MULTIPOINT COVALENT IMMOBILIZATION OF MICROBIAL LIPASE ON CHITOSAN AND AGAROSE ACTIVATED BY DIFFERENT METHODS

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Abstract

In this work *candida antarctica* type B lipase (CALB) was immobilized on agarose and chitosan and the influence of activation agents (glycidol, glutaraldehyde and epichlorohydrin) and of the immobilization time (5, 24 and 72 h) on thermal and alkaline stabilities and hydrolytic activity of the biocatalyst were evaluated. Protein concentration and enzymatic activity in the supernatant were determined during immobilization process. Best results of hydrolytic activities were obtained for agarose-glycidol, 845 U/g of gel - after 72 h of immobilization, chitosan-glutaraldehyde, 1209 U/g of gel and agarose-glutaraldehyde, 2716 U/g of gel - after 5 h of immobilization. High degree of derivatives stabilization was achieved as agarose-glycidol-CALB, chitosan-glutaraldehyde-CALB and agarose-glutaraldehyde-CALB and chitosan-glycidol-glutaraldehyde-CALB were, respectively, 20-fold, 18-fold, 21-fold and 58-fold more thermal stable than the soluble enzyme. The stabilization degree of derivative increased with the immobilization time, an indication that a multipoint covalent attachment of the enzyme to the support had really occurred.

Introduction

In its natural environment lipases (EC 3.1.1.3) catalyze the hydrolysis of triglycerides. However, under appropriate experimental conditions, these enzymes are very active and catalyze the esterification of fatty acids, alcoholysis and transesterification reactions (Lutz, 2004). The stereo specificity, high enantioselectivity, high activity and stability in non-aqueous environments of *Candida antarctica* lipase B (CALB) make them attractive for applications in biochemical and industrial fields (McCabe et al., 2005).

The possibility of enzyme recovery and re-usability is an attractive for industrial applications. Immobilization of lipase on solids supports using different methods is a strategy to insolubilize enzyme and to improve its operational stability. Moreover, immobilized enzymes offers some operational advantages over free enzymes, for instance, easier operational control, larger flexibility of reactor design and ease removal from the reaction medium (Tardiolli et al., 2003). Immobilized enzymes can be used under extreme conditions of temperature and pH, as well as in the presence of organics solvents or any other distorting agent. The improvement of enzyme stability is one of the main issues for the implementation of enzymes as industrial biocatalysts (López-Gallego et al., 2005).

Immobilization, however, does not guarantee stabilization of the enzyme three-dimensional structure. Stabilization would only occur if the enzyme-support interaction results in several multipoint bounds between enzyme and support, increasing rigidification of the enzyme structure

(Palomo *et al.*, 2003). Multipoint immobilization is a strategy proposed by Guisán (1988) and it results from several covalent bounds between an enzyme molecule and the activated surface of the support, allowing the enzyme stabilization by increasing the rigidity of a small part of its surface (e.g., 10-20%) what is translated to its overall three-dimensional structure (Galvão, 2004).
The aim of this work was study the influence of three different activating agents on CALB activity and stability after immobilization on chitosan and agarose.

Material and Methods

Materials
Native lipase B from *Candida antarctica* B was purchased from Novozymes Latin America Ltd. Agarose gels CL 6% were obtained from Amershan Bioscience (Uppsala/SU) and powdered chitosan, was purchased from Polymar Ind Ltda. Glycidol, epichloridrine, methyl butyrate and bovine serum albumin were acquired from Sigma-Aldrich. Glutaraldehyde was from Vetec/SP.

Methods
Hydrolytic Activity was determined by methyl butyrate hydrolysis using pH-stat method (Bastida et al., 1998). In this work, one unit (U) of enzymatic activity was defined as the amount of enzyme that hydrolysis 1 μmol of methyl butyrate per minute at pH 7.0 and 25 ºC.

Protein concentration: Protein concentration was measured according to Bradford (1976).

Glyoxyl supports were prepared by etherification of agarose or chitosan with glycidol and further oxidation of the resulting support-glyceril (Guisán, 1988; Beppu et al., 2004).

Determination of the aldehyde content on agarose: Was determined by quantification of the amount of sodium periodate consumed during oxidation (Guisán et al., 1988).

Glutaraldehyde-agarose was prepared from glyoxyl-agarose (Cardias, 1999).

Chitosan-glutaraldehyde gels: Chitosan gels were suspended in buffer, pH 10, containing 5 % (v/v) of glutaraldehyde. The mixture was maintained under agitation for 60 min at 25 ºC.

CALB was immobilized on agarose and chitosan gels, after activation with glycidol, epichloridrine or glutaraldehyde. The immobilization was carried out in 100 mM bicarbonate buffer, pH 10.5, at 25ºC and different incubation times (5, 24 and 72 h), under gentle stirring. After immobilization, sodium borohydride (0.5 mg/mL of reaction medium) was added to system and kept at ice bath.

Thermal stability: Samples of free or immobilized CALB were incubated in 25 mM sodium phosphate buffer, pH 7 at 50ºC. Periodically, samples were withdrawn and their residual activities were assayed. The deactivation constant and Half-life ($t_{1/2}$) for each immobilized derivative was calculated according to the model proposed by Henley and Sadana (1987).

Alkaline stability: was investigated by incubation of samples in an alkaline solution, pH 12 at 25ºC. Samples were withdrawn at selected time intervals and residual activities were determined.

Immobilization parameters: Immobilization yield (IY) was calculated by measuring the difference between protein concentration of the supernatant before (Ct_0) and after (Ct_t) immobilization, according to equation 1:

$$IY\% = \frac{Ct_0 - Ct_t}{Ct_0} \times 100 \quad (1)$$

In this work, recovered activity is defined as the ratio of the apparent hydrolytic activity, obtained by the direct measure of derivative activity, and the difference between hydrolytic activity of the

supernatant before and after immobilization, which can be considered as the derivative theoretical activity. Therefore, recovered activity can be considered as the biocatalyst effectiveness.

Results and Discussion

Influence of activation method and incubation time in the activity of immobilization efficiency

Table 1 shows the immobilization parameters, recovered activity (A_{Rec}), immobilization yield (IY) and derivative activity (A_{Der}), to the different immobilization methods and supports studied in this work. It can be observed that best results of immobilization yield were obtained when glutaraldehyde was used as activating agent, 89% and 90% to agarose-glutaraldehyde and chitosan-glutaraldehyde, respectively. Furthermore, this activating agent promoted very fast protein retention.

Similar results were obtained to hydrolytic activities when immobilized derivatives were prepared by chitosan and agarose activation with glutaraldehyde and enzyme loading of 3 g/g support.

To glyoxyl activation (glycidol and epichloridrine), chitosan-glyoxyl derivatives are less active than agarose-glyoxyl derivatives. The possible reason is the competition between amino groups of enzymes and chitosan for aldehydes groups from the support.

Table 1: Effect of time on yield immobilization (IY) and recovery activity (ARec) of CALB immobilized derivatives. ARec = ADer /AtSup (disappeared on supernatant). ADer is activity measured on the immobilized derivative; IY percentage of protein disappeared on supernatant. Enzyme loading offered: 3mg protein/g support.

Immobilized Derivative	**Incubation time (h)**								
	5			**24**			**72**		
	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)
Agarose-Glycidol	63	180	81	72	214	71	75	144	86
Chitosan-Glycidol	5	8	5	37	13	12	38	16	15
Agarose-Glutaraldehyde	89	330	68	92	300	62	92	300	61
Chitosan-Glutaraldehyde	90	402	82	90	295	58	78	306	59
Agarose-Epichloridrine	58	106	57	73	157	68	73	130,7	24
Chitosan-Epichloridrine	5	23	38	15	45	63	17	44,8	36

After 72h of incubation was observed loss of activity of all the immobilized derivatives, except to chitosan-glycidol and chitosan-epichoridrine. This strengthens the hypothesis of competition.

The loss of activity of the immobilized derivatives is reflected in the recovery activity.

The activation degree on supports may also have promoted the loss of activity of the immobilized derivative with the increase of incubation time. In agarose activation with glycidol the activation degree was 115 μmol of aldehydes groups/mg support. This means that the support have high surface density of reactive groups (Tardioli *et al.*, 2003). Glutaraldehyde and epichloridrine also was used in excess, but the several experimental conditions and the chemical reactivity of each activation agent promoted the formation of supports with different activation degree.

The presence of strongly reactive amino groups in chitosan suggests that several parallel reactions might proceed simultaneously. Therefore, the loss of activity observed may be due to very intensively multipoint-attachment causing conformational changes in the enzyme structure.

In the immobilized derivatives prepared on support-glyoxyl (glycidol or epichloridrine) was observed kinetic immobilization slower than in support-glutaraldehyde. In the first case, the equilibrium was reached after 24 h and incubation times longer cause loss of activity for immobilized derivatives prepared on agarose support.

An explanation for low hydrolytic activity in immobilized derivatives prepared by activation of chitosan with glycidol and epichloridrine is the competition of amino groups of the enzyme and

chitosan for aldehydes groups. Moreover, these activation agents might to promote cross linking of hydroxyl groups of chitosan (Gonçalves *et al.,* 2005).
A detailed study to derivatives chitosan-glyoxyl was carried out by bonding of amino groups using formaldehyde, glyoxal and glutaraldehyde, these results are presented in next section.

Effect of the reaction between chitosan amino and different aldehydes on the immobilized enzyme activity

In order to investigate the influence of amino groups of chitosan on the CALB immobilization, this support was first activated with glycidol or epichloridrine to formation of glyceryl groups. Then, the chitosan-glyceryl was treated with aldehyde solution.
The treatment of chitosan-glyceryl with formaldehyde and glyoxal was carried out using experimental conditions where no reaction occurred with hydroxyl groups and only amino groups were consumed (Li and Bai, 2005). Finally, oxidation with periodate was carried out and the support was used to CALB immobilization. In Table 2, the experimental conditions and values of hydrolytic activity obtained are showed.

Table 2: Immobilization parameters and half-life ($t_{1/2}$) of the several CALB-chitosan derivatives produced with one or two activation agents. $t_{1/2}$ CALB free = 0,5 h.

	CALB Derivative (immobilized enzyme)	Parameters			
		IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	$t_{1/2}$
With free amino groups	**chitosan-glycidol**	5	8	5	1,8
	chitosan-epichloridrine	5	23	38	0,4
	chitosan-glutaraldehyde	90	402	82	4,9
With amino groups bonded with different aldehydes	**chitosan-glycidol-formaldehyde**	18	3	3	-
	chitosan-glycidol -glyoxal	8	7	4	-
	chitosan-glycidol-glutaraldehyde	73	245	62	12,6
	chitosan-epichloridrine -glutaraldehyde	60	256	100	5,4

It can be observed that the reaction of amino groups with formaldehyde and glyoxal was not efficient to increase the activity of CALB derivatives prepared on chitosan-glyoxyl. Nevertheless, the reaction of amino groups with glutaraldehyde increased thirty times the activity this CALB derivative when compared with those prepared with chitosan-glycidol.
Higher thermal stability was observed for CALB derivatives prepared on chitosan-glycidol-glutaraldehyde ($t_{1/2}$ = 12,6 h with incubation time of 5 h) when compared with those prepared with chitosan-glutaraldehyde.
Different physical properties are observed when chitosan is treated with glutaraldehyde or formaldehyde and glyoxal (Hirano et al., 1999). The possible reason is the different degree of cross linking, which lead to different porosity and different water retention capacity of the support. When two activation agents are used simultaneously, multipoint immobilization is favored and higher thermal stability is observed. On the other hand, in some area of the support surface this high activation degree might cause conformational changes in the enzyme structure and consequently cause loss of activity. Moreover, the high degree of cross linking might hinder the entrance of enzyme within of the pores of support.

Effect of the reduction with sodium borohydride on the activity of CALB immobilized

The use of reducing agents, e. g., sodium borohydride, makes the produced derivative suitable to use in drastic reaction conditions (temperature, pH and organic solvent).
However, some enzymes might lose catalytic activity by reduction of disulfide bonds when the derivatives are submitted to treatment with sodium borohydride under drastic reaction conditions (Blanco and Guisán, 1989).

In this work, it was investigated different reduction conditions using sodium borohydride aiming at to prepare at stable immobilized derivatives and also avoiding significant loss of catalytic activity. The Table 3 shows its results.

Table 3: The influence of the reduction temperature and $NABH_4$ concentration on the agarose-glyoxyl - CALB activity.

Experimental reduction condition	A_{Der} (U / g)	Residual Activity (%)
unreduced	350 ± 10	100
0,5 mg/mL (4ºC)	355 ± 30	100
0,5 mg/mL (25ºC)	238 ± 3	68
1,0 mg/mL (4ºC)	289 ± 0	83
1,0 mg/mL (25ºC)	240 ± 12	69

It can be observed that higher the temperature and the $NABH_4$ concentration, lower is the residual activity of the immobilized enzyme. At 4°C and using the smallest $NABH_4$ concentration the derivative remains fully active after reduction.
The effect of non-specific hydrolysis was carefully investigated in the reaction that was used in this work to measure the enzyme activity (hydrolysis of methyl butyrate). An assay control was carried out by addition of activated support (without enzyme) to the reactor. No reaction was observed, therefore this support showed to be inert in the experimental conditions of hydrolysis (temperature, pH and agitation) used in this work.

Effect of incubation time on the thermal stability of immobilized CALB

Table 3 shows the values of half-life calculated using model for thermal inactivation. This value for native CALB is 0,5 h. All the produced derivatives using 72 hours of immobilization time showed higher half-lives than the ones obtained using 24 hours, except chitosan-glycidol. These results suggest that multipoint covalent attachment has occurred for these derivatives, since the protein concentration measured in the supernatant was constant after 24 h of immobilization. When multipoint immobilization occurs, although the first binding is very fast, the multinteraction process requires long time to achieve the correct alignment of the reactive groups of enzyme and support (Mateo et al., 2005). Therefore, the fact of longer the additional incubation time, higher stabilization of immobilized derivatives had occurred indicates that CALB is attached to the support by more than one bond.

Table 4: Half-life of CALB derivatives obtained under different immobilization times ($t_{1/2\ free\ CALB}$ = 0.5 h)

Immobilized derivatives	Half-life (t1/2 / h) for different Incubation time		
	5 h	24 h	72 h
Agarose-Glycidol	3,7	5,5	10,2
Chitosan-Glycidol	1,8	1,4	-
Agarose-Glutaraldehyde	4,3	6,3	9,0
Chitosan-Glutaraldehyde	4,9	5,4	10,6
Agarose-Epichloridrina	2,9	3,9	4,8
Chitosan-Epichloridrina	0,4	0,2	0,8

Figure 1 show the residual activity of different immobilized derivatives incubated at 50ºC for 72 h. Thermal inactivation curves of agarose-glutaraldehyde and agarose-glycidol show similar profile. This behavior suggest the presence of subpopulations of enzymes immobilized binding through two or more residues to support (Palomo, 2003). Arroyo (1999) has obtained similar stabilization of CALB immobilized.

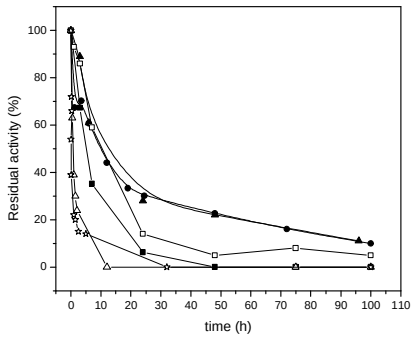

Figure 1 : Thermal inactivation profile of immobilized derivatives obtained after 72 h of incubation at 50ºC. (●) agarose-glutaraldehyde; (■) agarose-epichloridrine; (▲) agarose-glycidol; (□) chitosan-glutaraldehyde; (Δ) chitosan-epichloridrine; (★) CALB free.

Effect of the activation method on the alkaline stability of the CALB derivatives

CALB presents maximum activity at pH 7.0, with a rapid inactivation bellow pH 6.0 and above pH 8.0 (McCabe *et al.*, 2005). The alkaline inactivation curves are presented in Figure 2. These results show that all derivatives are more stable than free CALB, except chitosan-epichloridrine.

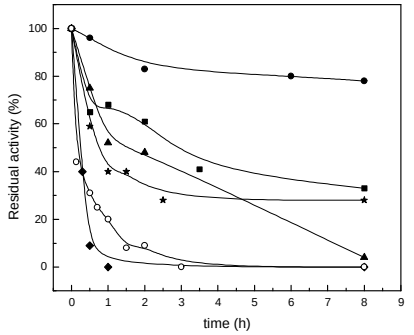

Figure 2 : Alkaline inactivation profile (pH 12). Enzyme load: 3mg of protein/g support, derivatives obtained after 72 h of immobilization. . (●) agarose-glutaraldehyde; (■) agarose-glycidol; (▲) agarose- epichloridrine; (♦) chitosan-epichloridrine; (✱)chitosan-glutaraldehyde; (○) CALB free.

The stability of three-dimensional structure submitted at treatment with different distorting agent (temperature, extreme pHs) is a strategy to confirm the formation of multipoint covalent attachment.

Effect of activation method on the amount of immobilized enzyme

The enzyme loads experiments were first performed using agarose support activated with glycidol. Two immobilization loads were offered, 5 and 10 mg of protein/g support. The lower enzyme load leads to derivative activity of 300 U/g. When 10 mg of enzyme/g support was offered, the derivative activity was 276 U/g, but was immobilized two times more protein. These results indicated the presence of impurities, which compete by the reactive groups of the support. In order to investigate the presence of smaller proteins in crude extract, electrophoresis was carried out (Figure 3).

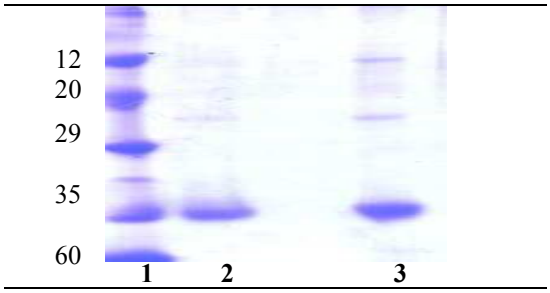

Figure 3 : Electrophoresis of CAL-B in 12 % polyacrilamide gels. Lane 1: molecular weight markers; 2: CAL-B pure; 3:crude extract.

The analysis of results indicates the need of purification of the crude extract before of the immobilization procedure. The crude extract was then submitted to dialysis, which was carried out

using a cellulose acetate membrane (Pinotti, 2003). After the purification step, it was possible to obtain CALB derivatives with activity of 845 U/g. Therefore, the removal of these impurities of the crude extract was very important, since the immobilization method is not selective and smaller proteins diffuse faster and consume preferentially the reactive groups on surface support.
The supports agarose-glycidol (72 h of incubation), agarose-glutaraldehyde and chitosan-glutaraldehyde (5 h of incubation) showed the best results as a trade-off of activity and stability of the derivatives produced and were selected to prepare CALB derivatives using high enzyme loads.
Figure 4-a shows the obtained CALB activity of the derivatives and Figure 4-b the immobilized protein/g support obtained for increasing enzyme loads offered to the activated support.

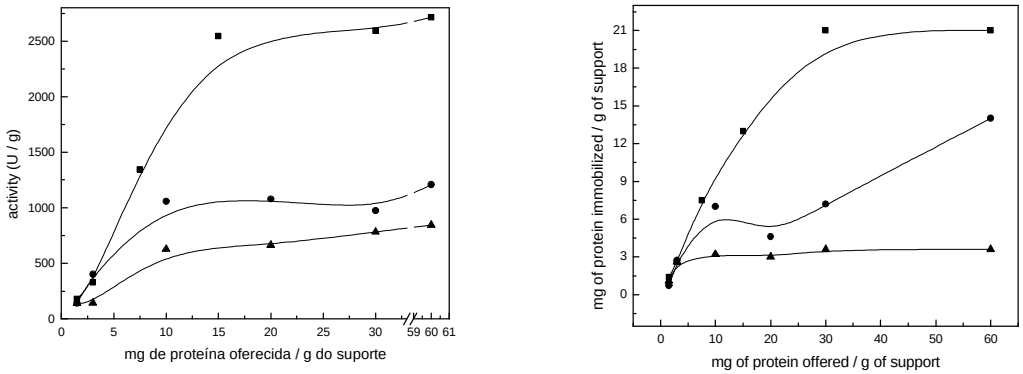

Figure 4 : Effect of enzyme load in (a) CALB activity immobilized and (b) protein immobilized. (•) chitosan-glutaraldehyde; (■) agarose- glutaraldehyde; (▲) agarose- glycidol.

The greatest protein amount immobilized and derivative activity (~ 20 mg of protein/ g support) were obtained using agarose-glutaraldehyde. The high reactivity of the glutaraldehyde lead to CALB derivatives with high activity using both chitosan and agarose supports. The derivatives with different enzyme load were submitted to test of thermal stability in order to check the presence of diffusion effects in these assays. The results are presented in Table 5.

Table 5: Immobilization parameters for several CALB derivatives obtained with different enzyme loads: disappeared protein in supernatant (IY), activity of derivative (A_{Der}), recovery activity (A_{Rec}) and half-life ($t_{1/2}$) at 50°C.

	Enzyme load (mg/g support)										
	1,5		**3,0**		**15**		**30**		**60**		
IMMOBILIZATION TIME/ DERIVATIVE	**A_{Rec} (%)**	**$t_{1/2}$**	**A_{Rec} (%)**	**$t_{1/2}$**	**A_{Rec} (%)**	**$t_{1/2}$**	**A_{Rec} (%)**	**$t_{1/2}$**	**A_{Rec} (%)**	**$t_{1/2}$**	**A_{Der} (U/g)**
72 hours/ Agarose-Glycidol-CALB	81	1,2	86	10,2	90	4,0	100	7,9	100	8,7	845
5 hours/Agarose-Glutaraldehyde	99	2,4	68	4,3	62	4,8	49	10,6	29	10,6	2716
5 hours/Chitosan-Glutaraldehyde	80	0,4	82	4,9	89	4,2	39	5,2	48	3,4	1209
24 hours/Chitosan-Glycidol-Glutaraldehyde	-	-	62	12,6	-	-	-	-	27	29	346

The CALB derivatives prepared on supports activated with glutaraldehyde showed increase in the stability as the enzyme load increased, confirming the hypothesis of the disguise of the obtained stability results when high enzyme loads are immobilized. The Thiele number increases with the reaction rate leading to smaller effectiveness factor of the reaction. Therefore, the thermal stability measured using a high amount of immobilized enzyme may be apparent. When evaluating the effect

of different immobilization strategies on the derivative stability, the research may be aware of this problem. The low values of the recovery activities presented in Table 5, when comparing to the amount of immobilized protein also indicate the presence of diffusive effects.
A significant improvement in the derivative activity, protein amount immobilized and apparent thermal stability was also achieved when chitosan activated with glycidol had the amino groups bonded with glutaraldehyde, the obtained values changing from 8 to 245 U/g support, from 0.15 to 2.2 mg of binding protein/g support and t1/2 from 1.8 to 12.6 hours, respectively, using enzyme load of 3 mg CALB/g support. Therefore, if the porous structure of chitosan could be modified the derivative activity would be higher than the one obtained in this work.

Conclusion

The use of different activation agents allowed the production of CALB derivatives with different properties. Agarose activated with glutaraldehyde lead to the highest derivative activity, 2.700 U/g and half-life of 10,6 hours. The most stable derivative was 58 fold more stable than the soluble enzyme and was obtained by immobilizing CALB on chitosan activated with glycidol, with the amino groups bonded with glutaraldehyde. The increase of the immobilization time increase the thermal stability of the derivative, indicating that multipoint immobilization has occurred. However, the longer the immobilization time, the lower the derivative activity. Therefore, the immobilization time chosen to produce the derivative is a trade-off between these two derivative parameters.

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