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Activity of covalently immobilised PGA in water miscible solvents at controlled a_w

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Abstract

Covalently immobilised penicillin G acylase is active in both apolar and water miscible solvents. Hydrated Celite R-640 added to water miscible solvents prevents the medium from stripping the water off the enzyme. The porous siliceous matrix has been used also for the dehydration and storage of the enzyme in apolar media.

Keywords: Water activity; Immobilised PGA; Water miscible solvents; Amide synthesis

1. Introduction

It has been largely demonstrated that the activity of enzymes varies dramatically as a consequence of different hydration and dehydration histories [1–3]. The hydration of immobilised enzymes employed in organic solvent is also critical, since the distribution of water molecules among the enzyme, the support and the medium must be evaluated by measuring and controlling the water activity of the reaction system [4].

In this work, a porous silica matrix, Celite R-640 [5], has been used to control the hydration of covalently immobilised penicillin G acylase (PGA-450) during (a) the preventive dehydration of the enzyme employed in organic solvent; (b) the storage of the enzyme at controlled a_w, (c) the use of the biocatalyst in water miscible solvents thus preventing the medium from stripping water off the enzyme [6].

2. Experimental

2.1. Materials and methods

PGA-450 (62.3% w/w of water content) is a generous gift of Boehringer Mannheim (Germany). It consists of PGA covalently immobilised on a polymer, the chemical structure of which is not disclosed by the supplier. One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1μmol of benzylpenicillin in 1 min at pH 8.0 at 37°C.

PGA immobilised on Eupergit (Eupergit-PcA®) is from Fluka.

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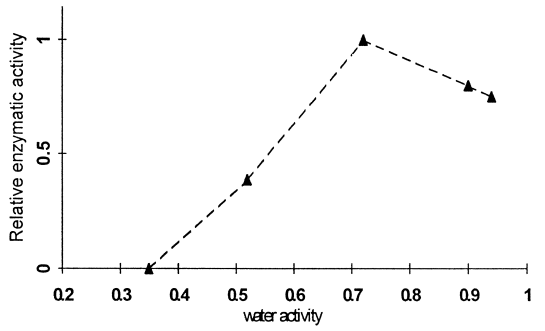


Fig. 1. Effect of a_w on the catalytic activity of immobilised PGA in toluene in the presence of hydrated Celite rods.

All solvents were dried over molecular sieves (4 Å). Ultrapure water was used in all experiments. Celite rods R-640 are from Fluka.

Water activity and relative humidity are measured using a hygrometer (Novasina MS1) equipped with a humidity–temperature sensor (enCR-3). The sensor is calibrated at 25°C at five different a_w values (0.12, 0.33, 0.52, 0.75, 0.90) using standard salt solutions. Measurements are carried out by sealing the sensor into the open end of 5-ml glass vials, thermostatted, until constant reading.

Samples were equilibrated in an air-bath type thermostatted orbital shaker (DARAI-Trieste, Italy).

Water content was measured by means of a Karl Fischer titrator (Mettler).

2.2. Adsorption isotherm of PGA-450 in toluene

The adsorption isotherm is obtained by mixing 1 ml of dry toluene, 62.5 mg of PGA-450 and water into 5-ml vials with screw caps and Teflon-lined septa. The system is equilibrated for 24 h in the thermostatted orbital shaker and water activity was measured by sealing the humidity sensor into the open end of the thermostatted vials, until a constant reading was obtained.

2.3. Dehydration and storage of immobilised PGA by means of dry celite rods

One hundred millilitres of dry solvent (*n*-hexane or petroleum ether) were added to 2 g of catalyst and 4 g of dry Celite. The components were gently mixed together and kept at 4°C in a carefully capped flask. After 7/14 days, the Celite was removed and the dehydrated enzyme was stored at 4°C.

2.4. Control of hydration in water miscible solvents

One millilitre of solvent was equilibrated for 24 h with 95 mg (three rods) of Celite R-640 hydrated with 30 μl of water. After measuring the water activity, the reaction was started by adding the reactants and maintaining the Celite rods in the vessel.

2.5. Enzymatic synthesis

Acylation was carried out using 62.5 mg of pretreated PGA-450 in 1 ml of solvent into a 5-ml glass vial. Water activity was measured after 24 h of equilibration and at the end of the reaction and no appreciable variation was observed. The reaction, as described in Ref. [5], was started by the addition of 80 mM of L-tyrosine ethyl ester and 100 mM of methyl phenylacetate. Reactions were monitored by RP-HPLC.

3. Results and discussion

PGA-450 is a covalently immobilised form of penicillin G acylase (PGA) which is available as hydrated beads (62.3% of water content) which ag-

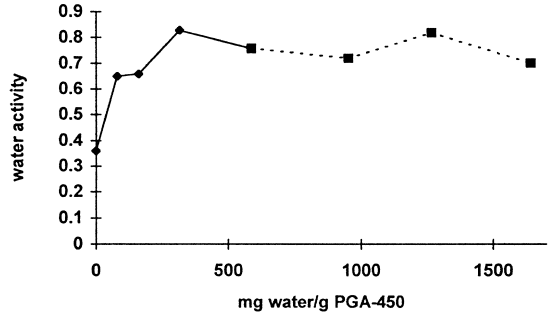
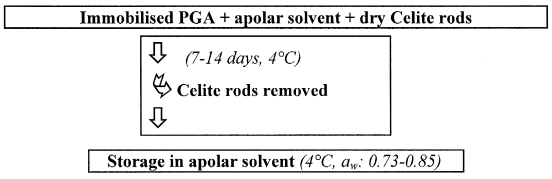


Fig. 2. Adsorption isotherm of PGA-450 in toluene at 30°C after 24 h of equilibration. The enzyme was previously dehydrated by rinsing with 2-propanol (residual water = 14.3% w/w).



Scheme 1. Dehydration of immobilised PGA by means of dry Celite rods.

gregate in organic solvent leading to water activities above 0.95 when suspended in toluene.

The excessive hydration is a frequent limit in the use of immobilised enzymes in organic media. However, some enzymes, such as PGA, are active in organic media only when hydrated [7]. The variation of catalytic activity vs. water activity is shown in Fig. 1.

Fig. 2 reports the adsorption isotherm in toluene of PGA-450 previously dehydrated by rinsing with 2-propanol [8]. The support is highly hygroscopic and in toluene can adsorb up to 550 mg of water per gram but larger amounts of water induce the formation of clusters.

In order to make the immobilised catalyst suitable for applications in organic solvent and with a reproducible level of hydration, it has been dehydrated by means of dry Celite R-640 rods in *n*-hexane or petroleum ether. Celite R-640 adsorbs large amounts of water (more than 90% of its weight) and maintains in organic apolar media the a_w constant within wide-defined ranges of water concentrations [5].

Scheme 1 illustrates the easy procedure for the dehydration of PGA-450 by means of dry Celite rods in *n*-hexane or petroleum ether. The enzyme and the rods are gently mixed together so that the excess water is adsorbed by the Celite which is removed after 7 days. After partial dehydration, the biocatalyst results as an easily handled powder, which can be stored in the same apolar solvent and samples are taken and weighted when required. The batches can be prepared on gram scale and there is no decrement in activity for at least 4 months. As required, enzymatic samples can be withdrawn and the volatile organic solvent used for the storage can be removed from the enzymatic samples at room temperature and atmospheric pressure without causing any detrimental effect to the catalyst.

The storage in apolar solvent prevents microbial contamination and loss of moisture. The a_w variates from 0.73 to 0.85 according to the dehydration time, namely, the time the Celite has been kept in contact with the enzyme. Values of water activity above 0.8 indicate that 7 days were not sufficient to reach the equilibrium between the Celite, the enzyme and the apolar medium. This is due to the large amount of water that has to be stripped from the polymeric support and adsorbed on the Celite, a process that is particularly slow in apolar media having very low water solubility.

After being dehydrated with Celite in apolar medium, PGA-450 gives a_w values (suspended in toluene, 24 h of equilibration) that are perfectly compatible with the enzyme activity in toluene (see Fig. 1).

Table 1
Activity of PGA-450 in water and in toluene after different dehydration treatments

Dehydration method	Time of storage	a_w[a]	Enzymatic activity in water (U/g of dry catalyst)	Activity in toluene: v_0[b] (μmol h^{-1} U^{-1})
Celite/petroleum ether (14 days)	–	0.73	401	8.2
Celite/petroleum ether (14 days)	1 month	0.73	401	7.6
Celite/petroleum ether (14 days)	4 months	0.73	401	8.7
Celite/*n*-hexane (7 days)	4 months	0.85	387	7.7
Celite/toluene (24 h)	–	0.84	490	10.7
Rinsing with 2-propanol	24 h (air)	0.35	10.2	No reaction
Vacuum	–	0.32	137	No reaction

[a] Measured in toluene after equilibrating the enzyme for 24 h.

[b] Initial rates of acylation of L-TyrOEt with methyl phenylacetate in toluene catalysed by PGA-450 dehydrated by means of Celite rods. Units calculated per dry gram of immobilised enzyme.

Table 2
Activity of PGA-450 in apolar solvents: acylation of L-TyrOEt with methyl phenylacetate

Apolar solvents			
Solvent	Water solubility[a]	a_w (30°C)	Conversion (%)
Toluene	0.046	0.73	99.2% (3 h)
Methylene chloride	0.42	0.89	90.6% (24 h)

[a]% (w/w) of water dissolving in a given solvent at 20°C [10].

Data in Table 1 compare the activity in water of the immobilised enzyme after different dehydration methods. Water evaporation under vacuum (8.3% w/w residual water) causes a considerable, irreversible loss of catalytic power. PGA-450 rinsed with 2-propanol (14.3% w/w residual water) is active in water only in the first hour after the treatment, whereas after 24 h the catalytic activity is almost completely lost. Both enzymatic preparations are inactive in toluene because of their low degree of hydration.

PGA-450 dehydrated with Celite rods in apolar solvents is very active also in toluene and it catalyses the complete acylation of L-tyrosine ethyl ester with methyl phenylacetate in 3 h. This is due to the fact that PGA-450 retains some water (about 300 mg/g) which is sufficient to maintain the PGA active in apolar solvents such as toluene (water solubility in toluene = 0.046% w/w).

Table 1 reports also the activity of PGA-450 after a fast dehydration treatment of 24 h in toluene and initial rates indicate that there are no considerable variations of activity due to the prolonged storage in petroleum ether or *n*-hexane.

It must be noted also that PGA immobilised on Eupergit (Eupergit-PcA®) can be dehydrated and stored following the same procedure and, in toluene, it gives an initial rate of 5.0 μmol h^{-1} U^{-1}, which is comparable to the activity previously demonstrated by this enzyme [9].

Tables 2 and 3 show how the catalytic activity of PGA-450 decreases moving from toluene to more polar solvents and almost disappears in water miscible solvents, which dissolve the water bound on the immobilised enzyme.

The enzyme becomes considerably less efficient moving to *tert*-amyl alcohol and acetonitrile, whereas it is not active at all in tetrahydrofurane and pyridine.

The very poor activity of the enzyme can be partially explained by the low a_w values measured in these solvents.

The a_w can be increased and adjusted at a value close to 0.7 by equilibrating the solvent with hydrated Celite rods. The increase of the a_w has a considerable positive effect on the reactions performed in *tert*-amyl alcohol and acetonitrile and the presence of the hydrated rods in the reaction vessel

Table 3
Activity of PGA-450 in water-miscible solvents: acylation of L-TyrOEt with methyl phenylacetate

Water miscible solvents				Water miscibile solvents + hydrated celite rods		
Solvent	Water solubility	a_w^a (30°C)	Conversion (%, 24 h)	a_w^b (30°C)	Conversion (%, 24 h)	Final conversion (%)
tert-Amyl alcohol	10.0[c]	0.48	4.7%	0.68	58.4%	93.0
Acetonitrile	miscible	0.57	7.8%	0.67	36.4%	88.2
Tetrahydrofurane	miscible	0.43	No reaction	0.67	1.2%	27.2
Pyridine	miscible	0.28	No reaction	0.34	No reaction	–
				0.53[d]	No reaction	–

[a]Water activity was measured after equilibrating 1ml of solvent for 24 h with 62.5mg of PGA-450.
[b]Water activity was measured after equilibrating 1ml of solvent for 24 h with 95 mg (three rods) of Celite R-640 hydrated with 30 μl of water.
[c]% (w/w) of water dissolving in *tert*-amyl alcohol at 20°C.
[d]Celite rods hydrated with 100 μl of water.

prevents the dehydration of the enzyme by controlling the partition of the water between the medium and the catalyst. However, there are effects on the enzyme activity, which are intrinsic to the solvents, which cannot be avoided. Consequently, tetrahydrofurane is a very poor medium and pyridine seems to be totally non-compatible with PGA activity, regardless of the a_w value. In the case of pyridine, the Celite rods are not able to control the hydration of the reaction medium. This could be ascribable to the ability of pyridine to strip and solubilise also the water adsorbed onto the rods.

4. Conclusions

The hydration of covalently immobilised PGA can be controlled in organic media by using a porous siliceous matrix, Celite R-640. The method is applicable to the preparation of biocatalysts having optimum hydration for biocatalysis in organic media. Finally, hydrated Celite R-640 rods are effective in preventing water stripping in water miscible solvents.

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