Enzymatic synthesis of ß-lactams: Constraints and control

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The constraint in synthesis of ß-lactams by penicillin acylase is the hydrolysis of the activated acyl donor and the synthetic product. The thermodynamically controlled synthesis of ß-lactams deals with direct acylation of a nucleophile (6-APA, 7-ACA or 7-ADCA) by free acids in acidic pH and high concentration of solvent to achieve good yield. On the other hand, the kinetically controlled synthesis with esters is much faster. However, success and yield of kinetically controlled synthesis depend on combined application of an insolubilized catalyst, optimum pH of the reaction, addition of suitable solvent and high concentration of activated acyl donor and ß-lactam nucleus. Water miscible solvents play an important role in the synthesis. The choice of solvent is guided by its inhibitory effect on enzyme activity and deleterious effect on enzyme stability. Synthetic yield of cefazolin in presence of water-miscible solvent is reduced, while the reaction carried in water-immiscible solvent markedly improves the yield of cefazolin, possibly due to reduction in hydrolysis of acyl donor and the product. Ethylene glycol inhibits more the hydrolysis of ampicillin than the hydrolysis of PGME and the synthetase/amidase ratio varies depending on the concentration of the solvent used. Hydrolytic vs synthetic activity of penicillin acylase is also influenced by water activity of the insolubilized catalyst. Shuffling of PA-encoding genes has been shown to improve the ß-lactam synthesis. Delineation of enzyme-solvent interaction needs close scrutiny to ensure further success in biocatalysis and synthesis of ß-lactams in particular.

Keywords: Immobilized penicillin acylase, ß-lactams, solvents, synthesis, water activity

Introduction

Penicillin acylases or penicillin amidases (PAs) (EC 3.5.1.11) are enzymes which cleave the acyl side chain of ß-lactams to furnish 6-aminopenicillanic acid (6-APA) and an acyl residue\(^1\) (Fig. 1). Penicillin acylases (PAs) are produced by bacteria, actinomycetes, yeasts and fungi\(^2\). Penicillin acylases are classified as Type-I (penicillin V acylases), Type II (penicillin G acylases) and Type III (ampicillin acylases). Type I acylases are composed of four identical subunits, each of a mass of 35 kDa. Type II acylases are heterodimers composed of a small subunit (16-26 kDa) and large ß-subunit (54-66 kDa). Again, Type II acylases are subdivided into Type IIA and Type IIB. Type IIA comprise of penicillin G acylases and Type IIB acylases are glutaryl acylases. Type-III acylases (ampicillin acylases) are reported to be homodimers consisting of identical subunits with a molecular mass of 72 kDa. However, substrate specificity study of penicillin acylase from *Streptomyces lavendulae*\(^3\) has revealed that it can hydrolyze penicillin F, penicillindihydro F and penicillin K. Although it has been described as a penicillin V acylase\(^4\), the results indicate that this enzyme should be considered as penicillin K acylase.

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Fig. 1—Penicillin acylase catalysed hydrolysis of penicillins to 6-APA
The availability of chromogenic analogs of penicillin dihydro F and penicillin K could allow screening of novel penicillin acylase-producing microorganisms and lead to a more detailed classification of this group of enzymes.

Modified Penicillin Acylases

Enzymes with improved properties can be developed either by classical screening, or by chemical modification of the existing proteins, or by using modern genetic and protein-engineering techniques. Chemical methods used for modification of proteins are not specific. They modify all accessible residues with common side chains or they are dependent on suitable amino acids to be modified, and often they are unable to modify amino acid difficult to reach, unless the enzyme molecule is unfolded. Moreover, chemical modification requires additional processing steps and chemicals to prepare the enzyme. Enzyme modification through mutagenesis of the encoding gene does not suffer from the problems mentioned above. Desired properties may include altered specificity, altered pH dependence or altered stability. Mutagenesis can be achieved either by random or by site-directed technique.

Random mutagenesis is usually done by treating whole microorganism with chemical mutagens or mutagenizing radiation. High probability of isolating desired mutant enzymes by random mutagenesis involves cloning of the encoding enzyme, mutagenizing in vitro or in vivo and expressing the encoded enzyme by recloning of the mutated gene in a suitable host. Also in this case suitable biological protocols should be available in order to select the desired mutant enzymes. Site-directed mutagenesis (SDM) is the most suitable way of obtaining modified enzymes, enabling specific substitution of one or more amino acids by any other desired amino acid.

Recombinant production and export of Bacillus megaterium ATCC 14945 penicillin G acylase into the growth medium has been reported for use in retrosynthesis of β-lactam antibiotics.

β-Lactamase-free Penicillin Acylase

Penicillin acylases produced by microorganisms are very often associated with β-lactamase. They are made β-lactamase-free by induced mutation. Three wild Escherichia coli strains, BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50, producing both penicillin G acylase (PGA) and β-lactamase were treated with acridine orange. The resultant mutants exhibited enhanced production of PGA and inactivation of β-lactamase. The mutant BDCS-N-M36 showed very negligible expression of β-lactamase activity and 2-fold increase in PGA activity. Isolation and characterization of a natural β-lactamase-free penicillin acylase from Alcaligenes sp. (Fig. 2) had been reported for the first time from the author’s laboratory.

The PA from Alcaligenes sp. shares properties of both type II and type III PA, although it is of type II origin. This enzyme is quite stable and it has been shown that the enzyme stability is not due to the presence of disulphide bond like PA from A. faecalis.

Structure of Penicillin Acylase

The mature E. coli PGA is a periplasmic 86 kDa heterodimer. The two monomer chains referred to as A and B contain 209 and 557 amino acid residues, respectively. The enzyme is synthesized as a precursor protein, from which an active enzyme is generated by proteolytic processing. The PGA precursor polypeptide consists of four segments: the signal sequence, the A-subunit (A1-A209), the spacer peptide (residues 210-263) and the B-subunit (B1-B557). The signal sequence directs the transport of PGA precursor into the periplasmic space, while the 54 amino acid spacer between A and B chains helps to ensure the correct folding of the polypeptide before cleavage to generate the mature enzyme. The N-terminal serine of the B chain reacts with phenyl methyl sulphonyl fluoride, which is consistent with a catalytic role for the serine hydroxyl group. Modification of serine to a cysteine inactivates the enzyme, whereas threonine, arginine or glycine substitution prevents in vivo processing of the

Fig. 2—Transmission electron micrograph of Alcaligenes sp.
enzyme, indicating that this must be an important recognition site for cleavage. The crystal structure of penicillin acylase from *E. coli* has been determined at 1.9 Å resolution\(^1\) and more recently crystal structure of PGA from *Providencia rettgeri* has been determined at 2.5 Å\(^1\). Analysis of the structure shows that two chains of the molecule are closely intertwined and form a pyramidal structure that contains a deep cone-shaped depression, at the bottom of which is the active site.

**Catalytic Mechanism of Penicillin Acylases**

Penicillin acylases are members of the N-terminal nucleophile (Ntn) hydrolase superfamily. These enzymes are characterized by a distinct fold and a catalytic residue in the N-terminal position\(^1\). Even though the nucleophilic residue differs (serine for penicillin G acylase and cysteine for penicillin V acylase), both these acylases share a striking similar arrangement of their catalytic environment. The typical fold of Ntn-hydrolase superfamily consists of a 4-layered catalytically active αβßα-core structure\(^1\). This core is formed by two anti-parallel β-sheets packed against each other, and these β-sheets are covered by a layer of α-helices on one side (Fig. 3). Like all Ntn-hydrolases, penicillin acylases go through a post-translational process\(^1\) that leads to an autocatalytically activated enzyme.

**Synthesis of β-Lactams by Penicillin Acylase**

Semi-synthetic penicillins are superior to penicillin G and V in respect of stability, absorption and side effects. They also represent a practical solution to the problem of adaptive microbial resistance to antibiotics. The semi-synthetic penicillins can be prepared by chemical and biological means. The enzymatic method is preferred because: i) the reaction is stereospecific, ii) reactants do not require side chain protection, iii) reduces environmental problem because of no need for organic solvent, iv) unlike chemical route less steps are required, vi) there is least amount of byproduct, and vii) extreme conditions of pressure and temperature are not required.

Semi-synthetic penicillins and cephalosporins are produced by condensation of appropriate D-amino acid derivative with β-lactam nucleus catalyzed by penicillin acylase\(^1\). This type of condensation can be done by either thermodynamically or kinetically controlled synthesis\(^1\).
b) hydrolysis of the activated acyl donor, and c) hydrolysis of the synthesized product (Fig. 4). Obviously, design of synthesis would be to favour the synthetic process and to reduce hydrolysis. This can be achieved by combined application of an insolubilized catalyst, optimum pH for the reaction, addition of suitable solvent and high concentration of activated acyl donor and β-lactam nucleus. Taking these into considerations, kinetically controlled synthesis proved to be equally successful for the synthesis of different semi-synthetic β-lactams.

Advantages of performing penicillin acylase-catalyzed synthesis of new penicillins and cephalosporins by enzymatic acyl transfer to the β-lactam antibiotic nuclei in the supersaturated solutions of substrates have been demonstrated. It has been shown that the effective nucleophile reactivity of 6-aminopenicillanic (6-APA) and 7-aminodesacetoxycephalosporanic (7-ADCA) acids in their supersaturated solutions continue to grow proportionally to the nucleophile concentration. As a result, synthesis/hydrolysis ratio in the enzymatic synthesis can be significantly increased due to the nucleophile supersaturation. In the antibiotic nuclei conversion to the target antibiotic, remarkable improvement (up to 14%) has been achieved. Methods of obtaining relatively stable supersaturated solutions of 6-APA, 7-ADCA, and D-p-hydroxyphenylglycine amide (D-HPGA) have been developed and syntheses of ampicillin, amoxicillin, and cephalaxin starting from the supersaturated solutions of substrates were performed. Higher synthetic efficiency and increased productivity of these reactions compared to the heterogeneous aqueous solution-precipitate systems were observed. It has been suggested that this approach seems to be an effective solution for the aqueous synthesis of the broad spectrum β-lactam antibiotics, i.e., amoxicillin, cephalaxin, cephadroxil and cephacorol.

Improvement in synthesis of β-lactam antibiotics has also been reported by shuffling of PA-encoding genes of E. coli, K. cryocrescens and Providencia rettgeri. Out of these three, the PA from E. coli possessed the best properties for the synthesis of ampicillin. Three clones with improved synthetic properties were selected and sequence analysis showed that the shuffled genes were hybrids of the PA-encoding genes from E. coli and K. crocrescens. The hybrid enzymes displayed a 40-90% increase in the relative rate of acyl transfer to the β-lactam nucleus during ampicillin synthesis. Similar improvements in acyl transfer were obtained for the synthesis of amoxicillin, cephalaxin and cefadroxil, making the new hybrid enzymes interesting candidates for the biocatalytic synthesis of several β-lactam antibiotics.

Enhanced synthesis of different β-lactams in ice at –20°C catalyzed by penicillin G acylase has been reported by Sheldon et al. The initial ratio between aminolysis and hydrolysis of the acyl-enzyme complex in the synthesis of cephalaxin increased from 1.3 at 20°C to 25 at –20°C. The free penicillin-G acylase could perform transformations in frozen media with predominant hydrolytic activity under above conditions. Relative rates of hydrolysis and synthesis of β-lactams can be modified by site-directed mutagenesis of the enzyme. In fact, mutation of phenylalanine at position 24 in the β-subunit to alanine of penicillin G acylase from E. coli yielded a mutant that showed a higher synthesis-hydrolysis ratio.

The transformation by enzyme has been found to be affected by various factors, viz., reaction medium, pH, ionic strength, temperature, organic solvent, water activity, stability of the catalyst with respect to reaction conditions and reactant and product stability. Methyl esters have been observed to be better acyl donor than free acid. The equilibrium of the reaction can be shifted in favour of acylation by changing the water activity of the catalyst with organic solvents, e.g., polyethylene glycol, methanol, ethanol, propanol, butanol and acetone.

**Synthesis of β-Lactams by Immobilized Penicillin Acylase**

The immobilized enzyme is superior to its free counterpart because of easy separation of enzyme from the product, reuse of the enzyme and enhanced stability. Immobilization methods can be divided into...
3 types: binding to a carrier, encapsulation in an inorganic or organic polymeric gel, or by cross-linking of the protein molecules by a bifunctional cross-linking agent. The hydrophilic resin, such as, glyoxylyl-agarose (activated with aldehyde groups) has been shown to be superior to hydrophobic acrylic-epoxy supports like Eupergrit C during N-acylation of 7-aminocephalosporanic acid by *Arthrobacter viscosus*. The use of cross-linked enzyme crystals (CLECs) as industrial biocatalyst had been introduced in the early 1990s for commercial purpose. CLECs proved significantly more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme or lyophilized powder. Later cross-linked aggregates (CLEAs) were preferred to CLECs because of productivity. In fact, penicillin G acylase CLEAs, prepared by precipitation with ammonium sulphate, proved to be the effective catalyst for the synthesis of ampicillin. Penicillin acylase from *E. coli*, *B. megaterium* or *A. faecalis* have been immobilized by different methods that range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders. In fact, covalent binding of penicillin V acylase from *Streptomyces lavendulae* to epoxy-activated acrylic beads (Eupergrit C) have shown improved production of 6-APA and enhanced pH and thermal stability. However, inspite of stabilization due to enzyme attachment to the support, immobilized penicillin G acylase on Eupergrit C exhibited lower turnover rates in penicillin G hydrolysis than the enzyme in solution. The hydrophobic acrylic epoxy-supports (Eupergrit C) also acted as a poor matrix and showed low turnover rate in kinetically controlled N-acylation of 7-amino cephalosporanic acid catalyzed by penicillin acylase from *A. viscosus*. This might be due to diffusion limitations of substrate and product inhibition. Mateo et al. reported a new epoxy-coated support called Sepabeads-EP, which showed excellent results as immobilizing agent. Later Phadtare et al. developed an enzyme (penicillin acylase)-fatty lipid biocomposite for more efficient immobilization. The modulation of penicillin G acylase (PGA) properties via immobilization techniques has been performed studying the acylation of 7-aminocephalosporanic acid with R-mandelic acid methyl ester. PGA from *E. coli*, immobilized onto agarose activated with glycidol (glyoxylyl-agarose), has been used for the design of a novel one-pot synthesis of cephamandole in aqueous medium and without isolation of intermediates, through three consecutive transformations catalyzed by D-amino acid oxidase, glutaryl acylase and PGA. All these techniques are oriented to reduce manufacturing costs and mass transfer limitations in order to increase their competitiveness for industrial applications. At present, worldwide demand of 9000 tons 6-APA is served by enzymatic means. Enzymatic production of 6-APA is only economically viable when immobilized biocatalysts are used.

Research is now focused on the preparation of very robust biocatalysts that may improve enzyme stability over a broad range of temperature, pH and solvents. Recently, whole cell immobilized penicillin G acylase have been reported to compete with biocatalysts prepared with isolated enzymes. In fact, whole cell of several strains of *E. coli* and its mutants have been entrapped within polymethacrylamide beads. The immobilized preparations were effective in the hydrolysis of penicillin G. Similarly, synthesis of β-lactam antibiotics has been affected by reacting an amino-β-lactam component with a corresponding amino group containing acylating side-chain component in presence of penicillin acylase from *E. coli* covalently immobilized on support particles. The resulting β-lactam antibiotic is solubilized by adding an acid, such as, sulfuric acid, to lower the pH to 1.0 at a temperature in the range of 0°C to +5°C. The immobilized penicillin acylase is substantially inactivated by the acid. After separating the product (β-lactam antibiotic), the immobilized enzyme can be reactivated for reuse in antibiotic synthesis by treatment with a buffer having a neutral pH. Antibiotics, viz., ampicillin, amoxicillin, cephalixin, cefaclor and cefadroxil, can be produced in this way.

Enzymatic synthesis of ampicillin, in particular, can be carried out by thermodynamically or kinetically controlled route. However, thermodynamic studies showed that synthesis is highly unfavourable for ampicillin due to low chemical energy of zwitterionic phenylglycine. Penicillin G acylase from *E. coli* requires that the PG carboxyl should be protonated, while at the same time, the amino group of 6-aminopenicillanic acid should be neutral, available for nucleophilic interactions. The kinetically controlled synthesis of ampicillin can be achieved by the use of activated substrates like phenyl glycine methyl ester (PGME) at pH values that are optimal for the enzyme. PGA from *E. coli* immobilized on an agarose-glyoxyl derivative or chitosan has been used for synthesis of ampicillin.
The presence and magnitude of interactions between reaction variables were examined. The best synthetic yield (56.9%) was at 4°C and pH 6.5. The highest productivity was achieved at 25°C and pH 6.5\textsuperscript{36}. Also, by controlling the pH, it is possible to inhibit the two undesirable hydrolysis reactions and increase the yield of synthesis.

**Synthesis in Water Miscible Solvents**

Synthesis of semi-synthetic penicillins by penicillin acylase is possible both by thermodynamically and kinetically controlled synthesis. While the former is slow, the latter is fast and in both the modes, i.e., water soluble solvents, play an important role on the yield of synthesis. The enzyme activity and stability account very much for overall yield of the product in synthetic/hydrolytic reaction.

In the thermodynamically controlled synthesis free acids act as acyl donor. The reaction is slow because of low pH and PGA is not so active, thereby affecting the yield. At the end, thermodynamically equilibrium is reached, which is convenient to stop the reaction. This approach works only with amidases, not with α-amino acid esterases, since these enzymes catalyse an α-amino acyl transfer. The equilibrium constant follows the charge state of the side chain, which depends on the Ks of the acid and pH value. The dissociation constant can be shifted to more favourable values by water soluble solvents as ions are less well hydrated. In contrast, the Ks is reduced by high ionic strength as ions are better stabilized. Water soluble solvents have also a direct influence on the equilibrium, since they reduce the water activity and therefore water, which is a by-product of synthesis, is apparently removed and drives the reaction forward. Also, increase in synthetic yield can be achieved by increase in concentration of β-lactic nucleus and side chain. Temperature and enzyme input have got influence on reaction time, but less on yields. Increase in yield is possible when the reaction is run in water miscible solvent at low substrate concentration. In contrast, at high substrate concentration, yields are reduced in synthesis run in water soluble solvents. Synthesis of cephalothin from thienylacetic acid and 7-aminocephalosporanic acid has been carried out in the presence of high concentration of organic cosolvents, e.g., 50% N,N-dimethyl formamide (DMF), under a variety of experimental conditions of pH and temperature by using penicillin G acylase. Synthetic yields are markedly increased (95%) compared to those obtained in fully aqueous medium under compromising reaction conditions\textsuperscript{37}. The kinetically controlled synthesis with esters is much faster, since Gibb’s energy of activated side chain is improved and more alkaline pH values may be applied, where the acylase is more active. The higher Gibb’s energy is also the reason for the yields better than thermodynamic equilibrium. These condensations have to be terminated in time, since the formation of antibiotic reaches a maximum. This is because of hydrolysis of the activated side chain followed by synthetic β-lactam hydrolysis. At the maximum yield, rate of synthesis is equal to the rate of hydrolysis of the condensed product. Very often these effects act controversial, e.g., a higher pH, higher temperature or more enzyme input increases the activity for hydrolysis and synthesis. For enzyme input, a compromise between reaction time and yield needs to be made. Also high substrate concentration will enhance the synthesis followed by faster hydrolysis, but better yields will be achieved at higher load. Water soluble solvent and ionic strength of the medium will have the same effect on the yield of the product as mentioned above. However, the overall performance of the enzyme in the synthetic mode is a combination of two effects (i) the inhibitory effect of the solvent on the enzyme and (ii) the desirable increase in the non-ionized form of the substrate in the presence of solvent. The ratio of the enzyme activity in its synthetic mode to its hydrolytic mode seems to be a useful guide for the selection of a suitable solvent\textsuperscript{38}.

**Synthesis in Biphasic Solvent**

The utility of synthetic route of penicillin acylase is often limited by the necessity of using organic solvents, which have an inhibitory effect on the enzyme activity and also a deleterious effect on the enzyme stability. The first limitation can be overcome by careful selection of the organic solvent so as to ensure that high synthetic yields are obtained without excessively reducing the activity of the enzyme derivative, and the second by using enzymes previously stabilized. Activation and stabilization of the enzyme can also be ensured by the addition of polyols, viz., glycerol and glycol. In fact, the effects of organic solvents on penicillin acylase catalyzed kinetically controlled synthesis of cefazolin ((CEZ) have been examined in various water-solvent
mixtures\(^3^9\). In the presence of water-miscible solvents, the initial rate and maximum yield of CEZ synthesis were found to be reduced. The extent of inhibition was increased with increasing hydrophobicity of the solvent in the reaction mixture. It is interesting to note that the reaction when carried in water immiscible solvent, e.g., ethylacetate or carbon tetrachloride (two-phase system), the yield of CEZ was markedly improved. It was suggested that enhanced effect of EtOAc or CCl\(_4\) on synthetic yield was possibly due to reduction of hydrolysis of acyl donor and product in the two-phase system rather than extraction of the product into the solvent phase. Similarly the effects of organic cosolvents on the synthesis of ampicillin from phenylglycine methyl ester (PGME) and 6-aminopenicillanic acid (6-APA) using immobilized \(B.\) \(megaterium\) penicillin G acylase have been examined. Several cosolvents were tested for their influence on the enzyme in terms of enzyme stability and hydrophobicity. Among the cosolvents tested, ethylene glycol was found to increase the yield of ampicillin by 39-50%. The effect of ethylene glycol on the pKa of PGME, the hydrolysis of ampicillin and PGME, and synthetase/amidase and esterase/amidase ratios was also been studied. In fact, ethylene glycol inhibited more the hydrolysis of the ampicillin than the hydrolysis of the PGME and the synthetase/amidase ratio varied from 0.2 to 0.88 when the concentration (v/v) of the cosolvent increased from 0 to 40%. The enhancement of the synthetic yield was mainly due to reduction in the hydrolysis of acyl donor (PGME) and product (ampicillin) in the water-cosolvent system\(^4^0\).

**Synthesis under Reduced Water Activity**

The real challenge in biocatalysis is the alteration of substrate specificity of the enzyme. Although the stereo-chemical outcome of a given enzyme-substrate reaction is predetermined, it got two options to alter the result of the reaction, viz., change of reactants or change of reaction conditions. With the former, redesigning of enzyme by site-directed mutagenesis is a powerful tool towards the goal; while in latter, reports are available on water activity (\(a_w = P/P_0\), where P and \(P_0\) are vapour pressures over a sample and pure water, respectively) which has been used to explain the results in biocatalysis.

Water activity is a much better choice than water content since microorganisms do not recognize the water content of a particular material but the amount of available water, which differs considerably depending on solutes. The water activity of the biocatalyst is usually estimated by isopiestic method devised by Bull and Bresse\(^4^1\). The penicillin acylase activity (hydrolytic and synthetic) of immobilized cells and periplasm tested under reduced water activity showed that, depending on water activity, the catalyst can be used for hydrolysis or synthesis of penicillins\(^4^2\).

**Conclusion and Future Trends**

Penicillin acylase is now an important alternative for the cost-effective synthesis of \(\beta\)-lactam antibiotics. Immobilized penicillin acylase is preferred to free cells because it can be re-used and product separation from the reaction mixture is easy. Again, synthesis is favoured over hydrolysis during kinetically controlled synthesis of \(\beta\)-lactams by the immobilized catalyst. Water activity of the immobilized catalyst is an important determinant during synthesis of \(\beta\)-lactams. Enzyme engineering, immobilized enzyme, non-aqueous biocatalysis, delineation of enzyme-solvent interaction are the areas which need to be defined precisely to ensure further success in biocatalysis and synthesis of \(\beta\)-lactams.

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