

Stability studies of a recombinant cutinase immobilized to dextran and derivatized silica supports

A. M. Gonçalves,* E. Schacht,** G. Matthijs,** M. R. Aires Barros,†
J. M. S. Cabral,† and M. H. Gil*

*Departamento de Engenharia Química, Universidade de Coimbra, Coimbra, Portugal, †Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Lisboa, Portugal and **Polymer Research Laboratory, University of Gent, Gent, Belgium

Recombinant cutinase from Fusarium solani pisi was covalently attached to dextran and two derivatized silica supports, Biosil-NH₂ and Biosil-Dextran-NH₂. Kinetic parameters were determined for all three systems as well as for soluble cutinase. Long-term stability in aqueous media was studied; dextran may have a stabilizing role not only due to the covalent links involved but also in the same way as other polyhydroxides in aqueous media. Differential scanning calorimetry analysis suggests an enhancement of conformational stability of the immobilized forms. © 1998 Elsevier Science Inc.

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Introduction

One of the major issues in protein immobilization is maintaining or eventually enhancing the structural stability and thermostability of the catalytic macromolecule in view of long-term applications.¹ When enzymes are involved, the biocatalytic behavior may indicate the efficiency of the immobilization procedure and reflect the delicate balance between the acquired conformational stability and the resulting microenvironment created around the biocatalyst.² Immobilization of biocatalysts through covalent multipoint attachment has been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases.^{3–7} Although there is a straightforward relation between the number of enzyme-support covalent links and its stability,^{3,4} the protective effect of covalent immobilization may not be due exclusively to this characteristic and may depend on other conditions of the system, i.e., geometrical congruence between the enzyme surface and that of the support,³ the surface charge of the support,⁸ the nature of the solvent involved, the presence of cosolvents

and/or other compounds,⁷ and perhaps the method of immobilization.^{5,6}

The immobilized enzyme used is a recombinant cutinase from *Fusarium solani pisi*. This biocatalyst presents many interesting applications both in predominantly aqueous or organic media for hydrolytic and esterification or transesterification reactions, respectively, due to the fact that it does not possess the “lid” domain^{9,10} typical to lipases and, hence, does not require a water-oil interfacial activation. In fact, this enzyme has been immobilized on solid supports¹¹ as well as in reversed micelles^{12–14} with interesting results concerning applications to organic media biocatalysis. Considering the stabilizing effects of covalent coupling and polyhydroxyl compounds, the present work serves as a preliminary study toward the selection of a stable covalently attached enzyme system for future aqueous and organic media applications.

Differential scanning calorimetry has proven to be a very useful tool for the analysis of conformational stability of proteins not only in solution¹⁵ but also in more complex systems such as adsorbed proteins,^{16,17} in the presence of added polymers and carbohydrates,^{18,19} and in organic solvents.^{16,20,21} This technique provides useful information on the thermal stability of a protein, and in this work it was used to measure the effects of the immobilization upon cutinase in terms of greater or lesser imposed conformational rigidity.

Address reprint requests to Dr. A. M. Gonçalves, Centro de Engenharia Biol e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1096 Lisboa Cedex, Portugal

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Table 1 Characteristics of immobilized cutinase

Support	Bound protein (mg g ⁻¹ support)	Immobilization yield (%)	Activity retention (%)	Specific activity (U mg ⁻¹ protein)	Activity g ⁻¹ support (U g ⁻¹ support)
Biosil-NH ₂	2.4	6.1	85	1.3	3.1
Biosil-Dextran-NH ₂	2.5	6.2	82	1.6	3.9

Materials and methods

Materials

Recombinant cutinase (MW 22,000) from the fungi *Fusarium solani pisi*, cloned in *Escherichia coli*, was produced and purified at the Centro de Engenharia Biológica e Química (I.S.T., Lisboa, Portugal). Dextran-CHO 40% (MW 70,000), Biosil-NH₂, and Biosil-Dextran-NH₂ were prepared and chemically treated²² at the Polymer Materials Research Laboratory, University of Ghent (Ghent, Belgium). The substrate, *p*-nitrophenyl-palmitate, was purchased from Sigma (St. Louis, MO). Acetonitrile (99.8% purity) was obtained from Merck (Dormstadt, Germany). All other chemicals used were reagent grade.

Methods

Immobilization of cutinase on Biosil-NH₂ and Biosil-Dextran-NH₂ supports. Either support (1 g) was treated with 10 ml of 5% (w/v) glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 7.0 for 5 min. Both supports were thoroughly rinsed with distilled water afterwards. Cutinase solution (10 ml; 4 mg ml⁻¹) in 50 mM Tris-HCl pH 8.0 was added to the activated support. Immobilization was performed at room temperature during 18 h in an orbital shaker (~80 rpm). After this time the material was filtered and rinsed twice with 5 ml of 50 mM Tris-HCl pH 8.0, and finally with distilled water. All supernatants and washing solutions were kept for protein determinations. The supports with immobilized cutinase were snap frozen and lyophilized, and posterosely stored in dry recipients at -20°C. Karl-Fischer titrations were done with a Mettler DL18 Karl Fischer Titrator and the titrating reagent (Hydranal Composite 5) was from Riedel de Haen (Spelze, Germany).

Coupling of dextran-CHO to cutinase. Dextran-CHO (500 mg) were weighed out and dissolved in 10 ml of 50 mM Tris-HCl pH 8.0. To this solution were added 4 mg cutinase. The mixture reacted for 4 h with gentle agitation. Uncoupled cutinase was separated from the mixture by gel filtration chromatography. The separation was performed on a column (85 cm × 1.5 cm) packed with Sephadex G-50 (Pharmacia), elution was done with 50 mM Tris-HCl pH 8.0 at a flow rate of 0.5 ml min⁻¹. Four fractions were collected and the protein content was determined by the Lowry method.²³ The fractions containing enzyme-dextran conjugate were lyophilized.

Table 2 Physical and chemical properties of Biosil-NH₂ and Biosil-Dextran-NH₂³⁶

Support	Biosil-NH ₂	Biosil-Dextran-NH ₂
NH ₂ content (μmol g ⁻¹)	215	32
% water sorption	69.3	56.2
Particle size (μm)	200–1,000	200–1,000
Porosity (%)	59.1	57.0
Pore size (nm)	19.4	19.1
Surface area (m ² g ⁻¹)	85	90

Activity assay. Enzyme activity was determined by the hydrolysis of *p*-nitrophenyl-palmitate (pNPP). Each assay (adapted)^{24,25} consisted of 4.9 ml of 50 mM Tris-HCl pH 8.0 (4.7 ml in the case of soluble cutinase), and a previously weighed amount of immobilized enzyme (or 0.2 ml of enzyme solution). The assay was initiated by the addition of 100 μl of substrate solution (2.65 mM in acetonitrile; final concentration in assay is 53 μM) and performed at 30°C for 5 min. The activity was calculated by measuring spectrophotometrically at 400 nm the release of *p*-nitrophenol (ε_M = 16,630 M⁻¹ cm⁻¹). An activity unit (U) is defined as the quantity of enzyme that catalyzes the formation of 1 μmol of *p*-nitrophenol min⁻¹.

Activity retention. The activity retention was calculated as the ratio between the specific activity of the immobilized catalyst (after determining the amount of protein present) and the difference between the specific activity of the enzyme solution before immobilization and the specific activity of the supernatant solution after immobilization.

Stability studies of soluble cutinase aqueous media. A stock solution of cutinase (20 μg ml⁻¹) in 50 mM Tris-HCl pH 8.0 was stored at 4°C without agitation. An aliquot of 200 μl was withdrawn at predetermined incubation intervals, and enzymatic activity was determined. A cutinase-dextran conjugate stock solution [2 mg ml⁻¹ (0.267 mg cutinase ml⁻¹)] in 50 mM Tris-HCl pH 8.0, was stored in the same conditions as the above. The same volume aliquots were taken at the same intervals for enzymatic activity determinations.

Stability studies of insoluble cutinase in aqueous media. Portions (4–8 mg) of dry support, Biosil-NH₂-cutinase or Biosil-Dextran-NH₂-cutinase, were stored in small sealed vessels to which was added 5 ml of Tris-HCl buffer. Each vessel was stored at 4°C for different periods of time without any kind of agitation. At the end of each incubation period, the buffer solution was filtered and a portion of wet support was used for an enzymatic assay with fresh buffer solution. The dry weight content of each assay was determined and the protein content was determined by a modified Lowry method.²³ The standard error for protein content determination was <5%.

Table 3 Kinetic parameters of immobilized and soluble cutinase

Enzymatic system	K _M (μM)	k _{cat} (min ⁻¹)	k (μM ⁻¹ min ⁻¹) specificity constant
Cutinase	16.9	0.478	0.0282
Cutinase-dextran	20.3	0.0969	0.00478
Biosil-NH ₂ -cutinase	13.1	0.134	0.0102
Biosil-Dextran-NH ₂ -cutinase	19.8	0.142	0.00718

All parameters were calculated by a software program available in Microsoft/Excel 95®

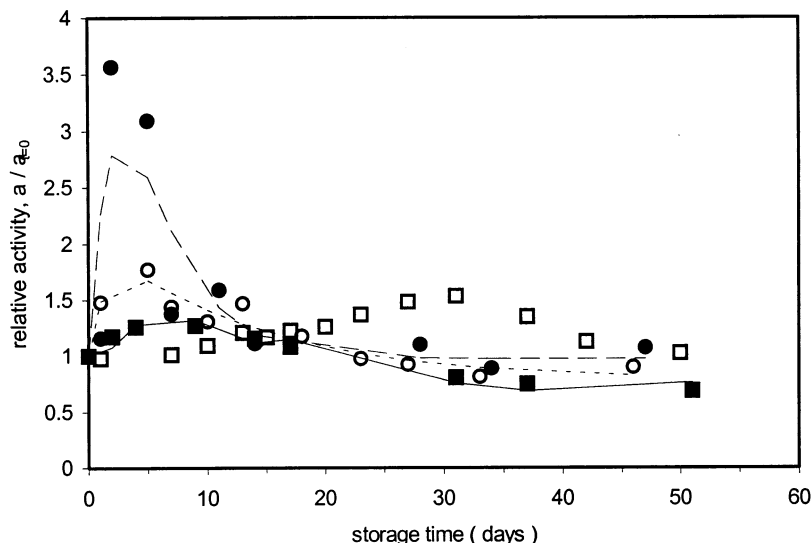


Figure 1 Storage stability of cutinase ($20 \mu\text{g ml}^{-1}$), cutinase-dextran conjugate ($0.267 \text{ mg enzyme ml}^{-1}$), Biosil-NH₂-cutinase ($2.42 \text{ mg enzyme g}^{-1}$ support) and Biosil-Dextran-NH₂-cutinase ($2.49 \text{ mg enzyme g}^{-1}$ support) in $50 \text{ mM Tris-HCl pH } 8.0$ at 4°C . The symbols correspond to experimental values while the lines correspond to values calculated by Sadana's model. For soluble cutinase (■, —), the Sadana model parameters are: $k_1 = 0.115 \text{ day}^{-1}$; $k_2 = 0.115 \text{ day}^{-1}$; $\beta_1 = 1.98$; $\beta_2 = 0.666$. For the cutinase-dextran conjugate (□), the model does not apply to the experimental values; for Biosil-NH₂-cutinase (●, ---), the calculated parameters are $k_1 = 0.342 \text{ day}^{-1}$; $k_2 = 0.342 \text{ day}^{-1}$; $\beta_1 = 6.2$; $\beta_2 = 0.974$. For the Biosil-Dextran-NH₂-cutinase (○, - - -), the calculated parameters are $k_1 = 0.0758 \text{ day}^{-1}$; $k_2 = 0.743 \text{ day}^{-1}$; $\beta_1 = 10.7$; $\beta_2 = 0.783$

Differential scanning calorimetry analysis. Calorimetric analysis was performed in a PL-DSC Polymer Technologies. Conditions of each run were as follows: samples were weighed out into aluminium "crucible" pans ($\sim 5 \text{ mg}$); the heat flux was $10^\circ\text{C min}^{-1}$; the nitrogen flux was 10 ml min^{-1} ; an empty aluminium pan served as reference. The measuring error of the apparatus is 1%. All data were treated with Plus V version 5.20 available with the DSC apparatus.

Results and discussion

In the present study, we compare two types of immobilization: one in which the enzyme is directly linked to the support (derivatized silica) and another in which the same solid support was treated with a layer of dextran and only afterwards attached to the enzyme. This porous support was treated with aminopropyltriethoxysilane in order to acquire the proper reactive amino groups (Biosil-NH₂).²² Coupling to the enzyme free amino groups was done via the bifunctional reagent, glutaraldehyde, as has been extensively described.^{5,6} In the second system, the enzyme was linked to the support treated with a layer of dextran (Biosil-Dextran-NH₂) containing amino groups obtained in a similar activation procedure. The aim is to observe if covalent immobilization to a solid support and the presence of a polyhydroxide (in the second case) promotes stability by inhibiting protein fluctuations leading to unfolding. Dextran is expected to exert the same behavior depicted by stabilizing additives²⁶⁻³⁰ such as polyalcohols [poly(ethylene) glycol, glycerol, sorbitol, lactitol] and carbohydrates (glucose, sucrose, etc.) along with the effects encountered in chemical cross-linking of enzymes.³¹ As a control, we simultaneously studied the free enzyme solution and the enzyme-polysaccharide conjugate. The enzyme-dextran conjugate was prepared initially by treating the polysaccharide with periodate, thus introducing aldehyde groups. This soluble polysaccharide is then placed in the presence of the protein, leading to the formation of Schiff's bases.

Characterization of cutinase immobilization

In both immobilization systems, Biosil-NH₂-cutinase and Biosil-Dextran-NH₂-cutinase, the covalent links involved, Schiff's bases, proved to be stable since no protein was released into solution during the enzymatic assays or even during prolonged incubation in aqueous media. Further stabilization of imide bonds by cyanoborohydride was therefore not necessary as has been stated elsewhere.³² Table 1 shows the characteristics of both immobilization methods. The low immobilization yields are typical of covalent immobilization procedures; however, the activity retention values are high and very similar for both cases. It would be expected to find a much higher value of bound protein for Biosil-NH₂ judging from the number of existing amino groups compared to Biosil-Dextran-NH₂ (Table 2). The similarity in values may be due to the limitation in number of free reacting amino groups on the enzyme surface rather than the maximum protein charge per support surface having been reached.

Kinetic studies

Table 3 shows the apparent kinetic parameters of immobilized and soluble cutinase calculated using ten different concentrations of substrate. In all systems, a Michaelis-Menten kinetic behavior was observed. One may notice that the apparent Michaelis constants, K_M , for Biosil-Dextran-NH₂-cutinase and cutinase-dextran conjugate are higher.

Table 4 Denaturing temperatures (T_M) and denaturing enthalpies ($\Delta_D H$)

Sample	T_M ($^\circ\text{C}$)	$\Delta_D H$ (mcal mg^{-1})
Cutinase	118.8	87.27
Cutinase-dextran	124.8	144.5
Cutinase (Biosil-NH ₂)	128.2	143.7
Cutinase (Biosil-Dextran-NH ₂)	127.9	154.2

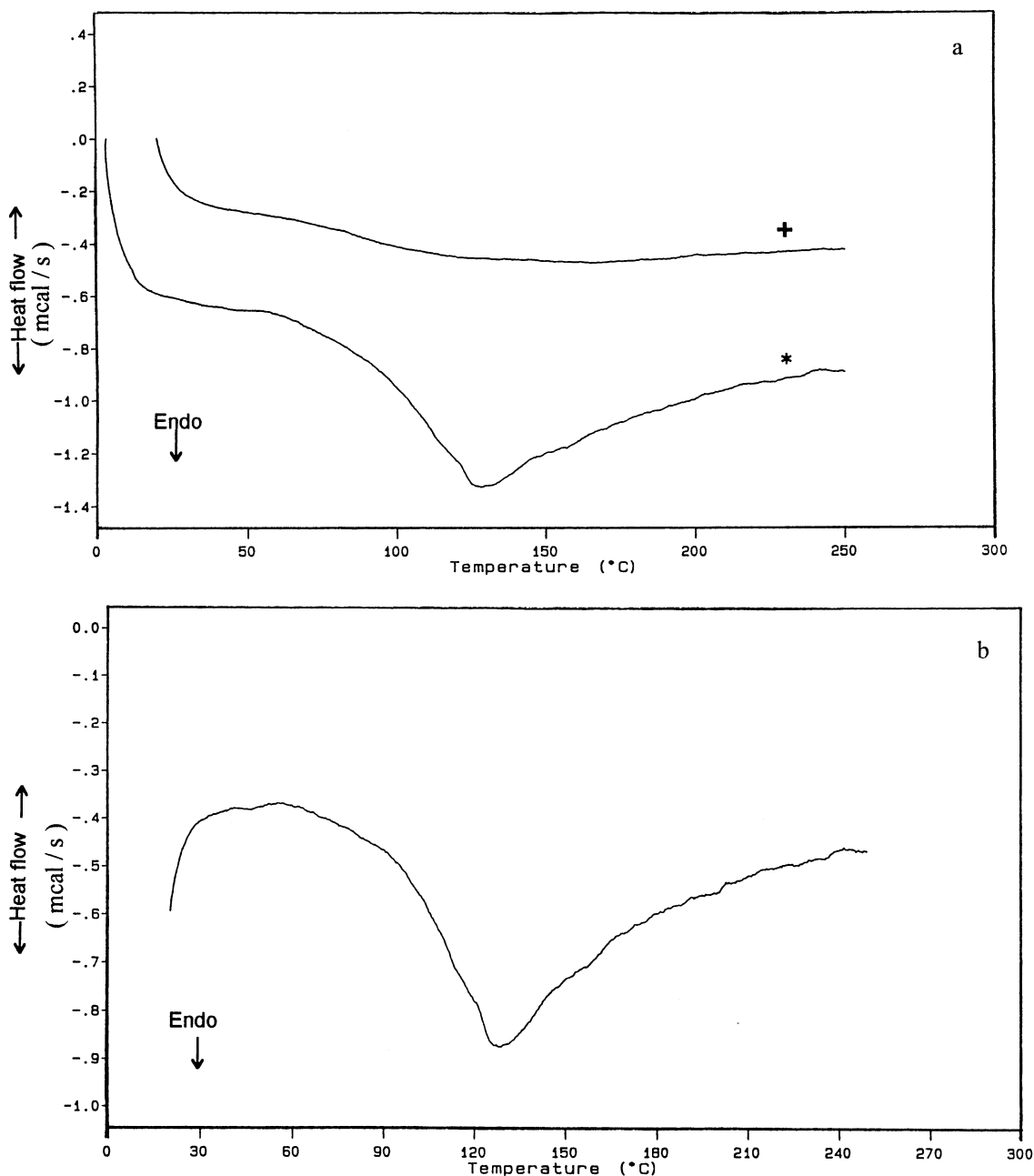


Figure 2 Thermal analysis of Biosil-NH₂ (+) and Biosil-NH₂-cutinase (*) (a). Analysis conditions are described in MATERIALS AND METHODS. Data treatment of the curves gives heat contribution given by the immobilized biocatalyst. Relative thermodynamic data are reported in Table 4 (b)

The presence of dextran in these systems may be responsible for the loss of specificity toward the substrate due to mass transfer and partition resistances. Furthermore, since cutinase has a molecular diameter of approximately 4.5 nm,⁹ and knowing that the interior of the pores in a support only become accessible when their diameter is 4–5 times that of the enzyme³³ (for cutinase that value would be 18–22.5 nm), it may be possible that immobilization of the biocatalyst also occurred inside the pores (Table 2). This occurrence will enhance mass transfer resistances. In any case, immobilization upon Biosil supports showed lower specificity constants in relation to native cutinase.

Stability studies

For storage stability studies in aqueous media, data were treated using the Sadana model,^{34,35} considering two sequential deactivations (Figure 1). This model consists in a series deactivation scheme involving two first-order irreversible steps. The model allows the calculation of the activity as a weighted function of the specific activities of the enzyme states. A different deactivation behavior for cutinase-dextran is noteworthy while the other three systems have approximately similar deactivation profiles. From the curve profiles, first-order kinetic deactivation

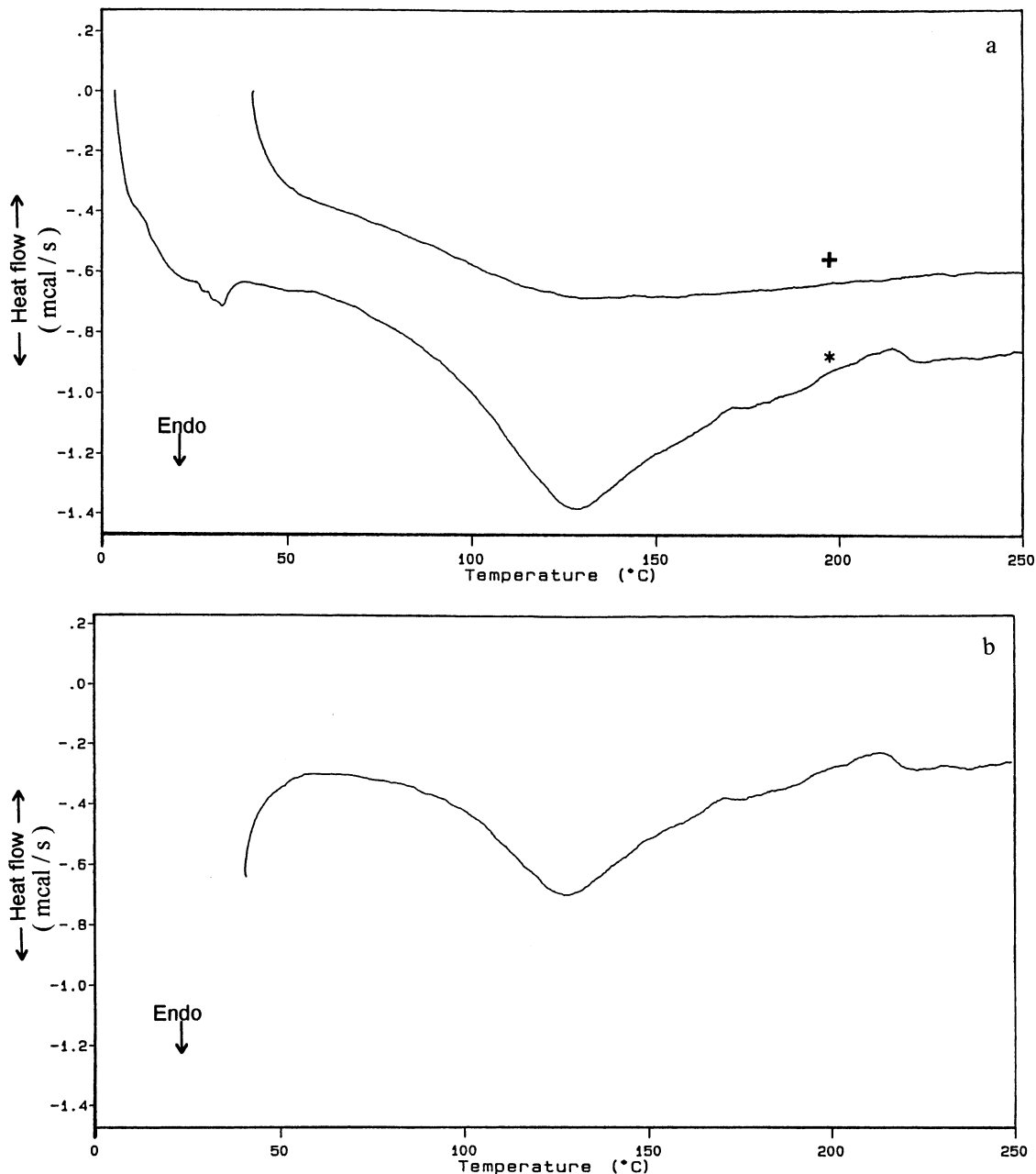


Figure 3 Thermal analysis of Biosil-Dextran-NH₂ (+) and Biosil-Dextran-NH₂-cutinase (*). (a). Analysis conditions are described in MATERIALS AND METHODS. Data treatment of the curves gives heat contribution given by the immobilized biocatalyst. Relative thermodynamic data are reported in Table 4 (b)

constants (k_1 and k_2) and activity ratios (β_1 and β_2 , ratio values of the residual activity at that state to the initial activity at $t = 0$) suggest that the immobilization upon the solid supports slightly enhanced the long-term stability relative to the soluble cutinase. At 50 days of incubation, Biosil-NH₂-cutinase exhibited an activity retention of 1.0 while Biosil-Dextran-NH₂-cutinase exhibited a value of 0.80 compared to 0.70 for soluble cutinase. By comparing Biosil-NH₂-cutinase and Biosil-Dextran-NH₂-cutinase, it may be seen from Figure 1 that it took 48 h and 5 days, respectively, for each system to achieve its most active form. The presence of dextran in Biosil-Dextran-NH₂-cutinase may

have a stabilization effect, minimizing possible distortion effects imposed upon the active site of cutinase. Additionally, it may be possible that dextran exerts a protective effect on cutinase, protecting the microenvironment of the immobilized enzyme and leading to a higher retention of its hydration level. Dextran may thus delay the mild denaturing effects that water has on biocatalysts. Karl-Fischer titration showed that both solid supports with immobilized cutinase had a very low water content (1–2% w/w) after freeze-drying which corresponds to the monolayer of hydrating water of the biocatalyst and a thin layer of water associated with the support surface. It may be that dextran, acting

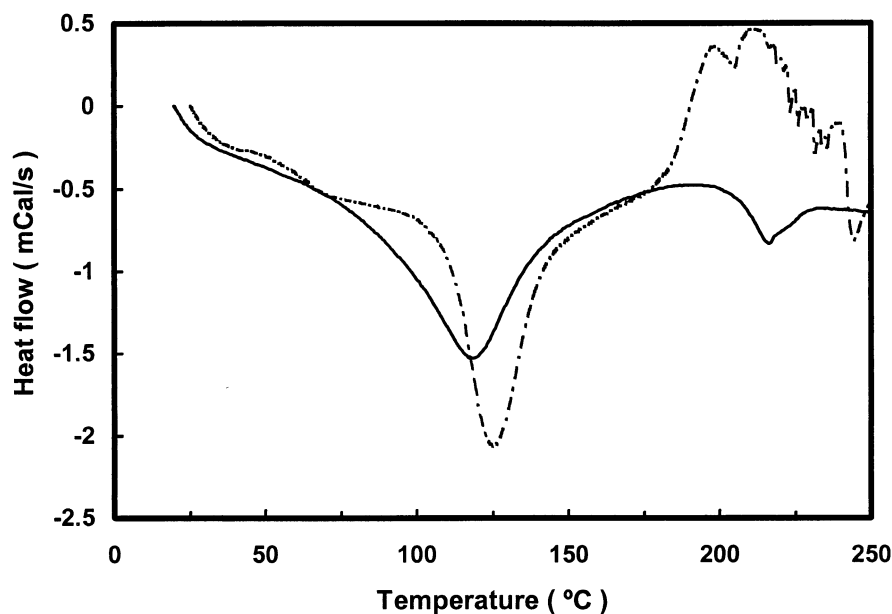


Figure 4 DSC analysis of cutinase (—) and cutinase-dextran conjugate (---)

through preferential hydration,^{26–30} “controls” the hydration layer of the enzyme as it gradually gains a new conformation and mobility imposed by the aqueous media.

The two-step deactivation model proposed for the other three cases did not apply for the values of activity retention given by the cutinase-dextran conjugate. Although there is not any indication of the polysaccharide/cutinase ratio for Biosil-Dextran-NH₂-cutinase, for the cutinase-dextran conjugate the ratio is 6.5 (w/w) which corresponds to 2.04 mol of dextran for each mol of cutinase. Since the polysaccharide has a 40% content of oxidized glucoside units, three out of five units have an intact structure containing three OH-groups; the other two units contain two dialdehyde groups. It is therefore quite possible that dextran may display preferential hydration upon the biocatalyst as well as conferring to it a certain rigidity by means of the covalent bonds involved. The stability observed in this system is probably a combined result of the double action of dextran on the enzyme. From *Figure 1*, the microenvironment surrounding the biocatalyst may be responsible for the different profile of deactivation since there is most probably a higher ratio of dextran/cutinase in the conjugate than in Biosil-Dextran-NH₂-cutinase, and therefore a higher number of free –OH groups.

Differential scanning calorimetry studies

The four systems were also analyzed in the dry form by differential scanning calorimetry. This technique provides information on the thermal stability of the protein by means of the transition temperatures resulting in the conformational changes the protein experiences when undergoing transition from the native state to the denatured state. *Table 4* shows the values of T_M and $\Delta_D H$ of all four systems.

The determination of the thermodynamic values for the two immobilized systems was done by subtracting the curve of each support without protein from the correspondent

curve with immobilized protein, thus giving the data exclusively for the biocatalyst (*Figures 2* and *3*). For cutinase-dextran, the heat contribution from dextran was subtracted from the total observed for the conjugate (*Figure 4*). All data manipulation was done by means of software programs included in the DSC apparatus.

Immobilization onto dextran and both solid supports made cutinase more resistant to heat, implying that the covalent bonds generated conveyed a higher conformational stability to the enzyme.

Conclusions

In aqueous media Biosil-NH₂-cutinase and Biosil-Dextran-NH₂-cutinase have a similar catalytic behavior as well as a similar stability behavior; however, the presence of the polysaccharide on the support surface and its potential action of preferential hydration of the enzyme microenvironment make this support attractive for nonconventional media applications, in particular for synthesis reactions (esterification and transesterification reactions). As calorimetric analysis demonstrated, immobilization on both solid supports made cutinase more heat resistant. The increment in T_M observed for Biosil-Dextran-NH₂-cutinase (9°C) is greater than that observed for cutinase-dextran (6°C) which is most probably related with the number of covalent bonds involved and not as much with the nature of the support. The increments observed for T_M in all three cases may reflect a gain in the conformational stability acquired by cutinase, resulting in an enhancement of long-term stability and potential for applications at higher temperature.

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