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Control of Enzyme Hydration in Penicillin Amidase Catalysed Synthesis of Amide Bond

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Abstract: Penicillin amidase catalyses the synthesis of amide bond in very high yield (>98%), using equimolar concentrations of the amine and the phenylacetic components. *In situ* hydrated phosphates were employed for controlling the water activity in a benzene/water system (97:3 v/v), where the water is taken up by the salt with formation of the hydrated species.
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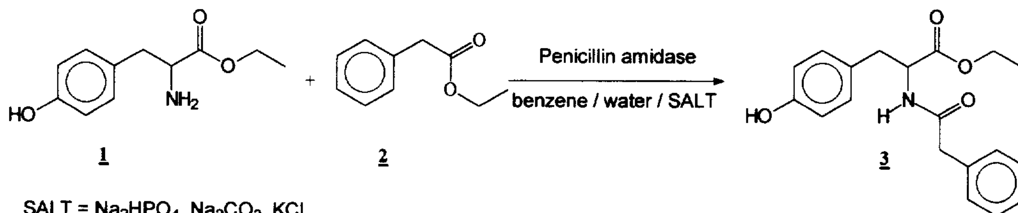
Penicillin amidase (EC 3.5.1.11)[1] can hydrolyse or synthesise esters and amides of phenylacetic acid selectively. The enzyme is industrially used to hydrolyse benzylpenicillin (penicillin G) to produce 6-aminopenicillanic acid (6-APA)[2]. Penicillin amidase is also a valuable tool for the synthesis of enantiomerically pure compounds and it has been used for the kinetic resolution of amines[3] and alcohols[4] both *via* hydrolytic and synthetic reactions[5]. Penicillin amidase have been employed for the synthesis of various semisynthetic β-lactam antibiotics, but yields obtained in the enzyme-catalysed semisynthesis cannot yet compete with yields from chemical procedures. In our previous statistical study on the influence of experimental conditions on the synthesis of ampicillin in aqueous media[6], we reported that the complete conversion cannot be achieved simply by adjusting the experimental factors.

The enzymatic synthesis of amide bond catalysed by penicillin amidase has been investigated by Luisi in aqueous solution, in organic solvent/aqueous buffer mixtures as well as in organic solvents containing reverse micelles[7]. Water-miscible organic cosolvents have been frequently used to increase the yield by influencing the equilibrium of the enzymatic reaction and also to enhance the conversion rates by improving the solubility of hydrophobic substrates[8]. Unfortunately organic cosolvents can have a denaturing effect so that it is highly desirable to find the most suitable cosolvent and the concentration which optimise the yield without causing a sizeable denaturation. Recently, Kim et al.[9] reported that in the penicillin amidase catalysed synthesis of pivampicillin almost no reaction was observed in nonpolar, water-immiscible solvents, and this was ascribed to the reversible inhibitory effect of the apolar solvent on the biocatalyst.

The crucial role of enzyme hydration in affecting the activity and selectivity of enzymes is largely documented[10-12]. Continuing our studies of the effect of the medium on enzyme activity[12,13], now we report the penicillin amidase catalysed synthesis of the amide 3 employing *in situ* hydrated phosphate salts for controlling the water activity[14] in a benzene/water (97:3 v/v) mixture. In these experimental conditions the enzyme catalyses the synthesis of amide bond in very high yield (>98%), using equimolar concentrations of the amine and the phenylacetic components.

A preparation of penicillin amidase and Na_2HPO_4 (1:9 w/w) lyophilised together[15] was suspended in a mixture of benzene and a small amount of water (3%), which is taken up by the salt with formation of hydrates "buffering" the water activity. The moles of water added to benzene were calculated in order to generate the couple of hepta- and dihydrated phosphates (Table 1, column 3). After equilibrating the system for 24h the amine (1) and the phenylacetic component (2) were added in equimolar concentrations. Complete conversion (>98%) was achieved in 5 days (v_0 = 1.1mM•h^{-1}). No competitive hydrolytic reactions were detected and hydrated Na_2HPO_4 does not exert any appreciable denaturing effect on the biocatalyst. Product 3 can be isolated very easily after centrifugation of the salt/enzyme preparation and evaporation of the solvent.



The homogeneous dispersion of the hydrated salt is crucial for the efficiency of the biocatalyst and higher yields were obtained when the salt and the enzyme were lyophilised together. When penicillin amidase was added directly to a benzene/hydrated salts system (0.2M of $Na_2HPO_4 \cdot 2H_2O$ and 0.2M $Na_2HPO_4 \cdot 7H_2O$), an appreciable amount of hydrolytic reaction was detected.

The same procedure was followed employing Na_2CO_3 or KCl[16] but the reactions stopped respectively at 49 and 62% of conversion. In the case of Na_2CO_3 the kinetic profile indicated clearly a progressive inactivation of the enzyme, probably ascribable to the detrimental effect of the basicity of the salt.

No reaction was detected when the salts were added to the reaction mixture in the absence of the enzyme.

In the absence of salts, and in experimental conditions similar to exp. 1 (benzene/water = 97:3 v/v) the splitting of the medium into two phases was visually evident, with the consequent solubilization of the enzyme and adhesion of the aqueous phase to the walls of the vial. An appreciable amount of hydrolytic reaction was also detected.

When the three salt-enzyme preparations were employed without previous hydration and equilibration, no or little reaction was detected (Conversion <5% after 120h). This is in accordance with the negligible reactivity observed by Kim et al.[9], using the immobilised enzyme in nonpolar, water-immiscible solvents without any hydrated salt.

Table 1. Conversion Observed after 120h Employing Different Salt/Enzyme Preparations[a].

Exp	Salt	Mole ratio of added water to salt	Conversion (%)
1	Na_2HPO_4	5	>98
2	Na_2CO_3	8	49
3	KCl	5	46

[a]Experimental conditions: T = 40°C, Organic cosolvent = benzene, [Salt-enzyme] = 50mg/mL, [1] = [2] = 75mM.

In conclusion, we report a system that allows to perform the penicillin amidase catalysed synthesis of the amide bond, working without any excess of the acyl donor and achieving a complete conversion[17]. These features make this process of practical applicability. Preliminary results from our laboratory indicate that a further improvement of the reaction rate can be achieved on the bases of a quantitative study of the effect of water activity on penicillin amidase's behaviour.

Pretreatment of penicillin amidase with salts. 50mg of penicillin amidase (Fluka) were dissolved in a 5mL aqueous solution containing 450mg of salt and then lyophilised.

Synthesis of amide 3. In a typical reaction 50mg of salt/enzyme powder were added to a benzene/water mixture where the water content was calculated on the bases of the salt concentration (see Table 1). The system was equilibrated for 24h inside a 5mL glass vial capped with a closure provided with a silicone membrane. The vessel was incubated in an orbital shaker (250rpm) thermostatted at 40°C. After equilibration tyrosine ethyl ester (1) (Sigma) and ethyl phenylacetate (2)[4b] were added both at a 75mM concentration. Samples were withdrawn, centrifuged and analysed by HPLC to monitor the reaction. All the operations were carried out paying the maximum attention to prevent exchange of humidity between the reaction vessel and the atmosphere. Kinetics were followed by reverse phase HPLC.

The product 3 was isolated concentrating the liquid phase of the system. Purity was checked by HPLC (>98%) and by ^{1}H-n.m.r. Amide 3 was characterised by ^{1}H-n.m.r., ^{13}C-n.m.r., and by comparison with a chemically synthesised standard.

^{1}H-n.m.r. ($CDCl_3$), δ (ppm): 1.23 (t, J = 7.1, 3H, CH_2CH_3), 2.90 (dd, J = 5.9, J = 13.9, -CHH-CH-), 3.00 (dd, J = 5.7, J = 14.0, -CHH-CH-), 3.55 (s, 2H, Ph-CH_2-CO-) 4.14 (q, J = 7.0, 2H, -OCH_2CH_3), 4.81 (m, -CH_2-CH-), 5.68 (s, 1H, -OH), 5.85 (d, J = 7.8, 1H, -NH-), 6.61 (d, J=4.2, 2H, H_3-Tyr), 6.75 (d, J=4.2, 2H, H_4-Tyr), 7.16-7.20 (m, 2H, Ph), 7.28-7.36 (m, 3H, Ph).

^{13}C-n.m.r. ($CDCl_3$), δ (ppm): 14.08 (-OCH_2CH_3), 36.91 (-CH_2CH-), 43.47 (Ph-CH_2-), 53.31 (-CH_2-CH-), 61.69 (-OCH_2CH_3), 115.56 (C_3-Tyr), 126.50 (C_4-Ph), 127.5 (C_1-Tyr), 129.04 (C_2-Ph), 129.42 (C_3-Ph), 130.17 (C_2-Tyr), 134.05(C_1-Ph), 155.61 (C_4-Tyr), 171.36 (C=O), 171.60 (C=O).

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