Conformational changes induced by immobilization of a recombinant cutinase on zeolites

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A recombinant cutinase from *Fusarium solani pisi* was immobilized by adsorption/deposition onto several zeolites. These preparations were used to catalyse the alcoholysis of butyl acetate with hexanol in isooctane. Ground state diffuse reflectance measurements were performed for the preparations previously equilibrated with salt solutions at well-defined optimum water activity. The corresponding cutinase fluorescence emission spectra were used to detect different conformational enzyme states, induced by different zeolite properties. Significant conformational changes were observed, with important consequences on the enzymatic activities.

KEY WORDS: zeolites; enzymatic catalysis; cutinase; enzyme immobilization; cutinase fluorescence

1. Introduction

Enzymatic catalysis in non-conventional media, with controlled water content, has been receiving increased attention [1]. In this domain, interesting results have been obtained with the utilisation of zeolites as supports for enzymatic reactions carried out in organic medium [2–5]. Nevertheless, the resulting catalytic properties are strongly dependent on the zeolite properties; these well-known microcrystalline silico-aluminate materials present selective adsorption affinities, acid–base and hydrophobic–hydrophilic character which can be regulated through the particular zeolite structure, its framework chemical composition, nature of the compensating cation and pore network [6].

It is important to characterize the immobilized enzyme in different supports in order to test the hypothesis that different zeolite properties induce different conformational states in the adsorbed enzyme. The three-dimensional structure of the enzyme is essential to its activity; any structural alteration, namely the unfolding or denaturation of the protein, is frequently responsible for the loss of activity. Fluorescence spectroscopy, one of the most sensitive techniques for studying protein conformation, reflecting changes in the surrounding microenvironment of the aromatic residues of the enzyme, should provide some insight in this characterization [7–9].

In this study a recombinant cutinase from *Fusarium solani pisi* was immobilized on several zeolites and some reference supports. The preparations were used to catalyse the alcoholysis of butyl acetate with hexanol in isooctane [3]. The ground state diffuse reflectance measurements were performed for the preparations previously equilibrated with salt solutions at well-defined optimum water activity ($a_w \approx 0.97$), presenting maximum enzyme activity in the mentioned alcoholysis reaction, and the corresponding cutinase emission spectra were carried out by excitation at 275 nm. The molecule of cutinase presents seven significant emissive residues, one tryptophyl and six tyrosyl, with absorption maxima near 275 nm [10]; for the proteins containing these two aromatic residues, both wavelength maxima emission shifts and fluorescence intensity changes reflect conformational alterations [11].

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2. Experimental

The cutinase from *Fusarium solani pisi* was prepared and immobilized by adsorption/deposition onto several zeolites and reference supports according to the procedure described elsewhere [3]. The enzyme is located at the external surface of the zeolite crystallite, since these are microporous materials. The samples were equilibrated with salt solutions at well-defined water activity at 30 °C [12] and used to catalyse the alcoholysis of butyl acetate (500 mM) with hexanol (400 mM) in isooctane at 30 °C [3]. Some blank tests were performed for this reaction system: when no enzyme was present no substrate transformation was detected; when free enzyme was used in isooctane, in the absence of the support, the observed enzyme activity was negligible. The amount of cutinase immobilized on the supports was measured [3] and presents similar values before and after the reaction, so that we can infer that no significant desorption of the enzyme during the reaction process occurs. The observed amounts of enzyme immobilized on the zeolite supports ranged from 12 to 18 mg enzyme/g support, without any noticeable sys-

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tematic variation, while a range from 10.5 to 17 mg enzyme/g support was observed for the other supports.

The ground state diffuse reflectance measurements were performed for the preparations with optimal water activity ($a_w \approx 0.97$), on an Olis 14 UV-VIS-NIR spectrophotometer with a diffuse reflectance attachment based on a 90 mm diameter integrating sphere [13]. The calibration of the apparatus was performed in the following way: a perfect reflector (100% reflection, $R = 1$) was obtained by the use of barium sulfate; as a black standard a finely divided carbon black (Cabot 2000) was used (0% reflectance, $R = 0$). The reflectivity for each sample was obtained by scanning the excitation monochromator from 250 to 600 nm, and the remission function $F(R)$ was obtained by calculating the Kubelka–Munk function for optically thick samples [14]:

$$F(R) = (1 - R)^2/2R.$$

The remission function was obtained for the preparations with the immobilized enzyme and for the support; the relative contribution due to the enzyme absorption was obtained by the expression [14]

$$F(R)_{\text{enzyme}} = F(R)_{\text{support+enzyme}} - F(R)_{\text{support}}.$$

The emission spectra were carried out on a fluorimeter described elsewhere [14], by excitation at 275 nm, which is the wavelength of maximum cutinase absorption. The intensity of fluorescence, I_F, is given by [14]

$$I_F = C\phi_F I_0^{\lambda e}(1 - R^{\lambda e})f,$$

where C is a constant dependent on the apparatus geometry, ϕ_F is the fluorescence quantum yield of the probe on the surface, $I_0^{\lambda e}$ and $R^{\lambda e}$ are the intensity of the exciting light and the reflectance at the excitation wavelength, and f is defined by $f = F(R^{\lambda e})_{\text{cutinase}}/F(R^{\lambda e})_{\text{total}}$. Since the fluorescence emission depends on the fraction of the exciting light absorbed at the excitation wavelength, $I_0^{\lambda e}(1 - R^{\lambda e})$, and on the fraction of the absorption due to the cutinase chromophores groups, f, the emission intensity of each preparation was normalized to the absorption of a given preparation at 275 nm (cutinase immobilized on zeolite NaY):

$$I_F^{\text{normalized}} = I_F^{\text{experimental}} \frac{(1 - R_{\text{NaY}}^{275})f_{\text{NaY}}}{(1 - R^{275})f}.$$

Zeolite A (NaA, with framework composition silicium/aluminium = Si/Al = 1), two Y zeolites (NaY with Si/Al = 2.7 and NaDY with Si/Al = 20), and two ZSM-5 zeolites (NaZSM-5 with Si/Al = 19 and 40), in their sodic forms, were used as supports; some of these zeolite samples are commercially available, and the other ones have been prepared by the commonly used ion-exchange processes and thermal treatments; details of the preparations can be found elsewhere [3]. The reference supports were polyamide accurel PA6, silica and alumina.

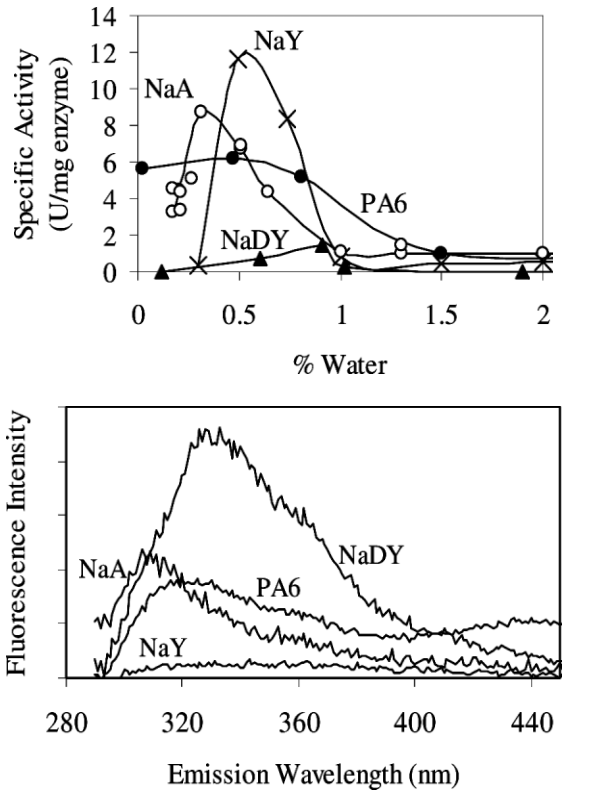

Figure 1. Specific enzymatic activities (U/mg enzyme) as a function of the water level and fluorescence emission spectra of cutinase immobilized onto zeolite NaA, NaY, NaDY, and PA6, prepared with the optimum water amount; U = 1 μmol of product formed per min; the water content (%) is expressed as the ratio between the amount of water in the preparation and the total volume of organic solvent.

3. Results and discussion

The influence of the water content on the obtained initial activity of the immobilized cutinase, for the alcoholysis reaction of butyl acetate with hexanol, is shown in figure 1 [3], and presents the well-known allure for this type of enzymatic transformations performed in organic medium: as the water content increases, the activity goes through a maximum and finally levels-off for higher water contents. For low water contents, the activity increases as the water amount increases, because the enzyme needs a certain amount of water to achieve a hydration level that allow it to function properly [1,15,16]. As more water is made available, the hydrolytic transformation of the ester begins to compete with the alcoholysis. In a previous work the immobilization of cutinase in some zeolites was also shown to be interestingly active for the hydrolysis of tricaprylin [2]. An optimal water level in the preparations for the alcoholysis reaction in the organic medium should then be a compromise between the required hydration of the enzyme and a low hydrolytic activity. From the measurements of the water activity in each preparation, it was shown that, despite the optimal water amount could be different for different supports, the correspondent water activity was always closely the same (0.95–0.97) [3].

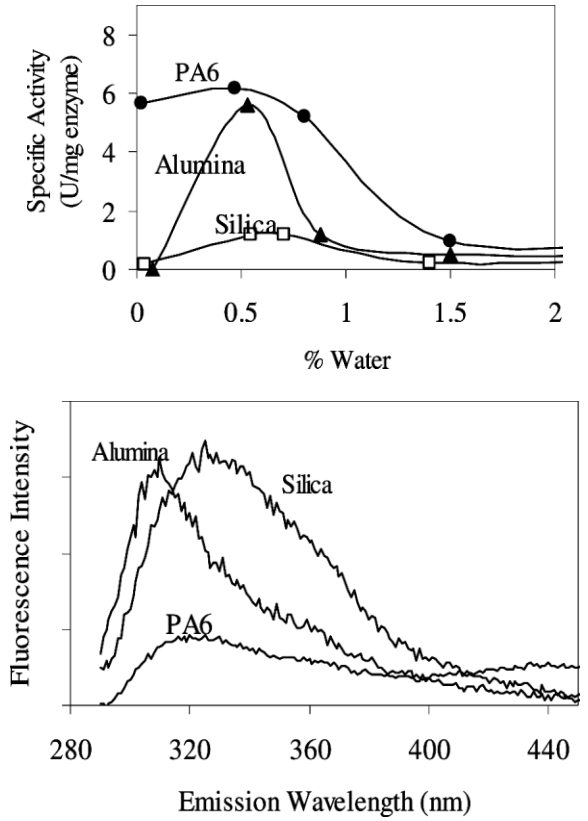

Figure 2. Specific enzymatic activities (U/mg enzyme) as a function of the water level and fluorescence emission spectra of cutinase immobilized onto alumina, silica, and PA6, prepared with the optimum water amount; U = 1 μmol of product formed per min; the water content (%) is expressed as the ratio between the amount of water in the preparation and the total volume of organic solvent.

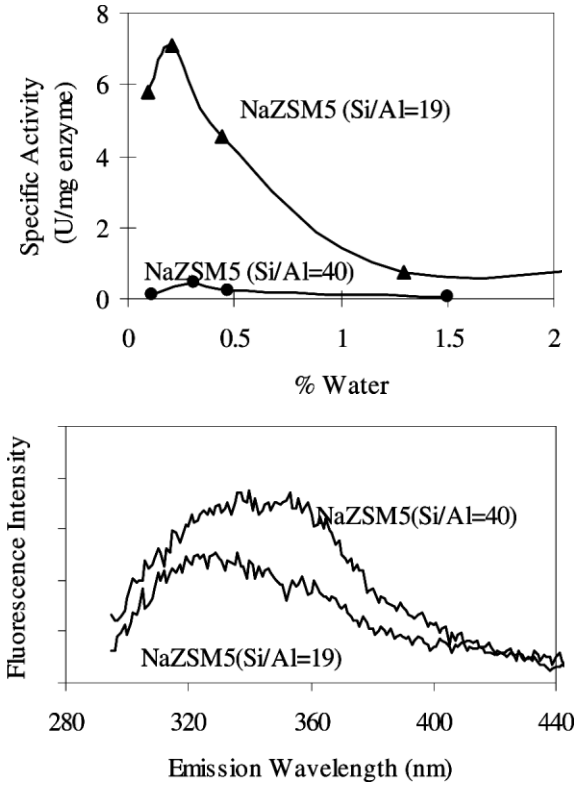

Figure 3. Specific enzymatic activities (U/mg enzyme) as a function of the water level and fluorescence emission spectra of cutinase immobilized onto zeolite NaZSM-5 (Si/Al = 19 and 40), prepared with the optimum water amount; U = 1 μmol of product formed per min; the water content (%) is expressed as the ratio between the amount of water in the preparation and the total volume of organic solvent.

The present preliminary study of the fluorescence emission characterization of cutinase immobilized onto zeolites was performed with the preparations presenting the optimum water activity for the mentioned alcoholysis reaction.

As can be observed in the results presented in figure 1 [3], for the immobilization of cutinase on the zeolites NaA, NaY, and NADY, there is a drastic reduction in the enzymatic activity for the most dealuminated zeolite NaDY. The cutinase emission spectrum presents a maximum at ∼306 nm when adsorbed on zeolite NaA and at ∼333 nm when adsorbed on zeolite NaDY; it was not possible to define an emission maximum for the preparation with the zeolite NaY.

A similar behavior, concerning the association of a drastic activity loss with a shift in the cutinase emission maximum from ∼306 to ∼333 nm, was observed when comparing the immobilization of cutinase on the reference supports alumina and silica (figure 2). In the case of the preparation with PA6, the spectrum presents an intermediate pattern.

A remarkable activity loss associated with an emission maximum shift is also observed when comparing the preparations with zeolite NaZSM-5 containing different aluminium levels: the immobilization in the most dealuminated sample, NaZSM-5 (Si/Al = 40) is almost inactive and presents an emission maximum at a higher wavelength, ∼333 nm (figure 3).

The cutinase emission spectrum in aqueous solution presents a maximum at ∼303 nm, when it is in a native conformation [17]. In this case the emission is mainly due to the tyrosyl residues and the corresponding emission intensity changes reflect eventual slight conformational alterations; the tyrosyl maximum emission wavelength is not very sensitive to changes in the microenvironment [18]. The tryptophyl residues are located inside the globular structure of the enzyme and the corresponding intensity emission is weak and blue-shifted (323 nm) in relation to the emission of free tryptophyl in aqueous solution (351–359 nm), so that its emission contribution to the spectrum pattern of cutinase is not important [17]. A progressive denaturation of the cutinase induces a maximum wavelength emission shift towards higher values, reaching approximately 335 nm when a strong denaturation is achieved [17]; this situation was ascribed to an unfolding of the protein, resulting from its denaturation, which will allow a more superficial localization of tryptophyl residues, in a more polar environment. The emission of the denaturated cutinase is now dominated by the tryptophyl contribution, since the tyrosyl residues were transformed into non-emissive tyrosinate species [17].

The heterogeneous zeolite surface presents multiple adsorption sites: network oxygens with negative charge, compensating cations, silanol groups, framework defects. Possible multipoint interactions between the enzyme molecule

and the zeolite surface can then be effective, with an electrostatic, hydrophobic, or hydrogen bond nature. Different zeolite properties induce changes in the nature and/or intensity of these enzyme–support interactions. The fluorescence emission characterization of the immobilized cutinase performed in the present study clearly shows that quite different enzyme conformations have been induced by the different supports, with important consequences on the enzymatic activities.

The results obtained with the almost inactive preparations (NaDY, silica, and NaZSM-5 (Si/Al = 40)) probably reflect a strongly denaturating interaction between the cutinase and the support, since the corresponding maxima of wavelength emission are close to that of the denaturated enzyme. For the active preparations the observed emission pattern corresponds always to a native (NaA, alumina, NaZSM-5 (Si/Al = 19)) or intermediate (PA6, NaZSM-5 (Si/Al = 19)) denaturation situation; in these cases the different enzymatic activities obtained depend not only on the conformational state but also on other parameters, such as a possible different partition of substrates and products between the organic and the aqueous phase induced by the support properties.

4. Conclusions

The enzyme–support interactions are in fact quite dependent on the support specific properties, inducing different conformational protein states. It seems that an inactive immobilization in the catalytic system studied here is achieved when the cutinase fluorescence emission is predominantly due to the tryptophyl residues contribution, reflecting a drastic conformational change of the cutinase by adsorption in the most dealuminated zeolites (NaDY, NaZSM-5 (Si/Al = 40)) and silica, with a strong denaturation of the protein. Native (NaA, NaY, alumina) or partially denaturated (PA6, NaZSM-5 (Si/Al = 19)) conformational states have been observed for the active preparations; in these cases the activity of a given immobilization depends on the conformational state of the enzyme and on other factors; very interesting enzymatic properties have been obtained with the cutinase immobilization onto zeolites, when compared to the preparations with the reference and commonly used supports.

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