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Penicillin G amidase in low-water media: immobilisation and control of water activity by means of celite rods

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Abstract

Penicillin G amidase (PGA) adsorbed on Celite rods (Celite R-640 from Fluka) catalyses, in toluene, the synthesis of amide bonds with yields > 98% using equimolar concentrations of reactants. The method allows the easy recovery of the product and the recycling of the catalyst. Experimental data have pointed out that Celite rods adsorb water in a unusual but useful way, maintaining the water activity of the reaction system constant within defined ranges of water concentrations. Adsorption isotherms of Celite rods are reported and further applications of the method are proposed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Celite R 640; Control of water activity; Organic solvent; Penicillin amidase; Synthesis of amide bond

1. Introduction

The crucial role of enzyme hydration in affecting the activity of native and immobilised enzymes is largely documented [1–10]. It has been demonstrated that enzymes differ in their behaviour in low water media, and some of them show no catalytic power when poorly hydrated.

One case is represented by penicillin G amidase (PGA; EC 3.5.1.11) which was reported to exhibit no or very low activity in pure apolar solvents [11]. In a previous work we have demonstrated that PGA is active in low-water media only when the water activity (a_w) is maintained sufficiently high and that in situ hydrated phosphate salts are effective in controlling a_w, maintaining the catalyst active in benzene [12].

The development of alternative methods for the use of PGA in low-water media could favour the application of PGA to the synthesis of β-lactam antibiotics [11,13–15], the resolution of amines and alcohols [16–19] and the protection of amino groups [20,21]. In fact, major limits for the use of PGA in synthetic reactions in aqueous media are the competition of hydrolytic pathways [14] and the poor solubility of hydrophobic substrates in water [16,22].

Immobilised enzymes offer several advantages over native lyophilised proteins when used in organic solvents, since diffusion limits are reduced significantly and enzyme molecules are more efficiently dispersed on a higher surface [23]. Celite powder is a common support for enzyme adsorption and many examples are re-

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ported in the literature of improved enzyme performances by means of adsorption of the catalyst onto fine Celite powder [23–25]. Adlercreutz has demonstrated that since Celite powder (30–80 mesh) adsorbs only minimum amounts of water it acts as a suitable support for chymotrypsin whereas other supports having a high water-binding capacity can compete for the available water, causing a decrease of enzymatic activity [25]. However, Halling has reported that when experiments are carried out at fixed a_w, instead of fixed water content, all components are able to take up water independently, in accord with their individual adsorption isotherm, and water competition effects do not influence the activity/a_w profiles [26].

Recently, we have reported the adsorption of PGA on hydrated Celite R-640 (rods) [27]. This type of porous Celite differentiates from Celite powder in its capacity of adsorbing water (more than 90% by Celite weight). Furthermore, we have observed that, unexpectedly, Celite rods adsorb and release water in such a way that water activity is maintained constant in the reaction system within defined ranges of water concentrations.

In this work the hydration of Celite R-640 is investigated and some novel applications of this support are described.

2. Experimental

2.1. Materials and methods

PGA from *Escherichia coli* (EC 3.5.1.11) was purchased from Fluka, as phosphate buffer solution (0.1 M, pH 7.5) having an activity, assayed in water, of 20 U mg^{-1} of protein. The enzyme was stored at 4°C as lyophilised powder having a residual activity of 5.88 U mg^{-1} of protein.

Celite rods R-640 were purchased from Fluka, which reports the following technical information for this product: mean pore diameter: 200 nm; pore volume 0.5 cm^3 g^{-1}; surface area 65 m^2 g^{-1}.

We determined the porosity independently by using a AutoPore III 9420 System, Micrometrics Instrum., Norcross, GA, USA., obtaining a cumulative pore volume of 0.8 cm^3 g^{-1}. Pore size distribution indicated that 55% of pore volume is given by pores having a diameter between 20 and 60 nm and a further 5% corresponds to pores with smaller diameter. The residual 40% of the volume is given by pores with a diameter between 70 and 10 000 nm.

Celite rods were stored over P_2O_5. The immobilisation of PGA and all adsorption isotherms were performed using rods with uniform weight (32 ± 2 mg each).

All enzymatic reactions and measurements of adsorption isotherms were carried out using toluene dried over molecular sieves (4 Å).

Tyrosine ethyl ester was purchased from Sigma and methyl phenylacetate was synthesised according to Ref. [18].

Enzymatic activity of the native and the immobilised PGA was assayed in water by automated titration of the phenylacetic acid formed in the hydrolysis of benzylpenicillin (Aldrich). One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1 μmol of benzylpenicillin in 1 min at pH 7.6 at 37°C.

Water activity was measured with a NOVASINA MS1 hygrometer equipped with a humidity–temperature sensor enCR-3 which was sealed into the open end of the thermostatted vials, until constant reading. The sensor was calibrated at 25°C at 5 different a_w values (0.12; 0.33; 0.52; 0.75; 0.90) using standard salt solutions.

2.2. Adsorption of PGA on Celite R-640

Adsorption on hydrated Celite: 500 mg of Celite rods were washed with 10 ml of ultrapure water and added to 25 mg of PGA (lyophilised powder) dissolved in 0.5 ml of 0.1 M phosphate buffer pH 7. In case of batch 2 the enzyme was dissolved in the same volume of ultrapure water.

The enzyme and the support were partially dried at room temperature for 4 h under reduced pressure. Batch 3 was dried for 7 h. The BCA protein assay (Pierce Europe, NL) was employed for measuring the residual protein not adsorbed on the support and then to calculate the adsorption yield for each batch (84–87%).

After drying, all preparations were weighed and stored in dry hexane at 4°C.

Adsorption on dry Celite (batch 4 and 5): PGA, 25 and 10 mg, respectively, were dissolved in 400 μl of ultrapure water and added to 500 mg of dry Celite R-640. The two preparations were kept in 25 ml flasks, carefully capped, for 24 h at 25°C until the enzymatic solution was completely adsorbed (adsorption yields: 94 and 96%).

2.3. Adsorption isotherms

2.3.1. Celite R-640 (rods)

One milliliter of dry toluene was added into 5 ml vials with screw caps and teflon-lined septa containing 3 Celite rods (95 mg). Different amounts of ultrapure water (0–150 mg) were added into the vials and the samples were incubated in a thermostatted orbital shaker for 24 or 48 h before measuring the a_w.

2.3.2. Celite R-640 (powder)

Different amounts of ultrapure water (0–40 mg) were weighed in 5 ml vials. Dry toluene (1 ml) was added together with 500 mg of Celite R-640, previously ground (> 200 mesh, determined by means of a Multisizer Accu Comp Coulter Counter). The a_w was measured at 28°C after 24 h of incubation.

2.3.3. Lyophilised PGA

100 mg of PGA (lyophilised powder previously stored over P_2O_5) were added into 5 ml vials containing toluene (1 ml) and different amounts of ultrapure water (0–30 mg). The a_w was measured at 28°C after 24 h of incubation.

2.4. Enzymatic synthesis of amide **3**

In a typical reaction PGA adsorbed on Celite was 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 the addition of tyrosine ethyl ester (**1**) and methyl phenylacetate (**2**). The a_w was measured at the starting of the reaction and after complete conversion of the substrates into the product. No product was observed in the absence of the catalyst.

Initial rates were determined by following the first 10% of conversion of the reaction. All initial rates were normalised on the basis of the enzymatic units employed in the reactions, which were determined in water for each enzymatic preparation. Samples were withdrawn and analysed by HPLC (Pharmacia) to monitor the reaction kinetics. The reagents and the product were eluted isocratically using a reverse phase C18 Chrompack column, with a flow of 1 ml min^{-1}. Eluents: acetonitrile/water = 70:30 and 0.025% of trifluoroacetic acid. Components were detected using a UV-visible spectrophotometer at a $\lambda_{max} = 275$ nm.

When equimolar concentrations of **1** and **2** were used the product **3** was isolated by removing the organic phase and evaporating the solvent under reduced pressure. Purity (> 98%) was checked by HPLC and by ^{1}H-NMR

Amide **3** was characterised by NMR and HPLC by comparison with a chemically synthesised standard [12].

^{1}H-NMR($CDCl_3$), δ(ppm): 1.23 (t, $J = 7.1$ Hz, 3 H, $-OCH_2C\underline{H}_3$), 2.90 (dd, $J = 5.9$ Hz, $J = 13.9$ Hz, $-C\underline{H}H-CH-$), 3.00 (dd, $J = 5.7$ Hz, $J = 14.0$ Hz, $-CH\underline{H}-CH-$), 3.55 (s, 2 H, $Ph-CH_2-CO-$), 4.14 (q, $J = 7.0$ Hz, 2 H, $-OC\underline{H}_2CH_3$), 4.81 (m, $-CH_2C\underline{H}-$), 5.68 (s, 1 H, $-OH$), 5.85 (d, $J = 7.8$ Hz, 1 H, $-NH-$), 6.61 (d, $J = 4.2$ Hz, 2H, H-Tyr), 6.75 (d, $J = 4.2$ Hz, 2H, H-Tyr), 7.16–7.20 (m, 2 H, Ph), 7.28–7.36 (m, 3 H, Ph).

^{13}C-NMR($CDCl_3$), δ(ppm): 14.08 ($-OCH_2$-$\underline{C}H_3$), 36.91 ($-\underline{C}H_2CH-$), 43.47 ($Ph-\underline{C}H_2-$

CO), 53.31 (–$CH_2\underline{C}H$–), 61.69 (–$O\underline{C}H_2CH_3$), 115.56 (C_3-Tyr), 126.50 (C_4-Ph), 127.50 (C_2-Tyr), 129.04 (C_2-Ph), 129.42 (C_3-Ph), 130.17 (C_1-Tyr), 134.05 (C_1-Ph), 155.61 (C_4-Tyr), 171.36 (C=O), 171.60 (C=O).

3. Results and discussion

3.1. Adsorption isotherms of Celite R-640 (rods)

Recently we have reported that reactions catalysed by PGA can be carried out at constant a_w by using the enzyme adsorbed on Celite R-640 [27]. This type of chemical inert matrix consists of diatomaceous earth calcined to create porous particles with controlled pore sizes [28].

Here we report further information on the hydration of Celite rods, gained from the study of the corresponding adsorption isotherms. Fig. 1 illustrates how a_w varies as different volumes of water are added to Celite rods in dry toluene. The samples were equilibrated for 24 or 48 h at two constant temperatures (23 and 28°C). In the absence of water the a_w of the system was 0.12. The pattern of the adsorption isotherms not only confirms that Celite R-640 can adsorb large amounts of water but also demonstrates that it is able to keep water activity constant within defined ranges of water concentrations.

After 24 h of equilibration at 23°C a_w is 0.48 ± 0.02 within a range of 100–400 mg of water per gram of Celite. At water concentrations between 700 and 1400 mg g^{-1} a second constant section ($a_w = 0.82 \pm 0.02$) can be observed in the plot while, between these two constant intervals, the system undergoes a steep increase of a_w upon minor variations of the water concentration. A longer time of equilibration (48 h) translates into a decrement of a_w values of the second constant section of the curve (0.75 ± 0.02).

At 28°C and after 24 h of equilibration the profile shows again a constant section at values of water activity close to 0.5 ($a_w = 0.51 \pm 0.01$) corresponding to a range of water content of 100–400 mg of water per gram of Celite. However, at this temperature the Celite shows a poorer ability for buffering the a_w at higher water concentrations. After 48 h of incubation a decrease of a_w values can be observed at high water concentrations, this confirming that Celite R-640 shows a more pronounced ability of controlling the water activity within a range of 100–400 mg of water added per gram of Celite.

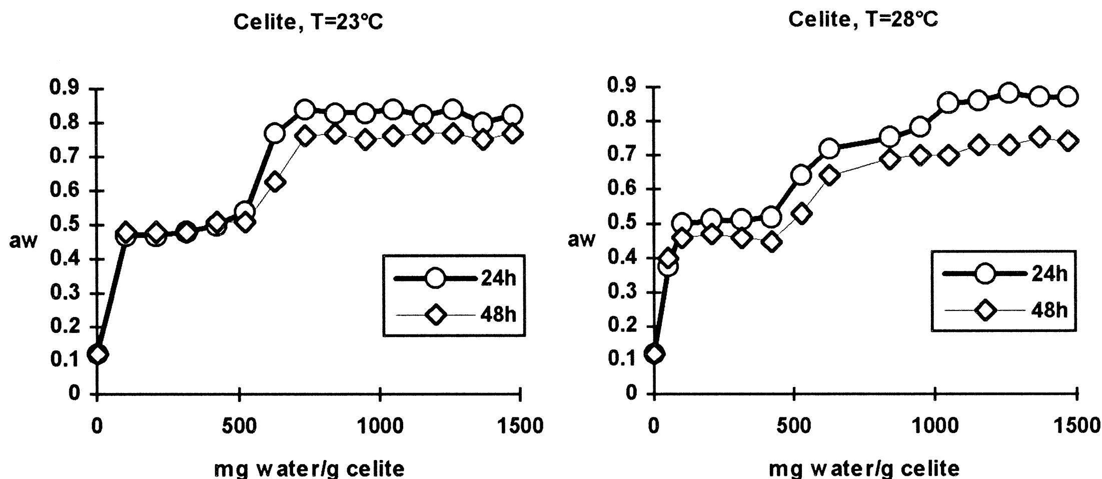


Fig. 1. Adsorption isotherms in toluene of Celite R-640 (rods) at 23 and 28°C after 24 and 48 h of equilibration.

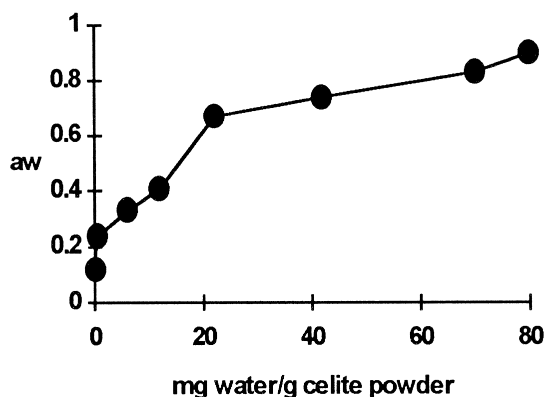


Fig. 2. Adsorption isotherm in toluene of powdered Celite at 28°C after 24 h incubation.

It is known that the existence of pores is the major factor affecting the adsorption and the motion of water molecules in silica based matrixes [29]. Motion of water molecules strongly depends on pores dimensions, since there are two types of water in the pores: one type of water behaves like bulk water, probably present in the central region of the pores, and the other is water perturbed strongly by the silica surface. Therefore, pores of different sizes have different relative amounts of bound and free water [30–32].

Porosimetry studies (see Section 2) showed that Celite R-640 has a cumulative pore volume of 0.8 cm^3 g^{-1}. Moreover, pore size distribution demonstrated that small size pores prevail, 60% of pores having a diameter below 60 nm.

It must be mentioned that previously Adlercreutz [25] had studied the hydration of several solid supports employed for enzyme adsorption and Celite powder (30–80 mesh) resulted to adsorb only minimum amounts of water (2 mg g^{-1} Celite). At least part of the difference in the adsorption capacity of the two matrixes can be ascribed to their different porosity, since the 30–80 mesh Celite has a pore volume more than one order of magnitude smaller (0.06 cm^3 g^{-1}) [33] than Celite R-640.

A further factor which differentiates the Celite R-640 from Celite powder is represented by the surface facing the external phase (air or solvent) and which is available for exchanging water molecules. In fact, Celite rods have a cylindrical shape, 5 mm height and with a diameter of 3 mm, whereas the size of the particles of Celite powder (30–80 mesh) is between 0.17 and 0.59 mm.

Indeed, when Celite rods were ground and reduced to fine powder ($>$ 200 mesh) Celite lost most of its ability of adsorbing water, as indicated by Fig. 2. The graphic reports a water activity as high as 0.83 upon the addition of only 70 mg of water per gram of powder, whereas with Celite rods the same value of water activity was reached by adding 1000 mg of water.

These results suggest that water can penetrate into very inner zones of the Celite R-640 rod, making water exchanges with the external phase very slow. As a result of the grinding, the adsorption properties change dramatically: although most of the pores remain still unbroken, their macroscopic three-dimensional organisation undergoes a partial destruction, so that pores once internal becomes superficial.

Therefore, the capacity of Celite R-640 of adsorbing water is mainly determined by its porosity as well as by the surface which is actually available for exchanges with the external phase.

3.2. Adsorption isotherm of PGA

The adsorption isotherm for the native, lyophilised PGA was also considered. Fig. 3

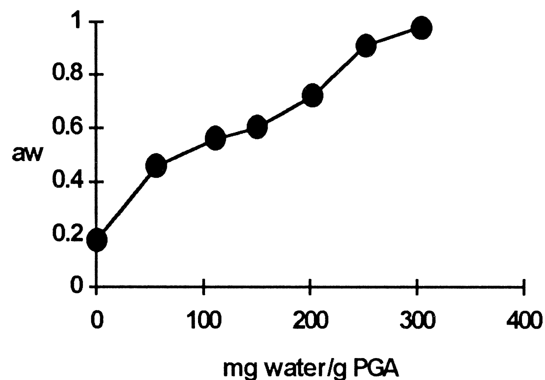


Fig. 3. Adsorption isotherm in toluene of lyophilised PGA at 28°C after 24 h equilibration.

Table 1
Enzymatic preparations of PGA adsorbed on hydrated Celite

Enzymatic preparation	% PGA[a] (w/w)	% H_2O (w/w)	a_w^b	Activity[c] (U per 100 mg PGA/Celite)
Batch 1	3.4	19	0.49	4.5
Batch 2	3.4	19	0.49	4.5
Batch 3	3.7	8.5	0.27	4.9

[a] Determined on the basis of adsorption yields (see Section 2).
[b] Water activity was measured after equilibrating for 24 h the adsorbed enzyme suspended in 1 ml of dry toluene at 30°C.
[c] Assayed in aqueous buffer after adsorption (see Section 2).

indicates that 1 g of lyophilised PGA can adsorb up to 300 mg of water.

Since a standard reaction employs about 5 mg of PGA adsorbed on 95 mg of Celite, by comparison of the corresponding adsorption isotherms it appears that in the PGA/Celite system the contribution of the enzyme in adsorbing water is negligible and we can disregard the presence of the catalyst in evaluating the behaviour of the Celite.

3.3. Adsorption of PGA on hydrated Celite R-640 (rods)

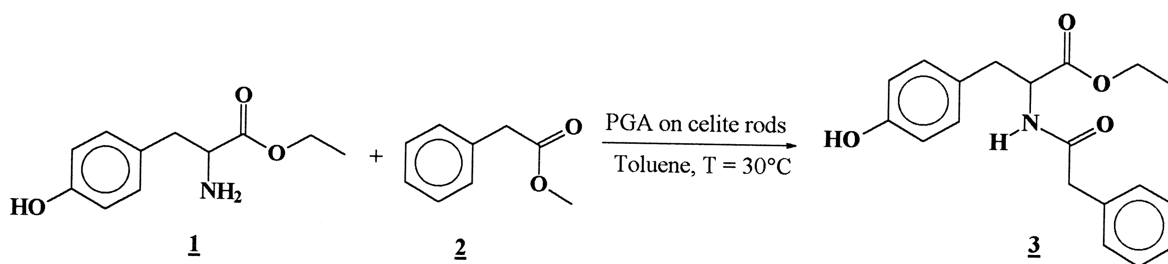
The enzyme was adsorbed on Celite rods previously washed with water. The water in excess was removed under reduced pressure.

Batch 1 was prepared dissolving the enzyme in a phosphate buffer, while ultrapure water was used for batch 2. Both enzymatic preparations had a final water content of 19%. Batch 3 was dried more extensively in order to reach a lower water content (8.5% w/w) and then to evaluate the effect of hydration on the catalyst.

All the preparations were stored in dry hexane, and this procedure showed to be effective in preserving both the hydration and the activity of the catalyst. Table 1 reports the final composition of the three enzymatic batches.

The efficiency of the different enzymatic preparations was evaluated by studying the acylation of tyrosine derivative **1** in toluene, as described in Scheme 1.

Water activity was measured in the reaction vessels after equilibrating for 24 h the adsorbed enzyme suspended in 1 ml of dry toluene. Constant values of a_w were measured before the starting of the reaction and after complete conversion. Hydration and equilibration were carried out into the organic solvent [34,35] and no deactivation phenomena were observed. Experiments 1–3 were performed adding hydrated phosphates into the reaction vessels (0.2 M of $Na_2HPO_4 \cdot 12H_2O$ and $Na_2HPO_4 \cdot 7H_2O$) [36,37]. Results in Table 2 indicate that batch 1 and 2 gave values of water activity close to 0.50, even in the presence of phosphate hydrates. It must be mentioned that at 30°C the couple formed by phosphate dodeca- and hepta hydrates should maintain, in theory, the water activity of the system at a constant value of 0.85. The observed a_w value cannot be explained by assuming the conversion of the phosphate salts into forms with a lower degree of hydration, since the corresponding couples of hydrates (7/2 H_2O; 2/0 H_2O) should buffer the a_w at 0.65 and 0.18, respectively.



Scheme 1.

Table 2
Initial rates for the acylation reaction (Scheme 1) obtained by using different preparations of PGA adsorbed on Celite and, when specified, in the presence of phosphate hydrates

Experiment	PGA/Celite rods[a]	[**1**] (mM)	[**2**] (mM)	Na_2HPO_4 12/7 H_2O (M)	a_w	v_0 (mM h^{-1} U^{-1})[b]
1	batch 1	80	80	0.2	0.50	5.35
2	batch 1 recycled (×1)	80	80	0.2	0.50	4.81
3	batch 1 recycled (×2)	80	80	0.2	0.50	4.43
4	batch 1	80	80	–	0.49	5.18
5	batch 2	80	80	–	0.49	4.97
6	batch 2	80	100	–	0.49	4.43
7	batch 2 recycled (×1)	80	100	–	0.48	4.21
8	batch 2 recycled (×2)	80	80	–	0.48	4.21
9	batch 3	80	80	–	0.27	–
10	batch 3	80	80	0.2	0.48	4.64

[a]All experiments were carried out using 120 mg of PGA/Celite per ml of toluene at 30°C.
[b]Initial rates are normalised on the basis of the enzymatic activity assayed in aqueous buffer (see Table 1).

In the case of batch 3, a lower value of a_w was measured ($a_w = 0.27$; Experiment 9). Water activity could be adjusted at a value of 0.49 by adding phosphate hydrates into the reaction vessel (Experiment 10).

Results reported in Table 2 indicate that batch 1 and 2 are catalytically active. All reactions carried out at a_w close to 0.50 have comparable initial rates, either working in the presence of the hydrated salts or with the immobilised catalyst alone.

Operating at $a_w = 0.48$ the synthesis of amide **3** (yield > 98%) was accomplished in 30 h using 5.4 PGA units, and in the presence of 25% molar excess of **2**. Complete conversion was achieved in 54 h employing equimolar amounts of reactants **1** and **2**. The increase of the concentration of the phenylacetic ester did not translate into any positive influence on the reaction rate. No competing hydrolytic reaction was detected in any reaction.

The pure product was isolated simply by removing the organic phase and evaporating the solvent under reduced pressure, since the complete conversion could be achieved working with equimolar concentrations of the reactants.

It must be mentioned that PGA lyophilised in the presence of Na_2HPO_4 and hydrated in situ to generate the hepta-/di-hydrated couple ($a_w = 0.73$) in benzene gave an initial rate of 1.1 mM h^{-1} (0.17 mM h^{-1} U^{-1}) at 40°C [12]. Therefore, PGA adsorbed on Celite R-640 leads to a reaction rate about 25 times higher and at a lower temperature.

The third enzymatic batch, which has a lower water content (8.5% w/w) corresponding to a water activity of 0.27, gave no detectable reaction after 48 h, this confirming the crucial role of hydration in maintaining PGA active in organic solvent. It is noteworthy that the activity of the enzymatic preparation was fully restored upon addition of phosphate hydrates into the reaction vessel and the acylation reaction proceeded at $a_w = 0.48$, with a rate comparable to the other reactions carried out at equal values of water activity.

At complete conversion the residual activity of the enzyme was tested. The catalyst was recovered simply by removing the organic phase and then washing the PGA/Celite with dry toluene. This procedure did not cause any appreciable variation of the hydration of the immobilised catalyst, as indicated by a_w values measured and reported in Table 2. Kinetic data are in agreement with this observation, since initial rates indicate that the enzyme is suitable to be recycled at least for three synthetic cycles. Only minor decrements of activity were observed in reactions performed in the presence of phosphate hydrates.

3.4. Adsorption of PGA on dry Celite R-640 (rods)

PGA was adsorbed on dry Celite rods by exploiting their hydration properties. A concentrated enzymatic aqueous solution was added to the dry rods, which adsorbed completely the aqueous solution and no further water was removed from the enzymatic preparation.

Two enzymatic preparations were obtained with different enzymatic loading (Table 3). Reactions performed with these highly hydrated immobilised PGA were characterised by a_w of 0.84 and 0.94, respectively. Therefore, reactions were carried out at a_w values at which Celite R-640 does not exert its ability of buffering the water activity of the system.

Initial rates indicate that variations in enzymatic loading translate in no appreciable influence over the observed activity of the enzyme.

The enzyme adsorbed on the dry support resulted to have an activity (determined in water) 40% lower than the PGA adsorbed on the hydrated rods under vacuum, probably because the adsorption onto the hydrated support ensures a more uniform distribution of the enzyme. Moreover, when PGA comes to direct contact with the dry support such strong interactions might establish (between the protein and the silicate based Celite) that the enzyme undergoes partial inactivation [3].

Table 3
Enzymatic preparations of PGA adsorbed on dry Celite rods and initial rates

Enzymatic preparation	% PGA[a] (w/w)	% H_2O (w/w)	a_w^b	Activity in water (U per 100 mg PGA/Celite)	v_0^c (mM h^{-1} U^{-1})
Batch 4	2.7	43	0.84	2.1	4.96
Batch 5	1	44	0.94	0.8	5.26

[a]Determined on the basis of adsorption yields (see Section 2).
[b]Water activity was measured after equilibrating for 24 h the adsorbed enzyme suspended in 1 ml of dry toluene at 30°C.
[c]Experiments were performed at 30°C using 185 mg of batch 4 and 167 mg of batch 5 per ml of toluene.

It must be mentioned that we tried, unsuccessfully, to remove some water from our enzymatic preparations by washing with 2-propanol, a water miscible solvent [38]. However, 185 mg of the batch 4 treated with 2-propanol (1 ml × 6) gave again an a_w value of 0.84 when suspended in toluene and equilibrated for 24 h. This value corresponds to a water content higher than 700 mg g^{-1} Celite (see Fig. 1) and it indicates that the partition of water between the Celite and the 2-propanol is negligible, at least in a time scale of minutes. The effect of 2-propanol was evaluated by assaying the activity in water of the Celite/PGA towards the hydrolysis of benzylpenicillin and the test indicated that there was no decrement of activity after the treatment.

4. Conclusions

Celite R-640 is a solid support suitable for enzyme adsorption and its use makes possible to carry out biocatalysed reactions in organic solvent at constant a_w, working within defined ranges of water concentrations. As a consequence, the addition of hydrated salts and the separate pre-equilibration of the reaction components are unnecessary.

The ability of Celite R-640 to buffer the water activity is mainly related to its porosity and it is not affected by the presence of the adsorbed PGA. Further investigations could define the potential application of Celite rods as an alternative to hydrated salts and their use in processes biocatalysed by native or covalently immobilised enzymes, as well as in bioreactors.

The great ability of Celite rods of adsorbing water does not prejudice enzymatic activity in organic media and this observation confirms that hygroscopic supports can be employed for enzyme immobilisation as long as the water activity of the system is controlled [26]. The remarkable adsorption capacity of Celite R-640 could be exploited in synthetic reactions liberating water to prevent the competition of hydrolytic reactions.

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