyl donor (e.g., PAA or derivative) reacts with the enzyme to form an intermediate that in turn transfers the acyl radical rapidly to an amine (e.g., 6-APA) water or an alcohol. The ester hydrolysis catalyzed by the *B. megaterium* enzyme is inhibited by anions of weak acids. The rate of ester hydrolysis as well as the rate of acylation of the acyl acceptor is also repressed by increasing phosphate concentrations. Similar results have been obtained by Kasche and Galunsky (*52*) while studying the effects of ionic strength and pH on the synthesis of Pen G. These and other observations have led Konecny (*70*) to propose a model to explain the mechanism of acyl transfer by PA, which implies that the yields of penicillins and cephalosporins depend on the extent of competition of the acyl receptors (6-APA and water) for the acyl-enzyme intermediate and of the ester and the produced amide for the enzyme. The ratios of these reaction rates obviously depend on the nature of reaction conditions, reactants, and the enzymes. Because the esters and the β-lactam ring are prone to decomposition under alkaline conditions, and also that the (thermodynamic) pH optima for the synthetic reactions lie in acidic pH (*66*), but the *E. coli* or the *B. megaterium* enzymes have lower stabilities under acidic conditions (*11*), the use of enzymes having greater stability to acidic pH (e.g., those from *X. citri, K. citrophila,* or *P. melanogenum*) has been suggested to be advantageous in the enzymatic synthesis of β-lactam antibiotics (*70*).

ACTIVE SITE STUDIES

Using chemical reagents that specifically react with certain amino acids, a preliminary understanding of the nature of the active site of PA

has been obtained (Table 3). Catalytic activity of the *E. coli* enzyme remains unchanged upon treatment with the sulfhydryl reagents *p*-chloromercurybenzoate or iodoacetate (*17,72*). Similar results have been obtained for the enzyme from *B. plumbea* also (*19*), indicating that the —SH group is not catalytically important. But the enzyme from *P. rettgeri* and *B. sphaericus* is inhibited to some extent by the —SH reagents (*18,55*). The *E. coli* as well as *B. megaterium* PA is strongly inhibited by phenylmethylsulfonyl fluoride (*17,70,71*), implying the involvement of serine at the active site. However, another serine-specific reagent, diisopropyl fluorophosphate, does not inhibit the *E. coli* PA (*17*). The observations with the *P. rettgeri* enzyme are again contradictory to this (*18*). Robak and Szewczuk (*18*) suggest the probable involvement of tryptophan at the active site of PA of *P. rettgeri* since *N*-bromosuccinimide causes a total inactivation and PAA protects the enzyme from this inactivation. Chemical modification of the *E. coli* NCIM 2400 enzyme using the tryptophan-specific reagents 2-nitrophenyl sulfenyl chloride as well as 2-hydroxy-5-nitrobenzyl bromide has shown the involvement of tryptophan at the catalytic site (*72*). However, ethoxyformylation of at least two histidines of this enzyme does not affect its catalytic activity (*73*). Thus the data so far accumulated from the chemical modification studies indicate the involvement of tryptophan at the active site of the *E. coli* as well as *P. rettgeri* enzyme. The role of serine is not very clearly understood. Further attempts with more specific reagents and rigorous characterization of the modified residues is essential to define the active site of this enzyme. Techniques other than chemical modification may also prove helpful.

In this respect, mention must be made of the studies carried out by Klyosov et al. (*74,75*) on the *E. coli* enzyme. These workers found that the *E. coli* PA is inhibited by a series of aliphatic alcohols, and the type and extent of inhibition changes with an increase in the carbon chain length and thus an increase in the alcohol hydrophobicity causes an increased inhibition of the enzyme. From the kinetic results obtained using two-component inhibition, these workers have concluded that the active center of PA has a complex structure that changes with the variation in the substrate structure. According to their proposed model, the hydrophobic active site of the enzyme in its ground state has a thermodynamically unfavorable contact with its environment as a result of hydrophobic interactions between the substrate and/or inhibitor and the enzyme. Similar predictions have also been made in another independent study by Iyengar et al. (*76*). These workers have designed an organic model catalyst for the hydrolysis of Pen G based on the concept of hydrophobic association of the aromatic ring of the substrate with the aromatic rings of the catalyst, which is the metal ion chelate of diphenylglyoxime or dibenzoylmethane. These organic catalysts have been found to be "active" in releasing 6-APA from Pen G at room temperature in phosphate buffer, pH 6.0. From all these observations, it appears that the catalytic site of PA has a complex structure involving hydrophobic domains.

TABLE 3
Effect of Organic Reagents on the Activity of Penicillin Acylases

Enzyme source[a]	Reagent[b]	Reaction conditions	%, Inhibition
E. coli ATCC 11105 (*17*)	PMSF	Enzyme treated with 1.3-fold molar excess of reagent for 30 min at pH 7.0	100
E. coli ATCC 11105 (*17*)	DFP	Reagent in 75-fold molar excess to enzyme; other conditions as above	16
E. coli ATCC 11105 (*17*)	TPCK	Reagent in 10-fold molar excess to protein; other conditions as above	10
E. coli NCIM 2400 (*72*)	NPS-Cl	Enzyme treated with 5 m*M* reagent in dark at RT in 0.1*M* acetic acid for 50 min followed by gel filtration	100
E. coli NCIM 2400 (*73*)	EFA	Enzyme treated with either 18-fold or 36-fold molar excess of reagent in phosphate buffer, pH 6.0, at RT for 60 min	None
E. coli NCIM 2400 (*72*)	HNB-Br	Reagent up to 10 m*M* in dioxane; protein in citrate-phosphate buffer, pH 4.0; at RT for 50 min	None
E. coli NCIM 2400 (*72*)	HNB-Br	Reaction in phosphate buffer pH 6.0; other conditions as above	100
E. coli NCIM 2400 (*72*)	HNB-Br	As above + 100 m*M* PAA	None
B. plumbea (*19*)	Iodoacetate	Reagent used up to 28 m*M*	No effect
P. rettgeri (*18*)	Iodoacetate	Protein treated with 1 m*M* reagent at pH 7.8 for 15 min at 37°C	27
P. rettgeri (*18*)	Iodoacetamide	Conditions as above	45
P. rettgeri (*18*)	PCMB	Conditions as above	37
P. rettgeri (*18*)	TNM	Conditions as above	42
P. rettgeri (*18*)	DFP	Conditions as above; reagent, 2 m*M*	100
P. rettgeri (*18*)	NBS	Reagent 2 μ*M*; conditions as above	100
P. rettgeri (*18*)	PMSF	About 300 pmol/acylase unit; other conditions as above	100

[a]Numers in parentheses are references.
[b]PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; TPCK, *p*-tosylamino-2-phenylchloromethyl ketone; NPS-Cl, 2-nitrophenylsulfenyl chloride; EFA, ethoxyformic anhydride; HNB-Br, 2-hydroxy-5-nitrobenzyl bromide; pCMB, *p*-chloromercurybenzoate; TNM, tetranitromethane; NBS, *N*-bromosuccinimide.

REGULATION AND GENETICS OF BIOSYNTHESIS OF PA

The fact that the understanding of the molecular mechanisms underlying the regulation of biosynthesis of an enzyme of such commercial importance would certainly be helpful in improving the cost-benefit ratio of acylase production has been recognized for quite some time. The initial attempts in this direction were restricted to the manipulation of fermentation parameters only. Now it is known that the regulation of acylase production in different organisms differs widely. The type I enzyme of bacterial origin is produced constitutively (*22,57,58*). The intracellular type I enzyme of *B. sphaericus* NCTC 10338 (*57*) as well as the *B. plumbea* enzyme are not repressed by glucose. Most of the type II acylases and the type I enzyme of *B. plumbea* (*19*), *P.chrysogenum,* and *Fusarium spp.* (*12*) are inducible. Phenylacetic acid is the best inducer for the type II acylases, whereas POAA is known to be the most efficient inducer for type I enzymes of fungal origin (*12,77*). In fact, Thadhani et al. (*77*) have observed a high degree of stereospecificity of the inducer for acylase production by *Fusarium spp.* However, many derivatives of phenoxyacetylamino acids with either hydroxyl or heterocyclic substitutions in the side chain, or even amides, have been shown to induce the enzyme production in *Fusarium spp.* (*77*).

Although biosynthesis of PA in *B. megaterium* is known to be induced by PAA (*15*), recently, Son et al. (*78*) have isolated a partially constitutive mutant of *B. megaterium* ATCC 14945 upon treatment with UV light. Acylase production by the parent strain is maximum at the inducer concentration of 0.15%. However, the mutant (designated as KFCC 10029 by these workers) produces substantial enzyme activity even in the absence of the inducer. Addition of PAA up to 0.3% causes a further threefold increase in the enzyme activity, thereby also indicating the increased tolerance of the mutant to PAA. Under submerged conditions, the mutant is claimed to produce 7–8 times higher enzyme activity within 20–24 h compared to the 70–72 h required by the parent strain. It is interesting to note that the acylases produced by various *E. coli* strains and *Proteus rettgeri* have similar substrate specificity (and hence are classified as type II), but the mechanism of regulation of their biosynthesis is entirely different. Whereas the enzyme production by *E. coli* is known to be affected by the temperature at which the organism is grown (*79–83*), acylase production by *P. rettgeri* is not subject to such temperature control (*54*). Many strains of *E. coli* produce a very low level of PA activity when grown in minimal medium, but inclusion of PAA causes induced enzyme synthesis, the extent of induction depending on the concentration and time of addition of the inducer (*82*). Of the many derivatives of PAA tested (*82*), none induced the enzyme synthesis, again indicating a high degree of specificity of the system. In *P. rettgeri,* the enzyme is formed constitutively and is not at all induced by PAA. The synthesis of PA in *E. coli* is fully repressed by increased dissolved oxygen, even in the

presence of the inducer (*83*). It is also subject to catabolite repression by glucose, maltose, and glycerol (*79–83*). But the production of PA in *P. rettgeri* is not under catabolite repression by glucose. On the contrary, addition of glucose and other energy-rich compounds such as glycerol or citrate results in the increased enzyme synthesis. However, when *P. rettgeri* is grown on media containing succinate, fumarate, or malate as the sole source of carbon, only a basal level of the enzyme activity is produced. Therefore, Daumy et al. conclude that these dicarboxylic acids repress the synthesis of PA in *P. rettgeri* (*54*).

Biosynthesis of PA in *E. coli* PCM 271 has been reported to be inhibited by 6-APA (*84*). Wojskowicz has also observed that PAA concentration above 0.2% is not toxic to, but only delays the growth of, the organism (*84*). Therefore, "self catabolite repression" of the enzyme by PAA is proposed. Recently, Sykita and Kyslik (*85*) have isolated several mutants of *E. coli* ATCC 9637 using continuous culture technique in the presence of amides as the only source of nitrogen. In addition, glucose has been used in the medium to generate mutants also resistant to catabolite repression. The mutants thus obtained are constitutive and fully resistant to repression by glucose and produce 8–20 times higher PA activity than the parent strain. The repression by glucose can also be overcome by cAMP (*82,83*). Moreover, cAMP itself is thought to induce the synthesis of the *E. coli* enzyme (*40*).

Recently, the technique of genetic engineering has been applied to clone the gene for PA from *E. coli* ATCC 11105 (*86,87*). The genomic library of the parent strain has been established by using the HindIII fragments of the *E. coli* chromosome ligated into the HindIII-cleaved plasmid, pJC 720. Introduction of these "cosmids" into the *E. coli* cells by virtue of direct physical selection has enabled Mayer et al. to obtain a clone, pHM 5. Subcloning of pHM 5 insert into pBR 322 yielded another clone pHM 6 and further UV mutagenesis of pHM 6 produced pHM 12 that shows 45 times higher acylase activity than the parent cells. Interestingly, enzyme synthesis by pHM 12 is totally constitutive and is insensitive to catabolite repression. The pHM 12-produced enzyme has similar K_m values to those of the parent strain enzyme when measured using Pen G as a substrate. Since the substrate profile of the pHM 12 enzyme is otherwise similar to that of the parent strain, these authors conclude that the structural gene for PA in the clone is not affected greatly during the series of genetic manipulations (*88,89*). However, the pHM 12 enzyme exhibits an additional activity namely the Pen V hydrolyzing activity, which is almost three times as that of *B. plumbea*.

CONCLUDING REMARKS

The unique position of PA in the enzyme industry in general and the production of semisynthetic antibiotics in particular, has led to accumulation of data mainly on the applied aspects of the enzyme. Questions

raised some 20 yr ago, regarding its physiological role (*1*), remain unanswered even today. The hydrolytic reactions catalyzed by various bacterial acylases are said to be rather nonspecific because these enzymes hydrolyze amino acyl esters in addition to penicillins and cephalosporins. However, the prerequisite for their hydrolytic ability seems to be the presence of a phenylacetyl group in the acyl side chain of the substrates (*11,33,62*), introduction of an oxygen function causing a dramatic decrease in the rate of hydrolysis of such compounds. In this sense, it would be appropriate to classify this group as having a "wide specificity" than to designate them as nonspecific. Nonetheless, a detailed kinetic analysis of the homogeneous enzyme preparations under well-defined conditions would give an overall perspective of the precise nature of these enzymes. We still do not know whether or not the Pen V hydrolyzing enzyme is structurally different from the Pen G hydrolyzing enzyme. Is it possible to induce structural changes in the enzyme molecule (e.g., by chemically modifying the protein) to modify the substrate specificity or to improve stability of the enzyme? A thorough structure–function analysis of this group of proteins may also be helpful in improving the reaction conditions. Moreover, such basic information may have direct commercial implication, as has been indicated by the thermodynamic studies of Svedas et al. (*66,67*). What is the molecular mechanism underlying the regulation of biosynthesis of PA in different organisms and especially, when different species in the same genera are capable of producing different types of enzyme (*15,57*)? The very striking observation made with the genetically engineered *E. coli* cells that unlike the parent strain, the 5K (pHM 12) cells can hydrolyze Pen V quite efficiently, raises the question whether these cells produce two different types of acylases. If so, why the parent strain does not exhibit this Pen V acylase activity? If not, what structural changes in the protein produced by the recombinants confer this "marginally altered" substrate specificity on the enzyme? The powerful tool of recombinant DNA technology should prove to be of great help in the study of such problems in these well-defined, isolated systems. Other areas of applications of PA (*90–92*) also need to be exploited.

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