Immobilization-Stabilization of *Candida antarctica* Lipase B in Agarose-Glyoxyl and Agarose-Octyl: Deactivation Kinetics

C. Belver[a], J.J. Tamayo[a], L. Molinero[a], M. Ladero[a], B.C.C. Pessela[b,] J.M. Guisán[c], F. Garcia-Ochoa[a].

[a]Dpt. Ingeniería Química, Facultad C.C. Químicas. Universidad Complutense de Madrid. Ciudad Universitaria 28040 Madrid. Spain.
[b]Dpt. Microbiología, Instituto de Fermentaciones Industriales, CSIC. C/ Juan de la Cierva, 3. 28006 Madrid. Spain.
[c]Dpt. Biocatálisis. Instituto de Catálisis y Petroleoquímica, CSIC. C/ Marie Curie, 2. Cantoblanco, 28049 Madrid (Spain).

Lipases are a well-known working-horse in the synthesis of several substances, mainly esters and amides. Their importance is increasing with time, with a high number of publications every year, in organic, analytical, and industrial chemistry, as well as in biochemistry and other health related sciences. CALB is the most used lipase in organic and industrial chemistry nowadays. On the other hand, glycerol is a good stabilizer for enzymes as well as a plentiful raw material whose market price is low due to the energy policy regarding biodiesel. This work is focused on the immobilization-stabilization of CAL-B for its use with glycerol, using both a mild and reversible immobilization, based on octyl-agarose, and an irreversible strong stabilizing immobilization on glyoxyl-agarose. The enzyme has been immobilized with and without a previous chemical modification based on the increase of amino groups on its surface. A kinetic study of the deactivation of the biocatalysts is presented to compare their performance in glycerol and other media, based on their activity and stability, being the octyl-agarose derivate the most promising for its use in glycerol.

1. Introduction

CALB is a well-known lipase used as a work horse in many biocatalytic applications, due to its flexibility in accepting many subtrates and to its relatively high activity, when compared to other lipases. An adequate immobilization technique is, in this situation, most convenient for getting a very stable and, sometimes, reversible biocatalyst (Mateo et al., 2007; Arroyo et al., 1999). On the other hand, glycerol seems a promising feedstock due to the biodiesel impact in the energy market.

In this work, the immobilization of CAL-B to agarose via glyoxyl (Mateo et al., 2006) and C8 groups (Fernández-Lorente et al., 2007; Torres et al., 2006), as well as the obtained thermal stability in several media, are studied. The enzyme was subjected, also, to chemical modification to increase the number of amino groups in its surface, so that more bonds can be formed and the stability increase because of the more rigid

protein-support structure created. After testing the retained activity, the thermal and chemical stability of the immobilized enzyme preparations, as well as that of the free enzyme were studied and the remaining activity data was used to select among several kinetic models by fitting them to the available experimental data using the integral kinetic methodology (Ladero el al., 2006; 2005).

2. Materials and Methods

2.1 Materials
The enzyme employed, lipase CALB-L, was kindly supplied by Novozymes (Bagsvaerd, Denmark). It is an enzymatic semi-purified solution in glycerol. Agarose was a kind gift from Hispanagar (Burgos, Spain), while octyl-agarose was purchased from Pharmacia Biotech (Uppsala, Sweden). P-nitrophenyl butirate, ethylcarbodiimide (ECI), ethylendiamine (EDA), sodium borohydride and Triton X-100 were purchased from Sigma Chem. Co. (St. Louis, USA). All other reagents and solvents were of analytical grade and provided also by Sigma Chem. Co.

2.2 Immobilization Methods
Amidation of the protein was performed after adsorption to octyl-agarose: the so immobilized protein is mixed with EDA and ECI in a relation 1 g: 10mL: 10mM. Afterwards, the enzyme is desorbed with with a 0.2% water solution of Triton X-100 and immobilized in glyoxyl-agarose at pH 9 and 10.5. Immobilization of the CALB on octyl-agarose was performed according to Torres (Torres et al., 2006). Immobilization of the enzyme on glyoxyl-agarose was carried out according as previously reported (Mateo et al., 2006). In all steps were enzyme is contacted with a support, 8 mg of enzyme were used per gram of support, being the protein always totally immobilized. Protein concentration was determined by the Bradford´s method (Torres et al., 2006). Activity was tested with p-NPB, as described in paragraph 2.4.

2.3 Deactivation assays
2 UI of enzyme (free or immobilized) were contacted with buffer phosphate 25 mM pH 7.0, dioxane, or glycerol, and incubated at 70 and 80°C when thermal deactivation were studied (buffer, glycerol) and at 25°C when the dioxane effect was studied. Several samples were withdrawn during the deactivation and activity tested as mentioned later.

2.4 Activity test
The enzyme activity test was performed by measuring the increase in the absorbance at 348 nm produced by the release of p-nitrophenol in the enzymatic hydrolysis of 0.4 mM p-NPB in 25 mM phosphate buffer at pH 7 and 25°C. One international unit (U) was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol p-NPB/min under the assay conditions describe above.

2.5 Mathematical methods
Integral method was applied in order to determine the kinetic model adequate in each deactivation run, and to optimize the kinetic parameters. Data were treated using the Aspen Custom Modeler, in the Aspen Program Package 11.1.

Table 1. Kinetic models fitted to deactivation data in phosphate buffer 25 mM pH 7.0 and in glycerine

Mechanism	Kinetic model	
1) Inactivation following elemental reactions from a unique enzyme at zero time		
$E \xrightarrow{k_1} D$	$a = e^{-k_1 t}$	Model 1
$E \xrightarrow{k_1} E^*$	$a = (1 - \beta^*) e^{-k_1 t} + \beta^*$	Model 2
2) Inactivation following consecutive reactions from a unique enzyme at zero time		
$E \xrightarrow{k_1} E^* \xrightarrow{k_2} D$	$a = e^{-k_1 t} + \beta^* \dfrac{k_1}{k_2 - k_1} \left(e^{-k_{21} t} - e^{-k_1 t} \right)$	Model 3
$E \xrightarrow{k_1} E_1^* \xrightarrow{k_2} E_2^*$	$a = \left(1 + \beta^*_1 \dfrac{k_1}{k_2 - k_1} - \beta^*_2 \dfrac{k_2}{k_2 - k_1} \right) e^{-k_1 t}$ $- \dfrac{k_1}{k_2 - k_1} \left(\beta^*_1 - \beta^*_2 \right) e^{-k_2 t} + \beta^*_2$	Model 4

3. Experimental results and discussion

Table 2 shows the results obtained with the different immobilization strategies, both with regard to the real final activity of the enzymatic biocatalyst refer to the free enzyme, and the stability of the biocatalysts in buffer phosphate, an aqueous solution where its stability is usually tested. It can be seen that the covalent bonding (glyoxyl-agarose) affects much more the retained activity compared to the free enzyme, as chemical bonding is much more prone to inactivate the enzyme during the immobilization process. Much milder conditions, as those used with the octyl-agarose supports, lead to an immobilized enzyme retaining much of the activity of the free enzyme (Torres et al., 2006). Consequently, the latter immobilization affects with lower intensity the structure of the enzyme, as expected with a reversible immobilization technique (Mateo et al., 2007). The lower interaction of enzyme and octyl groups leads, in this situation, to a lower stabilization when biocatalysts are subjected to high temperature in this buffer. However, dioxane does not affect neither the free enzyme nor any of the immobilized biocatalysts, so, seemingly, deactivation of the enzyme involves always polar species that solvate the outer region of the enzyme. Glyoxyl immobilization involves always a lower deactivation rate, and, in the case of not modifying the surface of the enzyme with amino groups, some activity seems to be retained even at high deactivation times. With the phosphate buffer media, deactivation always proceeds rapidly following a first-order reaction, usually towards a totally deactivated protein. In Figure 1, the fit of the selected kinetic models to experimental data is shown, being it reasonable in every case.

Table 2. Retained activity after immobilization and kinetic modelling of the thermal deactivation of free and immobilized CAL-B in buffer phosphate 25 mM pH 7.0 at 80°C

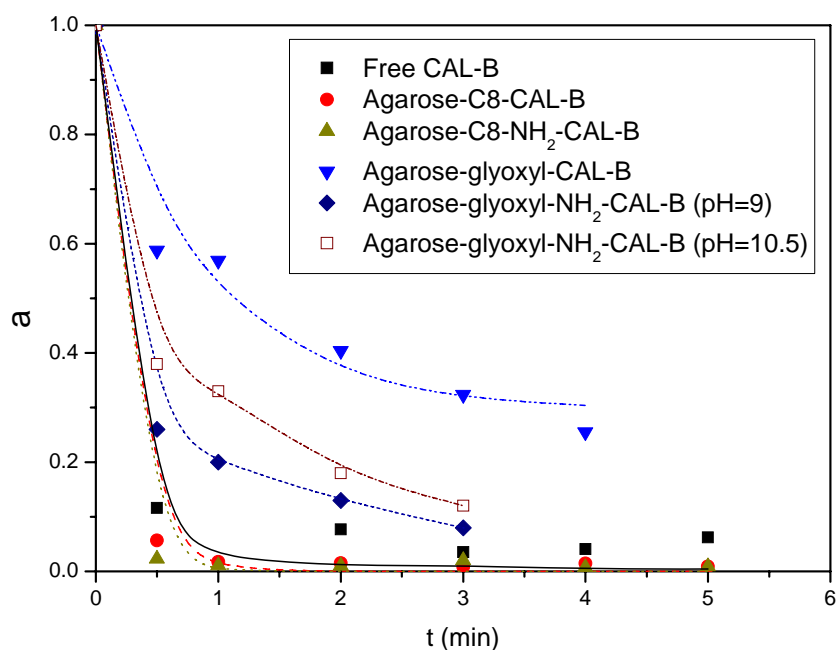| Enzymatic biocatalyst	Retained activity (%)	Model	Kinetic and statistical parameters			
			k_d	β	SQR	F
Free CAL-B	100	1	4.30± 0.87	-	0.046	399
Agarose-C8-CAL-B	58	1	5.68± 0.39	-	0.105	77
Agarose-C8-aminated CAL-B	52	1	7.50± 0.96	-	0.105	78
Agarose-glyoxyl-CAL-B	39	2	1.21± 0.29	0.30± 0.05	0.530	75
Agarose-glyoxyl-CAL-B aminated (pH 9.0)	9	1	0.98± 0.08	-	0.067	67
Agarose-glyoxyl-CAL-B aminated (pH 10.5)	16	1	0.56± 0.08	-	0.078	56

Figure 1.- Thermal deactivation at 80°C of several biocatalysts based on CAL-B on agarose in buffer phosphate 25 mM pH 7.0. The fitting of the models is shown by the lines.

When the biocatalysts are subjected to deactivation in glycerol, a promising alcohol that can act both as a substrate and a solvent (i.e. solvent-less esterifications to render monoglycerides and structured triglycerides), the stabilizing effect of this polyol is observed both in the more complex reaction scheme and in the lower values of the kinetic constants (at 80°C). The two reactions involved are first-order and are consecutive reactions. At any temperature tested, the initial lipase evolves towards a more active intermediate species. This enzymatic intermediate can be deactivated totally (at 70°) or partially (at 80°), yielding, in this latter case, an enzyme that sometimes has still a high activity, as when octyl-agarose is used with the native enzyme.

Deactivation in glycerol is always slower than in phosphate buffer and, what is more, the biocatalyst that was more stable in glycerol was that with the octyl moieties linked to the agarose chains. This can be due to the fact that glycerol can, as other higher carbohydrates, interact with both the support and the enzyme, yielding a more stable biocatalyst. Thus, though the initial linkage leading to immobilization is of an hydrophobic nature, hydrophilic interactions during deactivation or reaction can be of importance for the stability of the immobilized enzyme. Glyoxyl bonding, being much stronger, does not mean a higher interaction, in this case. Thus, it seems that stability depends much on the solvent used, polar or apolar, capable of interactions (hydrogen links or others) or not.

Surprisingly, deactivation at lower temperature, though at lower rate, proceeds towards a totally inactive protein. As enzyme deactivation involves a complex mechanism, with dissociation, coagulation, disruption of internal interactions and other phenomena involved, different temperature values can mean a different final species (Ladero 2006, 2005).

Table 3. Kinetic modelling of the thermal deactivation of free and immobilized CAL-B in glycerol at 70°C.

Enzyme biocatalyst	Model	Kinetic and statistical parameters				
		k_{d1}	k_{d2}	β	SQR	F
Free CAL-B	3	0.36± 0.08	0.020± 0.002	2.01± 0.11	0.045	738
Agarose-C8-CAL-B	3	0.01± 0.001	0.002± 0.0004	1.10± 0.15	0.034	922
Agarose-C8-aminated CAL-B	3	0.04± 0.003	0.011± 0.003	1.65± 0.15	0.112	543
Agarose-glyoxyl-CAL-B	3	0.01± 0.002	0.002± 0.0003	1.43± 0.15	0.043	846
Agarose-glyoxyl-CAL-B aminated (pH 9.0)	3	0.24± 0.014	0.015± 0.004	0.42± 0.08	0.145	436
Agarose-glyoxyl-CAL-B aminated (pH 10.5)	3	0.01± 0.002	0.002± 0.0007	1.62± 0.12	0.095	518

Table 4. Kinetic modelling of the thermal deactivation of free and immobilized CAL-B in glycerol at 80°C

| Enzyme biocatalyst	Model	Kinetic and statistical parameters					
		k_{d1}	k_{d2}	β_1	β_2	SQR	F
Free CAL-B	4	0.89± 0.24	0.89± 0.09	5.95± 0.24	0.15± 0.059	0.097	352
Agarose-C8-CAL-B	4	0.78± 0.24	0.012± 0.003	1.76± 0.38	0.43± 0.059	0.048	428
Agarose-C8-aminated CAL-B	4	0.06± 0.004	0.011± 0.003	1.15± 0.13	0.79± 0.032	0.086	396
Agarose-glyoxyl-CAL-B	4	0.83± 0.34	0.22± 0.022	1.49± 0.04	0.45± 0.019	0.032	1637
Agarose-glyoxyl-CAL-B aminated (pH 9.0)	4	0.72± 0.21	0.042± 0.009	0.35± 0.13	0.05± 0.003	0.048	1134
Agarose-glyoxyl-CAL-B aminated (pH 10.5)	4	0.73± 0.36	0.014± 0.005	0.61± 0.11	0.30± 0.024	0.039	1395
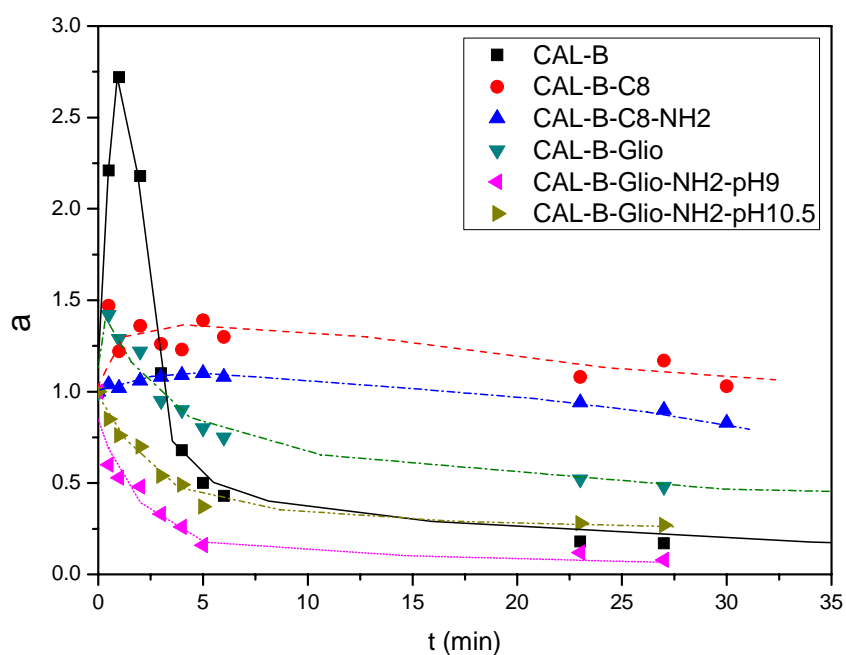
Figure 2.- Thermal deactivation at 80°C of several biocatalysts based on CAL-B on agarose in glycerol. Lines show the fitting of the selected kinetic models.

4. Conclusions

The obtained results show that the enzyme is stabilized both by the immobilizing strategy and the deactivation media, being dioxane the less harming for the enzyme. In phosphate buffer 25 mM pH 7.0, the enzyme was always scarcely stable when subjected to a high temperature, while much higher stability is observed in glycerol. In fact, the octyl derivative is much more stable in glycerol than in buffer phosphate, showing that the interaction of this substance with both the support and the enzyme is important for the stability of CAL-B. Moreover, even the stronger interaction due to chemical bonding in glyoxyl-agarose is not as strong as the myriad of little interactions in the octyl derivative when glycerol is the deactivation medium. The modification of the enzyme via amidation decreases both its activity and stability compared to the non-modified CAL-B. Thus, native CAL-B immobilized on octyl-agarose seems a promising biocatalyst to use with glycerol when this polyol is both the solvent and one of the substrates.

5. Nomenclature

a= remaining activity
D=inactive enzyme
E=active enzyme
F= Fischer's F
k_d, k_{d1}, k_{d2} = kinetic constants for deactivation
SQR = Sum of squares of the residuals
t=time (min or h)
β, β_1, β_2 = activities relative to that of the original enzyme

6. References

Arroyo, M., Sanchez-Montero, J.M., and Sinisterra, J.V. Thermal stabilization of immobilized lipase B from *Candida antarctica* on different supports: effect of water activity on enzymatic activity in organic media. Enz. Microb. Technol. 24 (1999) 3-12.

Fernandez-Lorente, G., Palomo, J.M., Cabrera, Z., Guisan, J.M., and Fernandez-Lafuente, R. Specificity enhancement towards hydrophobic substrates by immobilization of lipases by interfacial activation on hydrophobic supports. Enz. Microb. Technol. 41 (2007) 565-569.

Ladero, M., Ruiz, G., Pessela, B.C.C., Vian, A., Santos, A., and Garcia-Ochoa, F. Thermal and pH inactivation of an immobilized thermostable β-galactosidase from *Thermus* sp. strain T2: Comparison to the free enzyme. Biochem. Eng. J. 31 (2006) 14-24.

Ladero, M., Santos, A., and Garcia-Ochoa, F. Kinetic modelling of the thermal inactivation of an industrial β-galactosidase from *Kluyveromyces fragilis*. Enz. Microb. Technol. 38 (2005) 1-9.

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M.,and Fernandez-Lafuente, Roberto. Improvement of enzyme activity, stability, and selectivity via immobilization techniques. Enz. Microb. Technol. 40 (2007) 1451-1463.

Mateo, C., Palomo, J.M., Fuentes, M., Betancor, L., Grazu, V., Lopez-Gallego, F., Pessela, B.C.C., Hidalgo, A., and Fernandez-Lorente, G., Fernandez-Lafuente, R., Guisan, J.M. Glyoxyl agarose: a fully inert and hydrophilic support for immobilization and high stabilization of proteins. Enz. Microb. Technol. 39 (2006) 274-280.

Torres, R., Ortiz, C., Pessela, B.C.C., Palomo, J.M., Mateo, C., Guisan, J.M., and Fernandez –Lafuente, R. Improvement of the enantioselectivity of lipase (fraction B) from *Candida antarctica* via adsorption on polyethylenimine-agarose under different experimental conditions. Enz. Microb. Technol. 39 (2006) 167-171.