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Journal of Molecular Catalysis B: Enzymatic 5 (1998) 241–244

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Activity of immobilised penicillin amidase in toluene at controlled water activity

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Received 26 September 1997; accepted 21 January 1998

Abstract

Penicillin amidase (EC 3.5.1.11) supported on hydrated celite rods was used at constant water activity in toluene for the synthesis of amide bond. This approach couples the advantages coming from the immobilisation of the catalyst and the control of water activity in synthetic biotransformations, providing penicillin amidase with the water necessary for maintaining the catalytic activity. Furthermore, the thermodynamic equilibrium is shifted toward synthesis so that no competitive hydrolytic reaction occurs. The supported catalyst preserves its activity also after recycling. These features make the biocatalytic system of practical applicability in organic synthesis. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Penicillin amidase; Biotransformations; Low-water media; Control of water activity

1. Introduction

The crucial role of enzyme hydration in affecting the activity of biocatalysts is largely documented [1–5] and several methods have been developed for carrying out biotransformations at controlled water activity (a_w). Penicillin amidase (PGA) is one of the most largely employed biocatalyst in organic synthesis and industry [6,7]. This enzyme can hydrolyze or synthesize esters and amides of phenylacetic acid selectively and it is a valuable tool for resolution of amines and alcohols [7–9]. PGA is also used in the synthesis of various semisynthetic β-lactam antibiotics. Major limits for the use of penicillin amidase in aqueous media are the hydrolytic pathways competing with synthetic processes and the poor solubility of hydrophobic substrates in water. The development of alternative methods for the use of penicillin amidase in low-water media can contribute to overcome the before mentioned limits. Unfortunately, water-miscible organic cosolvents, which are often used to solve these problems, generally have denaturing effects [10,11]. Furthermore, it has been reported that PGA is not active when used in pure nonpolar water-immiscible solvents [12].

Recently, we demonstrated that in order to maintain penicillin amidase active in low-water media it is sufficient to control the water activity of the system. In our previous paper we have reported that penicillin amidase catalyses the synthesis of the amide **3** in benzene in 5 days by using in situ hydrated phosphate salts for controlling the enzyme hydration [13].

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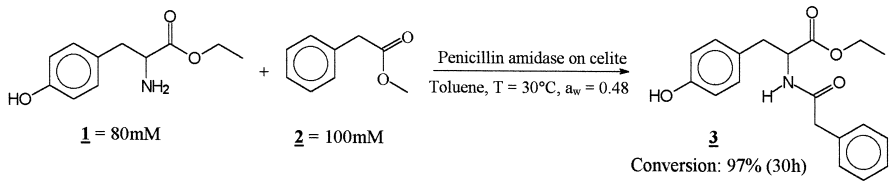
1381-1177/98/$19.00
PII: S1381-1177(98)00092-7



Scheme 1.

Now, we describe an alternative approach for controlling the water activity of biocatalytic systems in low-water media, which leads to a significant improvement of enzyme performance. Penicillin amidase was immobilised on celite rods which are able to absorb considerable amount of water (90% by weight). PGA supported on hydrated celite catalyzed the synthesis of the amide **3** in toluene in 30 h, with 97% conversion (Scheme 1). No competing hydrolytic reaction was detected. The system exploits the ability of celite to maintain the water activity of the system at constant values within a defined, wide range of water concentrations, as was confirmed experimentally. The immobilised enzyme is also suitable for recycling, without undergoing any appreciable decrease of activity.

2. Materials and methods

2.1. Measurement of water activity

Water activity was measured with a NOVASINA MS1 hygrometer equipped with a humidity–temperature sensor enCR-3 sealed into the open end vial, thermostat at 30°C. The sensor reading was calibrated between a_w 0.12 and 1.00 using standard salt solutions and pure water.

2.2. Assay of celite hydration

One milliliter of toluene dried over molecular sieves (4 Å) was added into a 5-ml vial with screw cap and teflon-lined septum containing 95 mg of celite rods (R-640 from Fluka, mean pore diameter 0.2 μm, water absorption: 90% by weight). Different volumes of water (5–60 μl) were added into the vials and the systems were equilibrated in an orbital shaker at 30°C. The a_w was measured after 24 and 48 h of equilibration. A constant value of $a_w = 0.48 \pm 0.02$ was measured in the vials containing from 10 to 40 μl of water. The a_w was constant also after removing the toluene, washing with 3 ml of dry toluene and equilibrating the system with 1 ml of dry toluene for 24 h.

2.3. Adsorption of penicillin amidase on celite

Batch 1 and 2: 500 mg of celite were washed with 10 ml of ultrapure water and added to 25 mg of PGA from *Escherichia coli* (EC 3.5.1.11, Fluka, 10 U/mg of protein) dissolved in 0.5 ml of 0.1 M phosphate buffer pH 7. The preparation was dried at room temperature for 4 h under reduced pressure. After drying the preparation weighed 650 mg and was stored in dry hexane at 4°C.

Batch 3: the aqueous suspension of celite and penicillin amidase was dried for a longer time (7 h).

*2.4. Synthesis of amide **3***

In a typical reaction, 120 mg of enzyme adsorbed on celite were added to 1 ml of dry toluene into a 5-ml glass vial. The system was equilibrated for 24 h at 30°C in an orbital shaker (250 rpm). The reaction was started by adding tyrosine ethyl ester (**1**) (80 mM) and methyl phenylacetate (**2**) [9] (80 mM) in the medium. Samples were withdrawn, centrifuged and analysed by reverse phase HPLC to monitor the

reaction kinetics. The a_w was measured at the start of the reaction and after complete conversion of the substrates into product. No appreciable variations were observed.

The product **3** was isolated removing the solvent under reduced pressure. Purity ($>98\%$) was checked by HPLC and by ^{1}H-NMR. Amide **3** was characterised by ^{1}H-NMR, ^{13}C-NMR, and HPLC by comparison with a chemically synthesised standard [13].

No product was detected when reactions were carried out in the absence of PGA, using 120 mg of celite treated as described in Section 2.3, in 1 ml solution of (**1**) and (**2**) in dry toluene.

2.5. Recycling of the immobilised penicillin amidase

After the reaction went to completion, the organic phase was withdrawn and the immobilised enzyme was washed with dry toluene (1 ml three times). Finally, 1 ml of dry toluene was added along with the substrates for a new cycle of reaction.

3. Results

We verified experimentally that a_w can be maintained close to a value of 0.48 at 30°C using celite rods hydrated with an amount of water varying from 10 to 40% of the celite weight. On the basis of this observation, PGA was adsorbed on hydrated celite obtaining a biocatalytic system able to keep $a_w \cong 0.48$ (Table 1, batch 1 and 2). The synthesis of amide **3** was accomplished in 54 h employing equimolar amount of **1** and **2**, and in 30 h using a slight excess of **2** (25%).

Comparable reaction rates were measured when the immobilised PGA was reused for two further synthetic cycles, a_w being still close to 0.48. Since water is poorly soluble in toluene (0.046% w/w, 20°C) the washings after each synthetic cycle do not cause any appreciable variation of the hydration of the immobilised catalyst and in the celite itself, as verified experimentally.

Table 1
Initial rate for the synthesis of **3** using different preparations of PGA adsorbed on celite[a]

PGA on celite	Initial rate (mM/h)	a_w
Batch 1	23	0.49
Batch 1 recycled 1 time	22	0.48
Batch 1 recycled 2 times	22	0.48
Batch 2	27	0.49
Batch 3	no appreciable reaction	0.27

[a] Reaction conditions: [**1**] = 80 mM; [**2**] = 80 mM; toluene; T = 30°C.

When the immobilised enzyme was employed at $a_w = 0.27$ (Table 1, batch 3), no product was observed after 2 days and this confirms the strong influence of a_w on PGA activity. It is noteworthy that the enzymatic activity was restored when a_w was adjusted to 0.48 by rehydrating the support.

4. Discussion

Biocatalysts are commonly used in nonpolar media with support particles which increase the interfacial area [14]. Improved mass transfer, dispersibility and activity are generally the consequences. In this work the support was chosen on the basis of its capacity of absorbing water. The advantage coming from immobilising PGA on highly-hydrated celite rods is double. While enlarging the interfacial area, the support also provides the enzyme with the water necessary for maintaining its catalytic activity, and allows to accomplish the biocatalysed syntheses at constant a_w.

Water activity indicates how water is partitioned in the system between enzyme, solvent and chemical reagents [15]. As a consequence a_w should be controlled in order to maintain the enzyme hydrated and active. At the same time, a_w determines the shifting of the thermodynamic equilibrium of the reaction towards synthesis.

A more detailed description of the celite properties as water activity buffer, as well as on the optimum water activity for penicillin amidase activity will be the subject of a forthcoming work.

Acknowledgements

Financial supports from M.U.R.S.T. (40% and 60%) (Roma) and from C.N.R. (Roma) to P.L. are acknowledged.

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