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Journal of Molecular Catalysis B: Enzymatic 1 (1995) 23–28

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Study of the stabilization of pure lipases: comparison of two different lipase-microgel derivatives

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Received 2 May 1995; revised 8 June 1995; accepted 3 July 1995

Abstract

The immobilization/stabilization of pure and very labile lipases was studied. Two types of lipase-microgels derivatives, which may be used in aqueous and/or organic media were designed and optimized. The first type consisting in the covalent linkage of the protein to the surface of a previously formed microgel. The second type was obtained in a reverse micellar system of AOT. The lipase was microencapsulated into the acrylic microgels formed after a polymerization process carried out in the micellar droplets. In this case, a crosslinking agent was simultaneously used to enhance the protein rigidity. Due to the distinct lipase localization the two microgel derivatives differ in their activities and stabilities: the microgel with the lipase at its surface had a similar activity and stability as the native lipase, while an important reduction of the conformational mobility of the protein was found when the lipase was microencapsulated, and it gave rise to a high stabilization factor. Thus, a new immobilization method which stabilizes by 352 times at 45°C the pure isolipase B from *Candida cylindracea* is described. These results were also better than those of the crude lipase stabilization by multipoint attachment to agarose gels.

Keywords: Lipases; *Candida cylindracea*; *Candida rugosa*; *Pseudomonas* sp; Immobilization; Stabilization; Microgel; Reverse micelles; Microemulsions; AOT; Microencapsulation

1. Introduction

Hydrophilic enzymes are not soluble in organic solvents, and most of the synthetic work in these media has been carried out with suspensions of finely powdered protein crystals. Chemical modification of proteins with chains of polyethylene glycol [1] and fatty acids [2] has been developed in order to increase their solubility in these systems. Reverse micelles have been used to co-solubilize both the enzyme and the apolar substrates in the aqueous droplet and the organic continuous phase [3]. In another approach, the enzyme may be covalently bound to colloidal cross-linked polymer particles [4] obtained by emulsion polymerization (microgels) [5], whose diameters vary between 40 and 300 nm. These microgel-immobilized enzymes may be dissolved in organic media as colloidal sols in the presence of a small amount of water (ca. 4%) [6]. A different type of enzyme–microgel derivative may be obtained by immobilization of the protein into permanent microcapsules formed after a polymerization process in a reverse micellar system. In this case the enzyme resides inside the microgel and its activity and stability properties may be changed.

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SSDI 1381-1177(95)00006-2

Recently, different polymerizations of acrylic derivatives in reverse micelles have been described [7]. The non-biodegradable polymers that start to form at the micellar interphase are spherical in shape, the microgel size and shape depending on the size of the micelles.

Crude lipases have been used to study most of the reactions in organic media. The entrapment of crude lipases in silica gel materials, showing excellent activities, stabilities and selectivities has been recently reported [8]. Recently, it has been reported that the enantioselectivity of the hydrolysis of (*R,S*)2-arylpropionic esters enhanced when a pure lipase from *Candida cylindracea* was used [9]. But, in general the stabilities of pure lipases are lower than those of these crude enzymes. Thus, the immobilization/stabilization of pure lipases is important to improve the selectivities of the bioprocesses.

In this paper, the immobilization/stabilization of pure and very labile lipases was studied. We designed and compared two types of pure lipase–microgel derivatives: (i) covalent binding of the pure enzyme to the previously formed microgel, and (ii) microencapsulated enzyme in a microgel formed by polymerization into the droplets of the AOT reverse micelles. Their activities and stabilities were studied in water. Due to the increment of the molecular weight of the enzyme–microgel derivatives, these may be used in membrane reactors, using membranes with large pore sizes which allow higher flow rates. Then both immobilization methods described here may have possible applications in industrial processes.

2. Experimental

2.1. Materials

Lipase from *Pseudomonas sp.* (PSL, 750 U/mg protein) was from Boehringer Manheim, lipase from *Candida cylindracea* (CCL, type VII), Sodium bis-(2-ethylhexyl)sulphosuccinate (AOT), *p*-nitrophenyl butyrate (pNPB), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from Sigma. Acrylamide and *N,N,N′,N′*-tetramethylenediamine were from Merck, *N,N*-methylene-bis-acrylamide from Serva. Other monomers were purchased from Aldrich and were freed from inhibitors before use by either distillation in vacuo or by swirling with dilute NaOH, separating the organic layer and drying, where appropriate, with $MgSO_4$.

2.2. Methods

Purification of lipase B from Candida cylindracea

Purification was carried out as previously described by Rúa et al. [10].

Microgel production

Microgels possessing carboxylic acid were prepared as described elsewhere [11]. The monomer feed compositions for the polymer synthesis was: methyl methacrylate, 45%; 2-ethoxyethyl methacrylate, 30%; ethyleneglycol dimethacrylate, 10%; acrylic acid, 15 mol%.

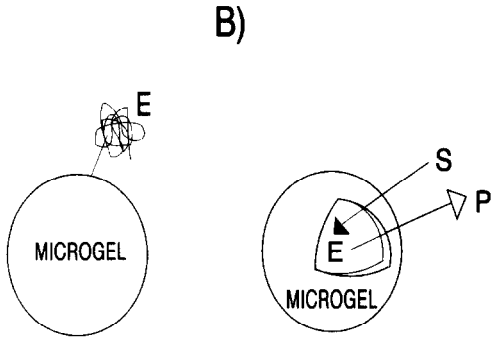
Conjugation of enzyme with polymer

Enzyme (2 mg) in water (1 ml) was mixed with the aqueous solution of polymer (1 ml) and the solution adjusted to the indicated pH. The mixture was kept for the indicated time with EDC (4 mg). Then, the enzyme–polymer conjugate was separated from the native enzyme and the urea by-product in a molecular exclusion column, Sephacryl S-200 or Fractogel TSK HW-65 (F).

The total protein was assayed by the Lowry technique [12]. The activities of native and immobilized lipases were spectrophotometrically assayed with 4-nitrophenyl butyrate, in phosphate buffer 0.1 M pH 7.2, at 30°C and 400 nm.

Permanent lipase microencapsulation

The permanent lipase microencapsulation was prepared in the supramolecular structure of a reverse micellar system, by addition of the indicated amount of acrylamide/*N,N*-methylene-bis-acrylamide solution (AA/BIS = 3.2) and



Scheme 1. Different enzyme-microgel derivatives. (A) Obtained by covalent linkage enzyme-support in water. (B) permanent microencapsulated lipase obtained in the supramolecular structure of a reverse micellar medium.

N,N,N′,N′-tetramethylenediamine to an AOT system ($W_o = [H_2O]/[AOT] = 3.3$) containing the lipase. The microencapsulated enzyme was extracted from the micellar medium to an aqueous phase after the indicated time of polymerization.

Measurements of the activity and stability

The enzyme catalyzed hydrolysis of PNPB in 0.1 M sodium phosphate buffer–0.1 M NaCl, pH 7.2, was used as a standard reaction for the activity and stability assays. This reaction was measured by following the accumulation of *p*-nitrophenol at 400 nm in a Uvikon 930 spectrophotometer from Kontron Instruments equipped with cells thermostated at 30°C.

3. Results and discussion

In this paper we designed and optimized two types of pure lipase-microgel derivatives. In both cases the microgels are acrylic polymers and they are represented in Scheme 1.

3.1. Covalent linkage of a lipase to a previously formed microgel

Coupling of lipase to a preformed microgel

Several pH values and temperatures were tested to obtain the lipase–polymer coupling. Table 1 shows the percentage of *Pseudomonas sp.* lipase which was immobilized under the different conditions studied. These values were obtained through protein assay by the Lowry method [12]. The percentage of immobilized lipase increased from 39% to 50% when the temperature was risen and the pH decreased. Optimal conditions were pH 6.8 and 30°C. Lower pH values and higher temperatures produced microgel precipitation and fast enzyme thermoinactivation, respectively.

Comparison of the kinetic parameters of native and immobilized lipases

The values of the specificity constant (k_{cat}/K_m) of the native and supported lipase were compared. The immobilized lipase obtained at pH 7.0 was chosen for this study. Their k_{cat}/K_m values in the *p*-nitrophenyl butyrate hydrolysis were $(7.5 \pm 4.5) \times 10^7$ and $(4.5 \pm 2.1) \times 10^7\ M^{-1}\ s^{-1}$, respectively. Thus, similar values of this parameter were found for the native and immobilized lipase. These results are in accordance to those obtained by Alcantara et al. [13] with subtilisin-microgel derivatives in water, where these authors reported the similarity in behaviour between the

Table 1
Properties of *Pseudomonas* sp. lipase (native and linked to the acrylic polymer)

Biocatalyst	Temperature (°C)	Time (min)	pH	Immobilization (%)	$t_{50\%}$(h)	Stabilization factor
Native	–	–	–	–	2.3	1.0
L-30,0,6.80	30	30	6.80	50	1.3	0.5
L-30,30,6.95	30	30	6.95	47	4.3	1.9
L-30,30,7.25	30	30	7.25	–	1.7	0.7
L-20,30,7.00	20	30	7.00	47	2.7	1.2
L-20,30,7.20	20	30	7.20	45	4.0	1.7
L-20,30,8.00	20	30	8.00	39	3.3	1.4

Immobilization conditions, the percentage of the immobilized lipases and their stabilities in 0.1 M phosphate buffer, pH 7.2 at 50°C (more details in the Experimental section.

native enzyme and the microgel-coupled one when employing an aqueous medium.

Stability studies

The themostabilities of the enzyme and the enzyme–polymer conjugates of Table 1 were studied at 50°C in 0.1 M sodium phosphate buffer, pH 7.2. This table shows the incubation time at which these biocatalysts lost 50% of their initial activities ($t_{50\%}$). In Table 1, the stabilization factor was defined as the ratio between the $t_{50\%}$ (immobilized)/$t_{50\%}$ (native). All the lipase-microgel derivatives had stabilities similar to those of the native lipase. In contrast, α-chymotrypsin conjugated with microgels possessing primary amino comonomer components or sulphonic acid with carboxyl functions showed a moderate stabilization versus the native protein, which was increased at high pH values [11]. The distinct effect of this immobilization method on the lipase and the protease stabilities may be attributed to the suppression of the protease autolysis.

3.2. *Permanent microencapsulation of a lipase by the in situ formation of the microgel containing the enzyme*

The non-permanent microencapsulations of two purified isoenzymes of CCL (CCL A and CCL B) in reverse micelles of AOT were previously studied by Otero et al. [14]. In these micellar media, CCL B had increased stability when the droplet size-related to W_o-decreased. Also, in a previous study [15] we used the micellar organization to develop the permanent microencapsulation method of this lipase, which improves its stability. In order to keep the maximum activity of the enzyme the polymerization process was studied at low W_o ($=3.3$). We showed that the permanent microencapsulation of CCL B in AOT micelles using acrylic prepolymers depended on the polymerization time and temperature [15], optimal conditions being 25°C and 1 h.

In this work the microencapsulation method of this lipase was optimized. The copolymerization of acrylamide + bisacrylamide in the presence of a crosslinking agent – glutaraldehyde – was studied. The influence of the monomers and glutaraldehyde concentrations in the reaction medium was investigated. Distinct lipase B microencapsulations where carried out at 25°C for 1 h.

Table 2 shows the residual activity and $t_{50\%}$ of the native and microencapsulated lipases. Immobilized lipases had low activities: 10–16% with respect to the native protein. Thus, Table 2 indicates the existence of possible diffusional limitations when the protein is covered by the acrylic microcapsule (Scheme 1, B). However, this method increased their stabilities, which were higher when higher concentrations of monomers (AA + BIS) were used. The addition of a crosslinking agent (G) enhanced the stability of the microencapsulated pure lipase. Thus, an acrylic microcapsule containing CCL B which was 352 times more stable than the native lipase, was prepared (Table 2).

Table 2
Activity and stability of the permanent microencapsulated lipase CCL B in reverse micelles of AOT

Biocatalyst	Residual[a] activity (%)	$t^{b}_{50\%}$ at 45°C	Stabilization factor
CCL B	100	45 min	1
CCL B-25°, 1H, [AA] = 0.24	12	12 h	17
CCL B-25°, 1H, [AA] = 0.4	11	2 days	67
CCL B-25°, 1H, [AA] = 0.32, [G] = 0.02%	11	5.5 days	176
CCL B 25°, 1H, [AA] = 0.24, [G] = 0.04%	14	7 days	224
CCL B-25°, 1H, [AA] = 0.32, [G] = 0.03%	10	11 days	352

Stability at 45°C in 0.1 M phosphate buffer, 0.1 M NaCl, pH 7.2.
[a]Residual activity of the enzyme after its immobilization.
[b]Time at which the enzyme activity is 50% of the initial value.

Previously, Otero et al. [16] achieved a 140 times higher stability with the crude CCL using a covalent multipoint attachment of the protein to Sepharose gels. But the stability of the native lipase decreases when the degree of purification rise [17], and the total inactivation of the pure lipase CCL B would occur during its linkage to Sepharose due to the high pH (9.5–10.0) required. As far as we know, the microencapsulation method described here is the only one which allows a stabilization factor for this pure lipase which is even higher than that previously described for this crude protein [16]. Moreover, the residual activity of the supported CCL on Sepharose (about 20%, [16]) was similar to the activity of microencapsulated CCL B (16%).

The micellar systems of AOT were not adequate to achieve the lipase stabilization by the simple action of a crosslinking agent such as glutaraldehyde in the protein [15]. However, we show here that the action of glutaraldehyde in the protein and the simultaneous formation of the microcapsule reinforced the stabilization effect of the microencapsulation method, without additional loss of activity (Table 2). An alternative lipase microencapsulation method has been recently described [18], which uses vesicles of a polymerizable surfactant. These authors showed the protection effect of the microencapsulation methods from the proteolytic degradation of the lipase. In this case, the enzyme activity was also decreased after microencapsulation according to our results of Table 2 [18]. In the present work the surfactant was not polymerized, but the polymerizable monomers which were added to the system were. The AOT is a good tensioactive because of the low polydispersity of its aggregates and their spherical shape. Thus, the microcapsules formed in micelles of AOT have quite homogeneous sizes and shapes [7].

Fig. 1 shows the thermoinactivation pattern of the native lipase, and two microencapsulated derivatives, in the presence and in the absence of the crosslinking agent. After a certain period of time the stabilities of the immobilized lipases rapidly decayed. The incubation time at which this effect appears depended on the thickness of the microcapsule [15]. This could be related to the fast delivery of the lipase to the solvent when the microcapsule is opened (or partly broken) after a certain incubation period [19].

The covalent linkage of an enzyme to a support usually requires high pH and/or temperatures; the simple adsorption of the protein to the matrix is not permanent, and its desorption takes place after a short time. In contrast, the permanent enzyme microencapsulation may be carried out at neutral pH and relatively low temperatures. Thus, in the case of pure and very labile enzymes, this microencapsulation could have in principle advantages over the more usual methods for its permanent immobilization. Also, these microcapsules are non-biodegradable and are adequate supports for biotransformations.

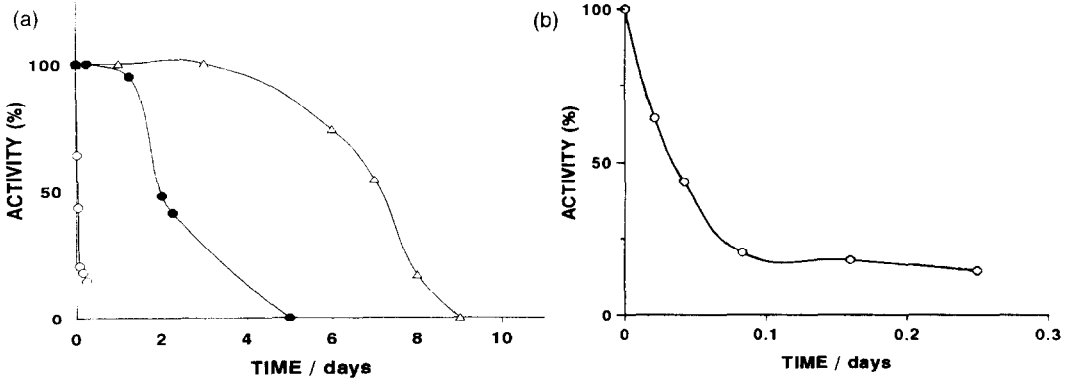


Fig. 1. (a) Stabilities of the native and microencapsulated CCL B at 45°C in buffer solution, pH 7.0. Conditions for the microencapsulation: $W_o = 3.3$; [AOT] = 0.1 M; [AA]/[BIS] = 3.2; 0.25 M sodium phosphate buffer, pH 7.2; 25°C; 1 h. (○) Non-microencapsulated CCL B; (●) [AA] = 0.4 mg/ml; (△) [AA] = 0.24 mg/ml + 0.04% glutaraldehyde. (b) The thermoinactivation curve of the native lipase.

Acknowledgements

This work has been financed by the Spanish CICYT (No. PB92-0495) and a predoctoral fellowship of the Spanish Ministerio de Educación y Ciencia.

References

[1] A. Takahashi, N. Nishimura, T. Yoshimoto, Y. Saito and I. Inada, Biochem. Biophys. Res. Commun., 121 (1984) 261.
[2] A.V. Kabanov, A.V. Levashov and V.Y. Alakhov, Protein Eng., 3 (1989) 39.
[3] P. Luthi and P.L. Luisi, J. Am. Chem. Soc., 106 (1984) 7285.
[4] A.K. Luthra, A. Williams and R.J. Pryce, J. Chem. Soc., Perkin Trans. II, (1987) 1575.
[5] A.I. Medalia, J. Polym. Sci., 6 (1949) 423.
[6] J.P. Davey, R.J. Pryce and A. Williams, Enzyme Microb. Technol., 11 (1989) 657.
[7] V. Vaskova, V. Juranicová and J. Barton, Makromol. Chem. Macromol. Sym., 31 (1990) 201.
[8] M.T. Reetz, A. Zonta and J. Simpelkamp, Angew. Chem., Int. Ed. Engl., 34 (1995) 301.
[9] M.J. Hernaiz, J.M. Sanchez-Montero and J.V. Sinisterra, J. Mol. Catal. A, Chemical, 96 (1995) 317.
[10] M.L. Rúa, T. Díaz-Mauriño, V.M. Fernández, C. Otero and A. Ballesteros, Biochim. Biophys. Acta, 1156 (1993) 181.
[11] D.J. Evans, A. Williams, A.R. Alcantara and R.J. Pryce, J. Mol. Catal., 81 (1993) 119.
[12] O.H. Lowry, N.J. Rosbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[13] A.R. Alcantara, J.V. Sinisterra, G.G. Guanti, S. Thea and A. Williams, J. Mol. Catal., 80 (1993) 137.
[14] C. Otero, M.L. Rúa and L. Robledo, FEBS Lett., 360 (1995) 202.
[15] L. Robledo, V. Vilas and C. Otero, Ann. NY Acad. Sci., 750 (1995) 89.
[16] C. Otero, A. Ballesteros and J.M. Guisan, Appl. Biochem. Biotechnol., 19 (1988) 163.
[17] C. Torres and C. Otero, J. Mol. Catal., 97 (1995) 119.
[18] E.W.J. Mosmuller, M.C.R. Franssen and J.F.J. Engbersen, Biotechnol. Bioeng., 42 (1993) 196.
[19] R. Langer and J. Folkman, Nature, 264 (1976) 797.