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Organically modified xerogels as novel tailor-made supports for covalent immobilisation of enzymes (penicillin G acylase)

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Abstract—A novel application of organically modified silicates for covalent immobilisation of penicillin G acylase is reported. The immobilisation is efficient and the enzymatic preparation shows high specific activity and thermal stability. The technique opens new perspectives for the preparation of innovative tailor-made supports matching specific requirements of enzymatic processes.
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Silicates and their organic derivatives have properties such as relative chemical inertness, constant volume, moderate cost and enormous potential for chemical modifications which make them particularly attractive as supports for the immobilisation of biological recognition molecules both for biosensor development and biocatalytic applications at the industrial level.[1,2]

Because of their high porosity, large surface area and low density the so-called aerogels and xerogels, prepared using the sol-gel processing, have been applied in advanced technologies, in analytical sciences[2] and also for biocatalysis.[3] Although adsorption and entrapment on sol-gel materials are suitable techniques for enzyme immobilisation,[4] they suffer from desorption of the protein (leakage)[5a] and from diffusion limitations,[5b] respectively.

Therefore, the covalent attachment of the catalytic protein on the support is generally preferred for industrial application. The covalent immobilisation of enzymes onto organically modified silicates, obtained with the sol–gel process, has been described for biosensor applications in nanotechnologies.[6]

Here we report the high stability and activity of penicillin G acylase, (PGA) one of most widely employed enzymes in industry, after covalent immobilisation on organically modified xerogels (OMX), thus opening new perspectives for the application of these materials in biocatalysis. OMX are prepared by co-hydrolysis and co-condensation of alkoxydes functionalised with an organic group[7] which, in principle, can be selected and modified to change the chemical and physical properties of the xerogels[8] and to match specific enzyme requirements in terms, for instance, of hydrophobic/hydrophilic character of the support.[9] A further advantage of the sol–gel process is given by the high reproducibility of the batches produced.

In the present study, PGA was immobilised on OMX prepared by using a functional amino derivative, (3-aminopropyl)trimethoxysilane, $(MeO)_3Si(CH_2)_3NH_2$, which was mixed with tetraethoxysilane $Si(OEt)_4$, (TEOS) water and methanol[†] following the procedure of Schubert.[10] After the air drying process that caused the gel to shrink, the physical appearance of the OMX was a heterogeneous mixture of fragments (particle size ranging from 20 μm to 2000 μm) containing up to 5% water. However, by preparing a 3 mm high film of wet gel, it was possible to avoid the shrinking of the matrix upon drying, thus obtaining a 0.5 mm thick surfaces of dry xerogel.

It must be noted that the OMX does not swell nor changes its morphology when incubated either in water

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[†] Preparation of OMX: 13.30 mL TEOS (12.42 g), 1.34 mL (3-aminopropyl)trimethoxysilane (1.38 g), 27.74 mL MeOH and 5.40 mL H_2O. The gel was dried at room temperature till constant weight. The water content of each preparation was determined by drying samples at 100°C.

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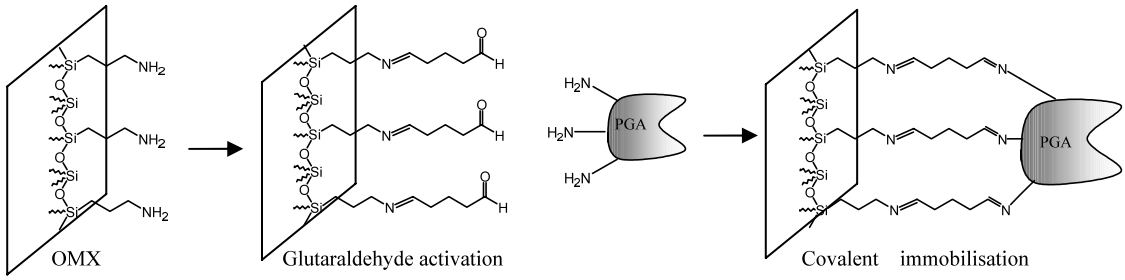

Figure 1. Structure of OMX and process of covalent multipoint immobilisation of PGA.

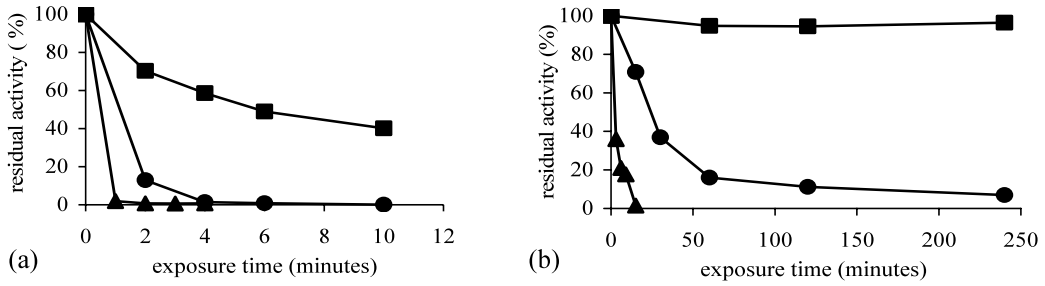

Figure 2. Thermal stability of native PGA (a) and of PGA-OMX (b) at 50°C (squares), 60°C (circles) and 70°C (triangles).

or in organic media. The fragments of OMX had a surface area of 165 m^2/g, that makes OMX particularly suitable for enzyme immobilisation since such a large surface greatly improves both the enzyme dispersion and the mass transfer during reactions. It must be underlined that the surface area of the OMX as obtained after air drying, is comparable to that reported for aerogels (184 m^2/g)[9] obtained starting from the same alkoxydes but dried using costly supercritical fluids.

OMX contained finally 0.3 mmol of amino groups per gram of dry support available for chemical modification (quantified by acid–base titration).[‡] PGA was covalently immobilised on the OMX by pre-activation of the matrix with glutaraldehyde followed by the reaction of the enzyme amino groups with the aldehyde groups.[§] In Figure 1, an outline of the process of covalent immobilisation onto OMX is reported.

The residual protein content on the supernatant after the immobilisation was calculated with the method of Pierce,[11] thus showing that 77% of the initial protein content was covalently immobilised onto the support. The enzymatic preparation PGA-OMX, with a final water content of 62% (w/w), had a specific activity of 110 U/g_{dry}, determined by measuring the hydrolysis of benzylpenicillin.[12]

Table 1. Kinetic data comparing the native enzyme and the covalent immobilised preparation PGA-OMX

Preparation	Activity (U mg protein^{-1})[a]	k_{in} (min^{-1})[b]		
		50°C	60°C	70°C
Native PGA	17.0	0.0876	0.6652	2.4244
PGA-OMX	13.5	0.0001	0.0185	0.2633

[a] The enzymatic activity was assayed by hydrolysis of benzylpenicillin using a previously reported procedure. One unit corresponds to the amount of preparation that hydrolyses 1 μmol of benzylpenicillin per min at 37°C in phosphate buffer, pH 8.0.[12]

[b] Determined by the linear regression (logarithmic transformation) of data reported in Figure 2. Mixtures containing 1 mL of phosphate buffer 0.02 M, about 20 mg of enzymatic preparation (or 20–30 μL of native PGA solution, 1.67 mg/mL) were incubated at 50°C, 60°C or 70°C. The residual activity (NIPAB activity) was measured by adding 1 mL of buffer and 1 mL of NIPAB 15 mM in buffer. After 25 min of mixing (150 rpm, 25°C) samples were filtered and analysed at 405 nm ($\varepsilon_{405}=9090$). One unit of activity was defined as the amount of enzyme required to hydrolyse 1 μmol of NIPAB per min at 25°C.[14]

[‡] A sample of the dry support (5 g) was packaged into a column and equilibrated with 20 mL NaOH 0.5N. The support was then washed with distilled water, until neutral elution. 60 mL of HCl 0.5N were passed through the column and the elute recovered into a beaker. The solution was diluted to 200 mL with water, then 20 mL were titrated with NaOH 0.1N.

[§] 5 g of dry OMX in phosphate buffer (0.1 M, pH 8.0, support/solvent: 1/4) were stirred for 15 min at 25°C, then filtered, rinsed with buffer, 0.02 M, pH 8.0, and a 2% solution of glutaraldehyde in buffer 0.02 M was added. The mixture was stirred for 15 min at 25°C, OMX was filtered and rinsed twice with buffer. A solution of PGA (1500 U in 20 mL 0.02 M Kpi buffer) was added and the immobilisation proceeded for 18 h at 40 rpm in a blood rotator, at rt. The stirring was stopped, OMX-PGA was filtered, and the protein content of the supernatant was checked with Pierce method. OMX-PGA was rinsed with buffer 0.02 M containing NaCl 0.5 M, to desorb no covalently bound protein, and the slurry was kept for 45 min under stirring and filtered. The protein content was checked and no protein was released. Finally, OMX-PGA was washed with buffer and the enzymatic activity was checked (NIPAB and benzylpenicillin activity).

The thermal stability of the PGA-OMX preparation and of the native PGA was studied at 50°C, 60°C and 70°C in phosphate buffer, pH 8.0, (Fig. 2) by assaying 2-nitro-5-(phenylacetylamino)benzoic acid hydrolysis (NIPAB, see footnote b in Table 1).

The kinetic constants of inactivation (k_{in}, Table 1), described by a first order model, were obtained from the logarithmic transformation of the data plotted in Figure 2.[13]

PGA-OMX maintained its initial activity after 4 h at 50°C, whereas native PGA lost almost 40% of its activity after 10 minutes of the same thermal treatment. A decrease of the residual activity of PGA-OMX was observed only at 60 and 70°C.

The high percentage of immobilised protein (77%) together with the high relative activity of PGA-OMX (almost 80% retained activity, see Table 1) demonstrates the effectiveness of the immobilisation technique which combines efficiency and low impact on the enzyme. Our results, in addition, demonstrate that this support is highly compatible for enzyme immobilisation, especially if compared with some examples previously reported of enzyme immobilisations on grafted silicas.[15]

The robustness of PGA-OMX was further confirmed when k_{in} (0.0185 at 60°C) was taken into account and compared with PGA covalently immobilised on widely used commercially available polymers such as Eupergit C ($k_{in}=0.0696$) and Eupergit C 250L ($k_{in}=0.0412$).¶

In conclusion, we report here the application of OMX to the covalent immobilisation of an enzyme applicable to productive biocatalysed processes. The high activity and thermal stability of this novel enzymatic preparation make OMX promising supports for immobilisation of enzymes, also on the light of the moderate cost of these materials. The possibility of synthesising matrixes for enzyme immobilisation with tailor-made properties, in terms of chemical nature and spacer length, is particularly attractive since it would enable to develop supports matching specific requirements of both the biocatalyst (e.g. hydrophobic/hydrophilic nature of the support; molecular size) and the biotransformation (e.g. nature of the reaction media).

We are currently investigating the use of different organically functionalised silicates as novel supports in the perspective of optimising the performance of PGA in different processes of industrial relevance, both in aqueous and in organic media.

¶ PGA was immobilised on the two supports, kindly donated by Röhm, according to the protocols provided by the producer and k_{in} were calculated as previously described.

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