Selective and oriented immobilization of (phospho) lipases from the Caribbean Sea anemone *Stichodactyla helianthus* (Ellis, 1768) by interfacial adsorption

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Key words: immobilization, interfacial adsorption, lipases, phospholipases, Stichodactyla helianthus.

RESUMEN. Las lipasas inmovilizadas por adsorción interfacial a soportes hidrofóbicos constituyen sistemas enzimáticos con potencialidades comprobadas para diversas aplicaciones como la bioconversión. Por otro lado, las enzimas de invertebrados marinos muestran especificidades de sustrato poco usuales, que las convierten en blancos atractivos para el desarrollo de biocatalizadores inmovilizados. En este trabajo se describe la inmovilización por adsorción interfacial al soporte Octyl-Sepharose CL 4B de dos (fosfo)lipasas: las Sticholysinas I y II, y una actividad esterasa de alto peso molecular, a partir del extracto total de la anémona del mar Caribe *Stichodactyla helianthus*. La inmovilización fue selectiva para las lipasas, que mostraron actividad esterolítica frente a *p*-nitrofenilacetato, β-naftilcaprilato y tributirina. Esta actividad esterolítica de las Sticholysinas frente a sustratos no fosfolípidos no había sido informada con anterioridad. La actividad enzimática específica máxima aparente frente a *p*-nitrofenilacetato fue menor en los derivados inmovilizados que en las muestras solubles. La inmovilización resultó además orientada, ya que aumentó la afinidad aparente por el sustrato, lo que indica una mayor accesibilidad a los centros activos. El 68 % de las (fosfo)lipasas inmovilizadas conservaron la actividad hidrolítica frente a la tributirina. Estas enzimas requieren el ion Ca^{2+} para hidrolizar *p*-nitrofenilacetato y se inhibieron a concentraciones de *p*-nitrofenilacetato mayores que 1,185 mmol/L. Concentraciones de Ca^{2+} iguales a 40 mmol/L eliminaron la inhibición por exceso de sustrato en el extracto inmovilizado. Estas características cinéticas sugieren un posible uso de los biocatalizadores obtenidos en bioconversión enzimática.

ABSTRACT. Immobilized lipases by interfacial adsorption on hydrophobic supports are enzymatic systems which show confirmed potentialities for diverse applications such as bioconversion. On the other hand, enzymes from marine invertebrates show unusual substrate specificities, which make them promising targets for the development of immobilized biocatalysts. In this work, the authors describe the immobilization, by interfacial adsorption on Octyl-Sepharose CL 4B support, of two (phospho)lipases: Sticholysins I and II, and a high molecular weight esterase activity from the whole extract of the Caribbean Sea anemone *Stichodactyla helianthus*. Immobilization was selective for lipases, which showed esterolytic activity towards *p*-nitrophenylacetate, β-naphtylcaprilate and tributyrin. This esterolytic activity of the Sticholysins towards non-phospholipid substrates had not been previously reported. The apparent maximal specific enzymatic activity towards *p*-nitrophenylacetate was lower in the immobilized derivatives than in soluble samples. Immobilization was also oriented, since it increased the apparent affinity by substrate, which indicates a higher accessibility to the active sites. 68 % of immobilized (phospho)lipases preserved tributyrin-hydrolyzing activity. These enzymes require Ca^{2+} ion to hydrolyze *p*-nitrophenylacetate and were inhibited by *p*-nitrophenylacetate concentrations higher than 1.185 mmol/L. Ca^{2+} concentrations of 40 mmol/L overcame the substrate-excess inhibition from immobilized extract. These kinetic characteristics suggest the potential use of the obtained biocatalysts on enzymatic bioconversion.

INTRODUCTION

Many successful industrial applications of lipases are based on immobilized forms of these enzymes.[1-3] The typical interfacial activation phenomenon[4] is technologically handled to selectively adsorb lipases on solid hydrophobic supports which resemble interfaces formed by natural substrates. This interfacial adsorption, which is produced at low ionic strength for very hydrophilic proteins, is not simply a result of hydrophobic interactions[5] and produces biocatalysts which show strongly adsorbed and highly active enzymes.[6] This methodology is simple, does not require expensive or toxic reagents, can retain or increase specific activity, can raise stereoselectivity and in several cases allows reusing the support, due to the occurrence of reversible interactions under some conditions.[7]

Marine invertebrates are a potential and relatively few explored source of lipases.[8] Therefore, interfacial adsorption-based biocatalysts derived from these organisms have certain probability of show unusual and helpful substrate specificities for industrial applications such as bioconversion. Phospholipase and lipase activi-

ties have been reported in the whole extract of *S. helianthus*.[8,9] The authors previously purified and enzymatically characterized two isotoxins with haemolytic and phospholipase A_2 (PLA_2) activities: Sticholysins I and II (StI and StII, respectively)[10] by affinity chromatography from this extract.[11] The purposes of the current study were to obtain Sticholysins-derived immobilized biocatalysts and to carry out their kinetic characterization in light of their potential application on bioconversion.

MATERIALS AND METHODS

Preparation of the extract of *S. helianthus* (Cnidaria; Anthozoa; Actinaria; Stichodactylidae) and phospholipase A-affinity chromatography

Whole animals, collected by apnea dive at the coast of Havana City, were homogenized in distilled water [1 : 2 (w/v) 4 °C].[12] Extracts were centrifuged twice during 1 h at 15 000 g and 4 °C. Supernatants were diafiltrated with 50 mmol/L Tris-HCl, 40 mmol/L $CaCl_2$, pH 7.5 buffer [1 : 1000 (v/v) 4 °C] using an Amicon ultrafiltration device (Millipore, Billerica, MA, USA) equipped with a membrane of cut-off 0.5 kDa. The whole extracts were kept at -20 °C until use.

The PLA-affinity matrix was obtained by the following procedure: Sepharose CL 4B (Sigma-Aldrich, St. Louis, MO, USA) was activated with glycidol (1,2-epoxy-3-propanol, Sigma-Aldrich, St. Louis, MO, USA) and oxidized with sodium peryodate as previously described.[13] The glioxyl-sepharose CL 4B was aminated with ethylenediamine (Merck & Co., Inc., Whitehouse Station, NJ, USA) according to Fernández-Lafuente *et al.*,[14] to obtain the monoaminoethyl-*N*-aminoethyl (MANA)-Sepharose CL 4B matrix. Phosphatidylcholine, purified from egg yolk (ePC) as described by Singleton *et al.*,[15] was oxidized with sodium peryodate according to Shimojo *et al.*[16] and immobilized to MANA-Sepharose CL 4B using 1-ethyl -3-(dimethylaminopropyl)carbodiimide (Sigma-Aldrich, St. Louis, MO, USA).[17] The matrix was validated with commercial *Crotalus durrisus terrificus* PLA_2 (Sigma-Aldrich, St. Louis, MO, USA).

Whole extract of *S. helianthus* (42 mg of protein) was applied onto the ePC-MANA-Sepharose CL 4B column (1.5 cm x 5 cm), previously equilibrated with binding buffer (50 mmol/L Tris-HCl, 40 mmol/L $CaCl_2$, pH 7.5) at 15.6 cm/h linear flow. As many secreted PLA_2 require Ca^{2+} to interact with phospholipid substrate, buffer binding contains $CaCl_2$. The column was exhaustively washed with binding buffer and elution was carried out with 50 mmol/L Tris-HCl, 40 mmol/L ethylen-diaminetetraacetic-acid (EDTA) buffer (pH 7.5) at 17.6 cm/h flow. Fractions of 3.5 mL were monitored at 280 nm. Eluted fractions were diafiltrated as above mentioned with 50 mmol/L Tris-HCl buffer, pH 7.5 [1 : 1000 (v/v)] to remove EDTA. Protein concentration[18] and enzymatic activity towards *p*-nitrophenylacetate (*p*-NPA; Sigma-Aldrich, St. Louis, MO, USA; see below) were determined in each fraction.

Protein immobilization by interfacial adsorption

Protein immobilization was performed according to Bastida *et al.*[6] Briefly: enzymatic samples were diluted [1 : 10 (v/v)] with 0.01 mol/L sodium phosphate buffer, pH 7.0, incubated with the Octyl-Sepharose CL 4B support (Sigma-Aldrich, St. Louis, MO, USA) during 5 h, at 4 °C and constant stirring, filtrated and washed with the immobilization buffer (0.01 mol/L sodium phosphate, pH 7.0). Protein loads during immobilization [protein (mg of protein / mL of support) were the following: 0.54 mg (extract) and 3.64 mg (affinity peak). Activity loads [enzymatic activity units (U)/ mL of support] were: 8 U (both samples with *p*-NPA), 15.80 U [extract with tributyrin (Merck & Co., Inc., Whitehouse Station, NJ, USA)] and 16.91 U (affinity peak with tributyrin).

The immobilization control parameters used and their respective mathematical equations are listed below. Protein concentration assays were performed according to Bradford.[18] Enzymatic determinations are described below.

Differential protein immobilization grade (immobilized total protein (mg of immobilized total protein / mL of support).[19]

diff. IG (prot.) = (mg of initial total protein / mL of support) – (∑ mg of initial total protein (foltrates + washes) / mL of support).

Differential immobilization grade (total units of immobilized enzymatic activity / mL of support).[19]

diff. IG = (initial total U / mL of support) – (∑ total U (filtrates + washes) / mL of support).

Direct immobilization grade (total units of immobilized enzymatic activity / mL of support).[19]

direct IG = total U / support mL.

Immobilization percentage. Fraction of the initial enzymatic activity that is immobilized (differential method).[6,7]

I (%) = [(diff. IG · mL of support)/initial total U] · 100

Functional activity retention percentage. Fraction of the initial enzymatic activity that is immobilized (direct method).[19]

FAR (%) = [(direct IG · mL of support)/initial total U] · 100

Over-expression of the enzymatic activity (hyperactivation) caused by immobilization.[6,7]

Hyperactivation = direct IG/diff. IG

Protein electrophoresis, immunoblotting and esterases-specific zimography

Protein samples (20 μg) were analyzed by polyacrylamide gel electrophoresis (PAGE), under reducing conditions with 15 % acrylamide and using sodium dodecylsulphate (SDS). Coomassie Brilliant Blue R-250 was used for staining.[20] SDS-PAGE-resolved proteins were transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA) and developed with a rabbit polyclonal antiserum anti-StII (1 : 200 dilution)[21] followed by an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1 : 10 000 dilution, Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA). The presence of Sticholysins was detected with 0.02 mmol/L diaminebencidine (Sigma-Aldrich, St. Louis, MO, USA) and 0.015 % hydrogen peroxide diluted in phosphate-buffered solution. The esterase-specific zimography was performed according to Hunter and Markeat.[22] Briefly: the electrophoresis gel, resultant of a SDS-PAGE under non-reducing conditions, was washed with 20 % Triton X-100, and the reaction was developed by incubating with 500 μL of 200 mmol/L β-naphtylcaprilate (Sigma-Aldrich, St. Louis, MO, USA) and 500 μL of 100 mmol/L Fast Blue RR (both in dimethylsulphoxide) in 100 mL of 100 mmol/L sodium phosphate buffer, pH 7.0, overnight, at 25 °C and darkness.

Kinetic assays

Kinetic assays were performed according to Sabuquillo *et al.*[5] with modifications. *p*-NPA-hydrolyzing activity was assessed by continuous and spectrophotometric measurement of the *p*-nitrophenol releasing at

a wavelength of 348 nm [$\varepsilon_{348\ nm}$ = 5150 $(mol/L)^{-1}\ cm^{-1}$].[23] Automatic determinations (Spekol, Germany) were performed under magnetic stirring, at 30 °C, in a 1 cm cuvette. The composition of the reaction mixtures were the following: 0.01 mg/mL final protein concentration for soluble enzymatic samples or 1 : 182 dilutions for immobilized derivatives, 1.185 mmol/L *p*-NPA and 40 mmol/L $CaCl_2$ for evaluation of immobilization, 0 - 5.13 mmol/L *p*-NPA and 0 or 40 mmol/L $CaCl_2$ for kinetic characterization, 25 mmol/L Tris-HCl buffer, pH 8.0.

Tributyrin-hydrolyzing activity was assessed by the pH-stat method.[5] Automatic determinations (Mettler-Toledo DL-21, Mettler-Toledo, Inc., Columbus, OH, USA) were performed under magnetic stirring at 30 °C, pH 8.0 and using 0.01 mmol/L NaOH as titrating solution. Tributyrin was emulsified with 3 % arabic gum (Sigma-Aldrich, St. Louis, MO, USA) and 10 mmol/L Tris-HCl, 40 mmol/L $CaCl_2$ buffer (pH 8.0) by mixing 10 min and sonicating 15 min in an ultrasonic bath (BRANSON 1200, Branson Ultrasonics Corporation, Danbury, CT, USA). Reactions were initiated by adding sample of 0.01 mg/mL final protein concentration to the reaction mixture. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmoL of *p*-nitrophenol or butyric acid per minute under the assay conditions. The results were expressed as specific activity (U/mg of total protein) as the mean of three parallel assays.

RESULTS AND DISCUSSION

Immobilization

Immobilization of the whole extract of *S. helianthus* by interfacial adsorption on the support Octyl-Sepharose CL 4B leaded to a decreasing in the heterogeneity of this sample (Fig. 1a). The most abundant component of the immobilized extract and the only resolved band of the immobilized affinity peak matched with the molecular weight (~ 19 kDa) of the isophospholipases previously purified by affinity chromatography.[11] Immunoblotting (Fig. 1b) confirmed that the immobilized 19 kDa-proteins are StI, StII or most probably both, which present immunochemical identity.[21] Adsorption of the isoenzymes on the interface provided by Octyl-Sepharose produced an enzymatically active biocatalyst, able to hydrolyze ester substrates (Fig. 1c). The extract of the anemone also contains, at least, a high molecular weight lipase which was not purified by PLA-affinity chromatography and, in consequence, it is absent in the immobilized affinity peak (Fig. 1c). Since Sticholysins are immobilized by interfacial adsorption, a lipase-selective immobilization method,[5-7] and they are able to hydrolyze phospholipids,[11] like phospholipases, and the general ester substrate β-naphtylcaprilate (Fig. 1c), the authors use the functional ambiguous term "(phospho)lipase" for these isotoxins.

This is the first report about the immobilization of the Stycholysins by interfacial adsorption. The high affinity of StI/StII by hydrophobic interfaces is an expected result. These isotoxins belong to a known group of sea anemones cytolytic polypeptides which insert spontaneously into membranes and promote cell lysis.[24] Interaction of StI and StII with cellular membranes to develop their biological function is required;[25] therefore they are interfacial proteins.[26]

Only 44 % of protein load from the heterogeneous extract was immobilized (Table 1). This is due to the presence of non-interfacial or non-hydrophobic components rather than a protein load excess, since the load of the affinity peak is 6.8 times higher and 100 % of protein immobilization was achieved. In contrast to the extract, the affinity peak only contains (phospho) lipases;[11] Figure 1 (a and b). The diff. IG (prot.) reached for the affinity peak is in agreement with the capacity of the Octyl-Sepharose CL 4B commercial support: 3-5 mg of an 18 kDa-protein by mL of gel.

Evaluation of the immobilization with the esterases-general substrate *p*-NPA showed the presence of esterases which are not lipases in the *S. helianthus* extract (38 % I, Table 1). Although the soluble extract only showed in the β-naphtylcaprilate-zimography the same two bands which were appreciated in the immobilized derivative (Fig. 1c), the presence of other esterases in both samples should not be discarded. Thus, the existence of other lipases in the immobilized extract or other lipases or esterases in the soluble extract, which cannot hydrolyze β-naphtylcaprilate in the polyacrilamide environment or do not renaturalize properly after of the Triton treatment, it is entirely possible.

The selectivity of interfacial adsorption for lipases was confirmed by the immobilization of all *p*-NPA-hydrolyzing enzymes from the homogeneous affinity peak and all tributyrinase activity from both protein samples (Table 1). Because tributyrin is a short-chain triacylgliceride, it is not a fully true lipase substrate. However, its hydrophobicity makes it a widely-used lipase substrate, and its little size allows the direct evaluation and characterization of the immobilized derivatives. This differential behaviour between esterase and lipase activities during interfacial adsorption has already been described by other authors. For example, octyl-sepharose-immobilization of a *Rhizopus niveus* lipase showed a 20 % of residual activity towards the

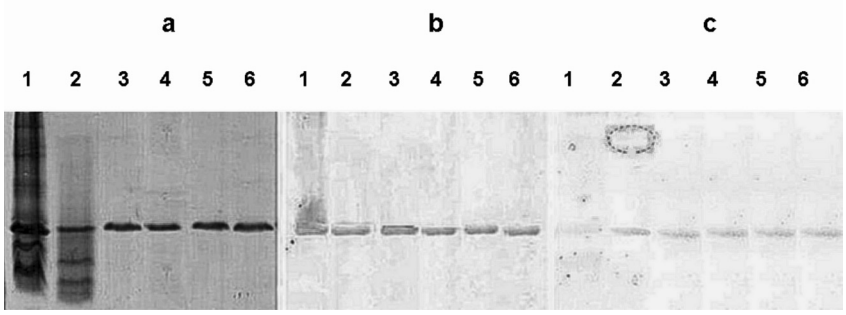

Fig. 1. *a) Reducing SDS-15 % PAGE with Coomasie staining. b) Immunoblotting with a polyclonal antiserum anti-StII. c) Esterases-specific zimography with β-naphtylcaprilate. Lanes 1. Soluble extract. Lanes 2. Immobilized extract. Lanes 3. Soluble affinity peak. Lanes 4. Immobilized affinity peak. Lanes 5. StI. Lanes 6. StII. Dotted circle: high molecular weight lipase.*

Table 1. Interfacial adsorption of the whole extract of *S. helianthus* and the affinity peak on Octyl-Sepharose CL 4B support

Protein sample	diff. IG (prot.) (mg prot. / mL supp.)	Substrate	diff. IG (U / mL supp.)	direct IG (U / mL supp.)	I (%)	FAR (%)	Hyper-activation
Extract	0.24 ± 0.03	*p*-NPA	3.04 ± 0.44	1.40 ± 0.09	38	17.5	0.46
Affinity	3.64 ± 0.36		7.94 ± 0.46	0.36 ± 0.04	99.3	4.5	0.05
Extract	0.24 ± 0.03	tributyrin	15.80 ± 1.62	4.35 ± 0.47	100	27.5	0.28
Affinity	3.64 ± 0.36		16.91 ± 1.5	11.46 ± 1.18	100	67.8	0.68

diff. IG (prot.) Differential protein immobilization grade. diff. IG Differential immobilization grade. direct IG Direct immobilization grade. I Immobilization (%). FAR Functional activity retention (%). Protein loads (mg of protein / mL of support): 0.54 (extract) and 3.64 (affinity peak). Activity loads (U / mL of support): 8 (both samples with p-NPA), 15.80 (extract with tributyrin) and 16.91 (affinity peak with tributyrin).

ester substrate: *p*-nitrophenyl-propionate, and a 100 % I with the lipase substrate: olive oil.[5] Using of interfacial adsorption method to selectively immobilize lipases from complex mixtures had already been applied to different biological samples with dissimilar purposes,[5-7,27,28] but this is the first report for proteins from *S. helianthus*.

Direct evaluation of the immobilized catalytic activity showed the highest values of FAR using tributyrin. This result is not only due to the selectivity of the immobilization method by lipases, since 100 % of esterase activity from affinity peak was immobilized with a very low percentage of FAR (Table 1). It is possible a substrate partition effect[29] that might produce an accumulation of hydrophobic tributyrin at the octyl-water interface, or a higher ability of the immobilized (phospho)lipases to hydrolyze tributyrin than *p*-NPA, as showed the highest FAR % and hyperactivation obtained for the affinity peak using tributyrin (Table 1). This result is in agreement with the documented capability of the physical adsorption methods to retain the functional activity in a high proportion.[30] The immobilized high molecular weight lipase seems to be a higher ability to hydrolyze *p*-NPA than tributyrin, as showed a hyperactivation near of 50 % for the extract towards *p*-NPA, in spite of the modest I %. Although it is known the ability of (phospho)lipases to catalyze the hydrolysis of *p*-NPA,[31] the esterolytic activity of the Sticholysins towards non-phospholipid substrates had not been previously reported (Fig. 1c; Table 1).

Kinetic characterization

The diminishing of the apparent maximal specific enzymatic activity (app.$_{max.}$ spEA) towards *p*-NPA in the *S. helianthus* extract in presence of 40 mmol/L $CaCl_2$ (optimum concentration for the PLA_2 activity of the Sticholysins,[11] Fig. 2a, Table 2) may be due to the existence of esterase activity in the heterogeneous extract that is inhibited by the ion. Since the Sticholysins absolutely require Ca^{2+} to hydrolyze *p*-NPA, the substrate-excess inhibition effect showed by the immobilized extract in absence of $CaCl_2$ may be due to the esterase activity of the high molecular weight lipase (Figures 1c and 2b). The soluble extract showed a Michaelis's classical kinetic, reflecting the masking of the substrate inhibitory effect by the contribution of non-interfacial esterases (Table 1; Fig. 2a).

Even though the adsorption techniques produce bioreactors which show high retentions of the catalytic activity,[30] this principle only is completely valid for pure enzymes. Immobilization of heterogeneous extracts using selective methods for specific enzymatic fractions produces more pure systems with a correlation of catalytic constants that is different to that the soluble material. In addition of the protein-support interactions, this fact influences on the overall enzymatic activity. The diminishing of the app.$_{max.}$ spEA of the extract caused by immobilization (Figure 2 (a and b), Table 2) reflects the mentioned disparity between the group of esterase present in the soluble extract and the group of enzymes that were immobilized from this sample.

It has been proposed that the active site of lipases stay buried in aqueous solution, avoiding substrate access. As a result, lipases are inactive in this state. In contrast, lipid-water interfaces provided by natural substrates stabilize the active conformation of lipases, in which the active site is accessible to the substrates. This means that the native structure of lipases is a dynamic arrangement as a consequence of the equilibrium between the "closed" structure, which prevails in homogeneous environments, and the "open" structure, which is stabilized at lipid-water interfaces. Hence, in presence of a lipid-water interface the equilibrium is displaced to the "open" structure and the affinity of lipases by their substrates increases.[32]

Solid hydrophobic supports, as Octyl-Sepharose CL 4B, resemble interfaces formed by natural substrates of lipases. Therefore, the interfacial environment provided by octyl groups of Octyl-Sepharose in aqueous media could cause the same effect on lipases that the natural lipid-water interfaces. In this fashion, interfacial adsorption could involve regions at protein surface distinct to the active site entrance, could produce a "freezing" of the enzyme structure in its "open" form[32] and, consequently, increases the apparent affinity of immobilized enzymes by their substrates, regarding the soluble molecules.[5] On the other hand, interfacial adsorption is not an affinity adsorption. The latter involves the active site and produces inactive immobilized derivatives, since the active site is occupied by a substrate analog.[7] According to this, the increasing of the apparent affinity towards *p*-NPA by the esterases from the extract, caused by immobilization (Fig. 2, Table 2), suggests that the interfacial adsorption of these enzymes on Octyl-Sepharose CL 4B support is an oriented process. In other words, during interfacial adsorption lipases might be immobilized by a region which does not involve the active site entrance and in a fashion that generates an active site more accessible for the substrates.[5]

The role of Ca^{2+} as cofactor of the *p*-NPA-hydrolyzing activity and the phospholipase activity[11] showed by the Sticholyisins, are in agreement with some reports in the PLA_2 topic.[33] The substrate-excess inhibition observed in the affinity peak (Fig. 2c), in contrast of the inhibition showed by the immobilized extract in absence of calcium (Fig. 2b), it is related to enzymes that need the ion for activity. Therefore, the Ca^{2+}-dependent elimination of the

substrate-excess inhibition in the immobilized extract was due to the direct influence of the cation upon the esterase activity of the high molecular weight lipase or another not-visualized Ca^{2+}-sensible esterase activity.

The diminishing of the app.$_{max.}$ spEA of the affinity peak caused by immobilization (Figures 2c and d, Table 2) was in agreement with the low FAR % and hyperactivation values of this sample using *p*-NPA (Table 1). As it is expected, taking into account the result corresponding to the immobilized extract and the report from Sabuquillo *et al*.,[5] immobilization of Sticholysins by interfacial adsorption increased affinity by the substrate (Figures 2c and d, Table 2).

CONCLUSIONS

Immobilization of Sticholysin I and II, as components of the heterogeneous extract of *S. helianthus* or together in the more pure affinity peak, by interfacial

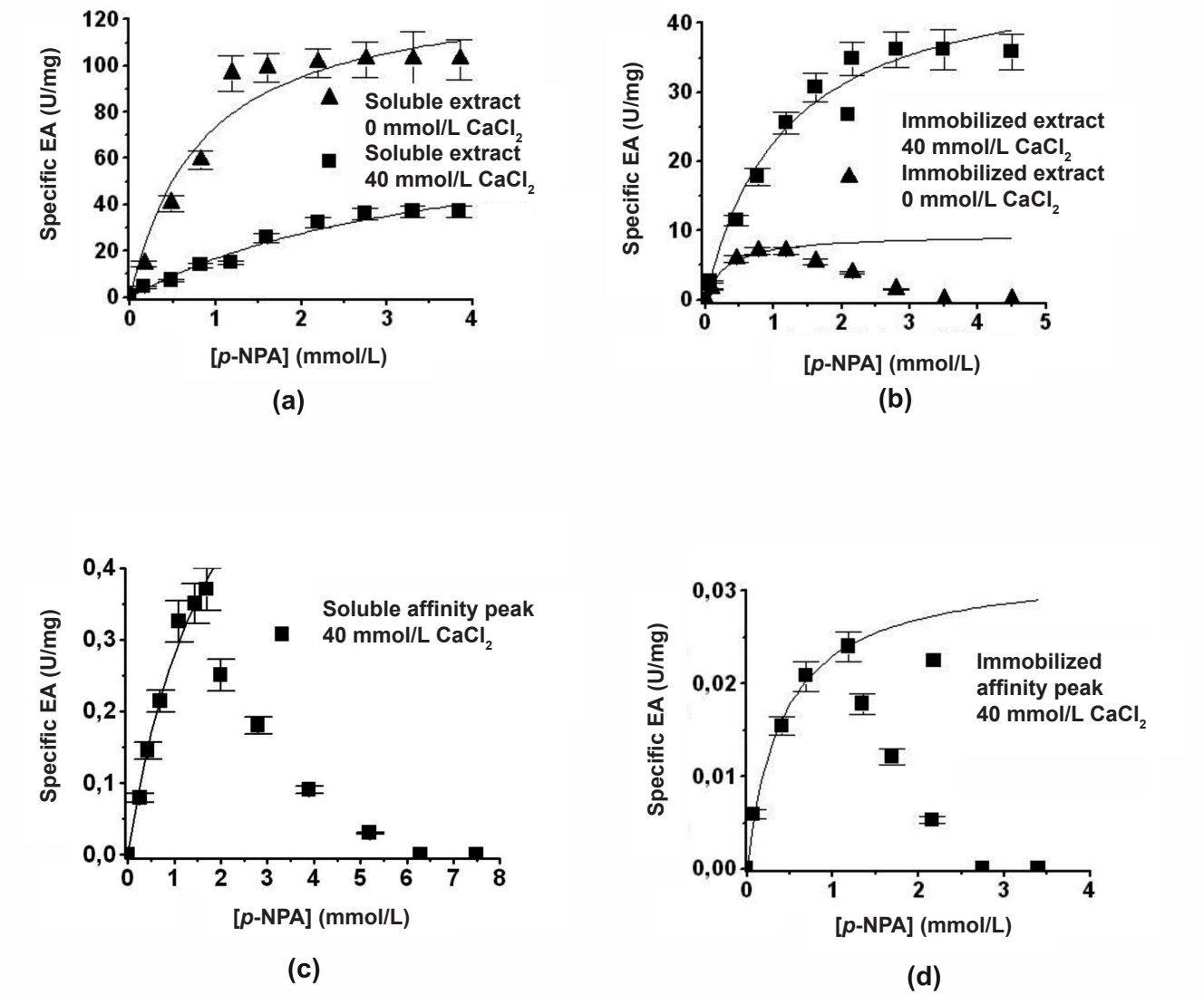

Fig. 2. *Michaelis-Menten's curves with p-NPA. Initial velocity values were expressed as specific enzymatic activities (specific EA). a) Soluble and b) immobilized S. helianthus extract. c) Soluble and d) immobilized affinity peak. Fitting to rectangular hyperbolas was made with an appropriate software. For fitting in samples that showed the substrate-excess inhibition effect, only the first 5 (b and d) or 7 (c) points were taken. Error bars indicate standard deviation of the mean for three independent measurements.*

Table 2. Values of app.$_{max}$. spEA and apparent K_M (app. K_M) for soluble and immobilized protein samples, in absence and presence of $CaCl_2$.

Protein samples		0 mmol/L $CaCl_2$		40 mmol/L $CaCl_2$	
		app.$_{max.}$ spEA (U/mg)	app. K_M (mmol/L)	app.$_{max.}$ spEA (U/mg)	app. K_M (mmol/L)
Extract	Soluble	134.6 ± 12.5	0.8 ± 0.2	77.8 ± 15.0	3.6 ± 1.2
	Immobilized	9.5 ± 1.0	0.3 ± 0.1	49.0 ± 3.4	1.2 ± 0.2
Affinity	Soluble	–	–	0.8 ± 0.1	2.0 ± 0.5
	Immobilized	–	–	0.033 ± 0.002	0.4 ± 0.1

Maximal velocity values were expressed as app.max. spEA.

adsorption on Octyl-Sepharose CL 4B support, produced enzymatically active biocatalysts, able to hydrolyze non-phospholipid ester substrates. This immobilization was selective for lipases, among other esterases, and oriented, since it increased affinity by *p*-NPA, indicating active sites more accessible for substrates. On the other hand, Sticholysins require Ca^{2+} to hydrolyze *p*-NPA. The kinetic characterization performed in this work indicates the potential use of these bioreactors on bioconversion of different active principles.

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