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α-Amylase kinetic parameters modulation by lecithin vesicles: binding versus entrapment

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Abstract

The effect of α-amylase–lipid interaction and entrapment inside lipidic soybean lecithin (PC) vesicles on the kinetic parameters of the enzyme was investigated. Human saliva was used as enzyme source and starch as substrate. Reaction kinetics were followed by measuring the consumption of starch as revealed by the decrease in the absorbance at 600 nm of the I_2–starch complex. The presence of PC induced a decrease in A_{600} at low concentrations; above 0.1 g PC/l light scattering became important and an increase in absorbance was observed as a function of lipid concentration. These effects were taking into account in the interpretation of results. Multilamellar vesicles (MLVs) and giant unilamellar vesicles (GUVs) were prepared by established procedures; enzyme was 'entrapped' by the inclusion of saliva, conveniently diluted, in the buffer used for lipid dispersion during the preparation of vesicles. The kinetic parameters K_M and V_{max} were determined by adjusting saturation curves to the Michaelis–Menten equation by a computer assisted nonlinear regression analysis by the least squares method. The presence of PC in MLVs induced an increment of the value of K_M of α-amylase for starch as the substrate both in the condition we designated as 'entrapped' and when the enzyme was applied to the already formed liposomes ('untrapped'). The effects exerted by PCs MLVs on K_M and V_{max} were statistically significant only at the highest PC/protein mass ratio used (28 g/g). GUVs affected amylase K_M only in some of the 'entrapped' samples at random suggesting certain aggresiveness of the GUVs preparation method. V_{max} decreased significantly only in the 'entrapped' samples. The higher effect of MLVs in reducing the affinity of the enzyme for starch compared with that of GUVs is probably due to a higher protein adsorbing capacity of the lower molecular packing, higher surface tension and more curved MLV surface compared with the GUV surface. The binding of the enzyme to the lipidic surface is reversible and can be modulated by the presence of high salt concentrations or with the pH of the media which affect the surface electrostatics of both, the lipidic and the protein surfaces. The conclusions drown from the present experiments represent the average behavior of all the isozymes that represent α-amylase from human saliva and may be useful for understanding α-amylase activity in heterogenous media of physiological and technological importance. © 2000 Elsevier Science B.V. All rights reserved.

Abbreviations: ANOVA, analysis of variance; GUV, giant unilamellar vesicle; MLV, multilamellar vesicle; PC, soybean phosphatidylcholine; SDS, sodium dodecyl sulphate; s.e.m., standard error of the mean; SUV, small unilamellar vesicle; T, trapped; UT, untrapped.

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1. Introduction

α-Amylase [1→4 glucan 4-glucanohydrolases, EC 3.2.1.1] is an endoenzyme that catalyses the breaking of α-1,4-glucosidic bonds in amylose and amylopectin molecules. This type of enzyme is widely used in the sugar, textile and brewery industries, one of its most important applications concerning the liquefaction of starch to oligossacharides for subsequent production of glucose syrups by glucoamylases (see Ref. [1]). It is incorporated as an additive in dough formulae with an effectiveness that depends on an optimum α-amylase/emulsifiers ratio (see Ref. [2]). Enzyme sources containing amylase have been proved to be effective in improving the utilization of starch from cereal grains in poultry diets, especially those for young animals [3]. This enzyme is present in animals, plants, bacteria and fungi [4]. In humans the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of the polymeric substrate into shorter oligomers. Later on in the gut these are further hydrolyzed by pancreatic α-amylase into maltose, maltotriose and small maltooligosacharides.

In most technological applications as well as in physiological conditions, α-amylase exerts the enzymatic catalysis of substrate hydrolysis in media of complex chemical composition and heterogeneous from the physical point of view. The former quality of the environment implies the possible enzyme coexistence with typical competitive or non-competitive soluble modulators (activators or inhibitors). The latter, adds the possibility of α-amylase adsorption to interfaces; this may be another modulating mechanism of its enzymatic activity.

In the present paper we investigated the effect of α-amylase–lipid interaction and α-amylase entrapment inside lipidic vesicles on the kinetic parameters of the enzyme.

2. Materials and methods

2.1. Materials

All the reagents and solvents used were of analytical grade. Human saliva diluted 4–40 times with bi-distilled water was used as enzyme source.

2.2. Preparation of liposomes and enzyme encapsulation

Giant unilamellar liposomes (GUVs) were prepared according to Moscho et al. [5]. Briefly, soybean phosphatidylcholine (PC) was dissolved in chloroform (0.1 M), and 20 μl of this solution was added to a 100 ml round bottom flask containing 980 μl of chloroform and 150 μl of methanol. The aqueous phase (7 ml of distilled water or buffer) was then carefully added along the flask walls. The organic solvent was removed in a Bucchi rotating evaporator under reduced pressure. After evaporation for 2 min, an opalescent fluid was obtained with a volume of ≈6.5 ml. The resulting aqueous solution contained GUVs. Enzymatic preparation was added to the buffer solution prior to evaporation of the organic solvent (trapped, T) or to the GUVs suspension (untrapped, UT).

In some experiments, multilamellar vesicles (MLVs) were used [6]; they were prepared by evaporating, under reduced pressure, a chlorophormic solution of PC. The dry lipid was suspended in water (1.5 g PC/l, final concentration) containing (trapped) or not (untrapped) enzyme preparation, by repeating six consecutive cycles of heating for 2 min at 21°C, which is a temperature above the corresponding T_c of the lipid, and vortexing for 1 min. In these conditions, PC self-aggregate into multilamellar vesicles [7].

2.3. Effect of lipid and detergents on the spectroscopic behavior of I_2–starch complex

Aqueous solutions containing: starch (0.19 g/l) and I_3^- (1.2 mM) ($[I_2]$/[starch] ratio 6.3 mmol/g): (i) in the absence or in the presence of; (ii) 0.12 g/l SDS; (iii) 4.6×10^{-4} g/l PC; or (iv) both were prepared and their visible-spectra recorded in a double beam Beckman DU 7500 spectrophotometer equipped with a diode array detector and 0.0001 AU sensitivity.

In a separate experiment, the effect of 1.3 g/l Triton X-100 on the visible spectra of I_2–starch complex and on I_3^- was tested in solutions containing: (i) 1.57 g/l starch and 2.94 mM I_3^-; or (ii) 2.94 mM I_3^-, and compared with the corresponding control sample without the detergent.

The effects of sodium desoxicholate (0.64 g/l) and Lubrol PX (0.64 g/l) were also tested, however, as well as 0.19 g/l Triton X-100, at a $[I_2]$/[starch] concentration ratio above 4.4 mmol/g, particularly in strong acidic medium, they precipitated.

2.4. Determination of enzymatic activity

Essentially, the method of Caraway [8] was used, which is based on the determination of the decrement in the concentration of starch, revealed by the formation of the blue colored I_2–starch complex in the presence of I_3^- ions. The incubation system containing saliva (28.5 μg protein/ml), starch ranging from 0.5 to 5 g/l (final concentration) in a final volume of 0.175 ml unbuffered bi-distilled water pH 5, was incubated for 15 min at room temperature. The reaction was stopped by the addition of 3 ml of I_2/KI solution (1.67 mM I_2 and 16.2 KI mM) in 0.11 M HCl, and the absorbance at 600 nm was measured 20 min latter. Starch concentration was calculated by interpolating the decrement in absorbance at 600 nm in a calibration curve of ΔA_{600} versus starch concentration. The optimal concentration of enzymatic preparation, incubation time and I_3^- concentration were determined in previous experiments.

2.5. Determination of protein concentration

Protein concentration was determined by the method of Lowry [9].

2.6. Determination of kinetic parameters of α-amylase and statistical analysis

The values of K_M and maximal velocity (V_{max}) were determined by adjusting the experimental data from the saturation curve to the equation of Michaelis–Menten by a computer aided nonlinear regression analysis by the least squares method. The effects on the kinetic parameters exerted by lipid concentration, type of vesicles (MLV or GUV) and the enzyme condition of 'trapped' or 'untrapped' were analyzed by a 3-way ANOVA test; the post hoc LSD test was used for individual comparisons [10].

2.7. Effect of enzyme–phosphatidylcholine interaction on the enzymatic kinetics

The enzymatic kinetic parameters were determined using an aqueous solution of the enzymatic preparation in the absence or in the presence of a suspension of either MLVs or GUVs or using MLV- or GUV-trapped enzyme. Final PC/protein ratio varied between 0 and 28 g/g. The effect, on the saturation curve, of 1.4 g/l SDS, was tested in the presence of an amount of enzymatic preparation equivalent to 28.5 mg protein/ml. So, the ratio SDS/protein was 49 g/g.

2.8. Distribution of protein and enzymatic activity between lipidic and aqueous phase

The enzymatic preparation (285.5 μg/ml) was added to a dispersion of MLVs (PC final concentration ranging from 0 to 5 g/l) and incubated for 20 min at 21°C and then, the mixture was centrifuged at $30\,000 \times g$ for 40 min. The pellet and the supernatant were separated and the α-amylase activity was determined in both fractions as indicated previously in the presence of 5 g/l starch (saturating concentration, see Fig. 5).

2.9. Effect of saline concentration and pH on the adsorption of α-amylase to phosphatidylcholine multilamellar vesicles

The enzymatic preparation (285.5 μg/ml) was incubated in the presence of PC at a [PC]/[protein] = 2 g/g in water at pH 5 containing NaCl 140 mM or at pH 5, 3 or 9 in the absence of salt. After an incubation for 20 min at 21°C, the samples were centrifuged for 30 min at 30 000 × *g*. The pellet was resuspended in 10 mM Tris–HCl buffer at pH 7 and the α-amylase activity was measured in the presence of 5 g/l starch.

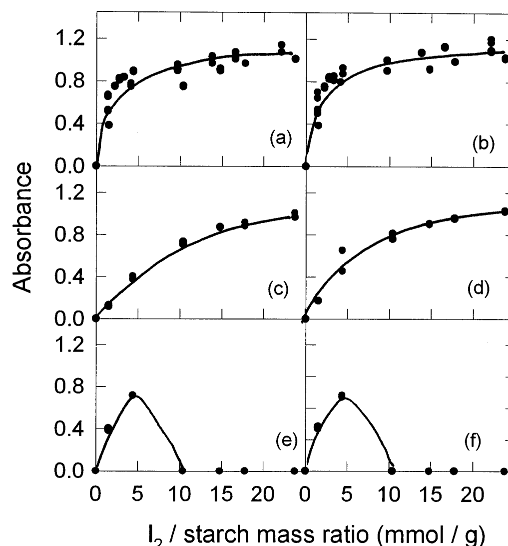

Fig. 2. Optimal I_2/starch mass ratio required for a quantitative determination of starch concentration in the presence and in the absence of PC and detergents. Samples contained the I_2/starch mass ratio diluted in water (a), (b), 0.12 g/l SDS (c), (d) or 0.19 g/l Triton X-100 (e), (f) in the absence (a), (c), (e) or in the presence (b), (d), (f) of 0.006 g/l PC. Concentrations are referred to the final volume after the addition of I_2/I^- solution.

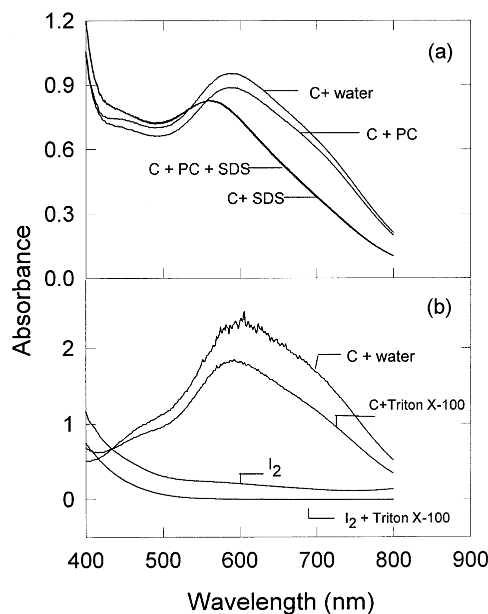

Fig. 1. Effect of soybean lecithin and detergents on the spectroscopic characteristics of I_2–starch complex. (a) I_2–starch complex control sample containing starch (0.19 g/l) and 1.2 mM I_2/12 mM KI alone (C + water) or containing 0.00046 g/l soybean phosphatidylcholine (C + PC) or 0.12 g/l sodium dodecylsulphate (C + SDS) or both (C + PC + SDS). (I_2/starch mass ratio = 6.3). (b) I_2: 2.94 mM I_2/ 29.4 mM KI alone (I_2) or in the presence of 1.57 g/l starch without (C + water) or with 0.19 g/l Triton X-100 (C + Triton X-100). (I_2/starch mass ratio = 1.87). Concentrations are referred to the final volume after the addition of I_2/I^- solution.

3. Results

3.1. Effect of lipid and detergents on the spectroscopic behavior of I_2–starch complex

Fig. 1 shows the visible absorption spectra of I_2–starch complex. Both PC (Fig. 1(a)) as well as Triton X-100 (Fig. 1(b)) induced an hypochromic effect on the absorbance of I_2–starch complex within the whole wavelength range tested; the hypochromic effect of SDS was observed at wavelengths above 570 nm; moreover, SDS induced an hipsocromic change in the λ_{max} value from 580 to 560 nm (Fig. 1(a)). The simultaneous presence of SDS and PC did not exert an additive effect. The absorbance of I_3^- was very low compared with that of the complex, and it also decreased in the presence of Triton X-100 (Fig. 1(b)) but not in the presence of SDS (not shown).

The absorbance values of I_2–starch complex at 600 nm increases in an hyperbolic manner as a function of I_2 concentration, measured as the

ratio $[I_2]$/[starch] (mmol/g), in the absence and in presence of PC and/or SDS (Fig. 2). The plateau indicates the achievement of starch saturation level with I_2 and corresponds to the maximal amount complex formation in the system. The effect of PC at the present concentration (equivalent to 0.05 g/l in the incubation system) (Fig. 2(b)) was negligible compared with the control in water (Fig. 2(a)); SDS decreased the complex absorbance mainly at low $[I_2]$/[starch] both in the absence (Fig. 2(c)) and in the presence of PC (Fig. 2(d)). These result suggest that SDS may affect the affinity of I_2 for starch. In the presence of Triton X-100 with (Fig. 2(f)) or without (Fig. 2(e)) PC, the absorbance of the complex increased up to $[I_2]$/[starch] = 4.4 mmol/g and at that point it suddenly decreased to zero, coincidentally with the appearance of turbidity in the assay tube. From these results, a $[I_2]$/[starch] relationship of 17.7 mmol/g was chosen for applying in the experiments that followed, in order to assured the measurement of the total amount of starch in the system, even at the maximal starch concentrations used in the saturation curves.

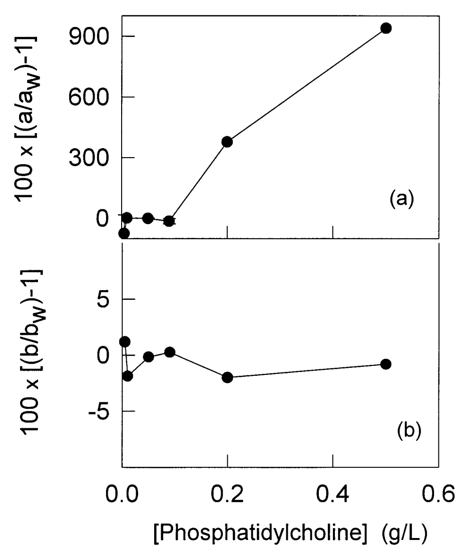

Fig. 3. Evaluation of the light scattering effect of PC vesicles as a function of PC concentration in the incubation system. Percentual increments, respect to the values obtained in water, of ordinates (a) or slopes (b) of calibration curves (absorbance vs. I_2/starch complex concentration) at different PC final concentrations in the incubation system. a and a_w: ordinate in the presence and in the absence of PC; b and b_w: slopes in the presence and in the absence of PC.

Calibration curves of absorbance of I_2–starch complex at 600 nm as a function of starch concentration (from 0.5 to 5 g/l) at varied PC concentrations (from 0 to 5 g/l) could be adjusted to straight lines. As shown in Fig. 3, the slopes of these curves showed minor changes within the PC concentration range tested however, the ordinate values remained approximately constant up to 0.1 g/l and increased at higher PC concentration values, suggesting that the effect of PC at high concentrations was associated with a light scattering phenomenon but not with the light absorption processes. The proper calibration curve was used in the following experiments in order to calculate, from the $\Delta A_{600} = A_{initial} - A_{final}$ values, the amount of starch hydrolyzed (initial minus final after enzymatic degradation).

3.2. Effect of enzyme–phosphatidylcholine interaction on the enzymatic kinetics

Optimal protein concentration of 28.5 μg/ml and optimal incubation time of 15 min were determined from the linear region of curves of amount of starch degraded as a function of protein concentration at constant incubation time and substrate concentration (Fig. 4(a)) and as a function of incubation time at fixed protein and substrate concentrations (Fig. 4(b)), respectively.

Typical curves of reaction rate (change in starch concentration per minute) as a function of starch concentration (0.5–5 g/l) are shown in Fig. 5. The hyperbolic shape of these curves indicates that the enzyme follows a Michaelian kinetics both in the presence and in the absence of 1.4 g/l SDS or 0.05 g/l PC MLV-untrapped. In the examples given in Fig. 5, the values of K_M were 0.855 ± 0.197, 1.04 ± 0.08 and 1.33 ± 1.76 g/l and those of V_{max} were 0.043 ± 0.003, 0.028 ± 0.004 and 0.02 ± 0.0079 g/l per min in the absence or in the presence of PC or SDS, respectively.

The effect on the kinetic parameters of α-amylase induced by lipid concentration, type of PC vesicles (MLV and GUV) and the untrapped or trapped enzyme condition, were investigated. The results of K_M and V_{max}, shown in Tables 1 and 2,

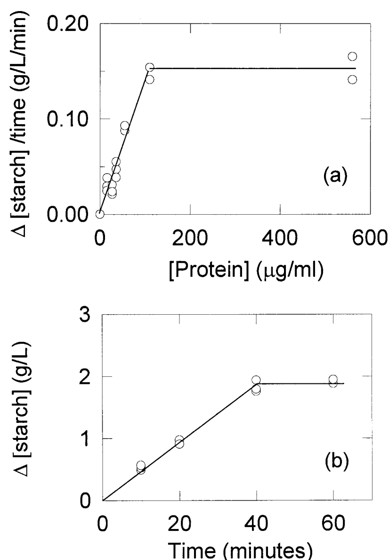

Fig. 4. Determination of the optimal values of enzyme concentration and incubation time. Reaction rate followed a pseudo first order regime up to 110 μg/ml protein concentration in the incubation system (a) and up to 40 min of incubation time (b).

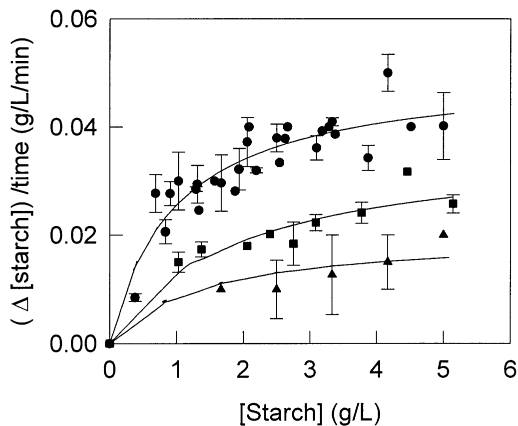

Fig. 5. Effect of PC and SDS on the saturation curves of α-amylase with starch. Change in starch concentration per unit of time was measured in the absence (●) or at a PC/protein mass ration of 28 g/g (■) or of 1.4 g/l SDS in the incubation system (▲). Symbols correspond to mean ± s.e.m. of experimental data; lines were calculated by adjusting experimental data to the Michaelis–Menten equation through a non-linear regression analysis by the least squares method.

respectively, were submitted to a 3-way ANOVA test as indicated in Section 2. Significant effects were found in the analysis of K_M values (Table 1) for the type of enzyme treatment (T or UT) ($F_{(2,15)} = 10.17$, $P < 0.0016$ but not for the type of vesicle (MLV or GUV) ($F_{(1,15)} = 3.14$, $P < 0.0965$) and for the lipid concentration ($F_{(3,15)} = 0.33$, $P < 0.8016$). The values of V_{max} (Table 2) were significantly dependent on lipid concentration and on the type of vesicles ($F_{(3,15)} = 22.98$, $P < 0.0000$ and $F_{(1,15)} = 10.51$, $P < 0.0055$, respectively) but not on the type of enzyme treatment ($F_{(2,15)} = 2.76$, $P < 0.0951$). The post-hoc test showed a significant decrement in V_{max} exerted by MLVs at PC/protein ration of 28 g/g (see Table 2) and by GUVs both in the 'trapped' enzyme samples. The presence of PC in MLVs induced a statistically significant increment in the value of K_M of α-amylase for starch as the substrate (Table 1) in the two conditions assayed (U and T) only at the highest PC protein mass ratio used, as revealed by the post-hoc test on K_M. GUVs affected amylase K_M only in some 'trapped' samples at random.

3.3. Distribution of protein and enzymatic activity between lipidic and aqueous phase

When saliva was incubated in the presence of PC in MLVs, between 20 and 40% of the proteins (depending on lipid concentration) could be pelleted, supposedly adsorbed to the liposomes, while about 60% or more remained in the soluble fraction (Fig. 6(a)). Compared with the activity of α-amylase measured in the whole sample (saliva in the presence of a suspension of PC liposomes), the α-amylase activity recovered in the pellet was significantly smaller and that in the supernatant was higher (Fig. 6(b)), suggesting that enzyme-membrane interaction impairs the activity of the enzyme.

3.4. Effect of saline concentration and pH on the adsorption of α-amylase to phosphatidylcholine multilamellar vesicles

The activity at pH 7 of α-amylase adsorbed to MLVs depended on the conditions of the media during the adsorption process, being lower in high salt concentrations or extreme pH values (pH 3 or 9) compared with pure water (Fig. 7).

4. Discussion

In the present paper, saliva was used as a source of α-amylase. The amylase from saliva is encoded by the amy one gene and is the same protein expressed in the mammary and lacrimal glands, while the pancreatic amylase isozyme encoded by the amy two gene is expressed only in the pancreas [11]. Early studies of electrofocusing indicated that human α-amylase in saliva could be separated into one major isozyme with an isoelectric point of pH 6.5 and two minor isozymes with isoelectric points of pH 6.0 and 6.9 [12]. Later on, by thin-layer gel isoelectric focusing in a pH 4–8 gradient, it was shown that the normal isozyme pattern of saliva consisted of five major isozymes together with a number of minor ones with seven variant isozyme phenotypes [13]. The complexity of this landscape indicates that the conclusions drown from the present experiment represent the average behavior of all the isozymes.

Early experiments were directed to the settlement of the conditions for enzymatic activity determinations. The changes induced by PC on the

Table 1
Effect of phosphatidylcholine on the K_M value of α-amylase[a]

[PC]/[Protein (g/g)	Control without PC	Multilamellar vesicles		Unilamellar vesicles	
		Trapped-enzyme	Untrapped-enzyme	Trapped-enzyme	Untrapped-enzyme
2	1.6 ± 0.22	1.31 ± 0.3	1.19 ± 0.16	2.56 ± 0.6	1.53 ± 0.39
4	1.49 ± 0.47	1.51 ± 0.4	0.915 ± 0.2	nd	nd
20	0.84 ± 0.21	1.135 ± 0.36	0.79 ± 0.29	3.36 ± 1*	1.01 ± 0.55
28	0.907 ± 0.045	2.02 ± 0.64*	1.04 ± 0.08*	1.77 ± 0.01	nd

[a] The values shown correspond to K_M in units of g/l and were determined by a computer aided nonlinerar regression analysis by the least squares method of the experimental data (reaction rate in g/l of starch hydrolized per minute as a funtion of starch concentration in g/l shown in Fig. 5) adjusted to the Michaelis–Menten equation.

* Indicates values statistically different from the control in the absence of lipid ($P<0.05$, post-hoc LSD test; the results from the ANOVA were detailed in the text). Values are the mean ± s.e.m. of two to four experiments each one performed in triplicate determinations. Experimental samples at each PC/protein ratio were compared with a control sample (no lipid) run in parallel experiments.

Table 2
Effect of phosphatidylcholine on α-amylase V_{max} values[a]

[PC]/[Protein (g/g)	Control without PC	Multilamellar vesicles		Unilamellar vesicles	
		Trapped-enzyme	Untrapped-enzyme	Trapped-enzyme	Untrapped-enzyme
2	0.055 ± 0.005	0.046 ± 0.013	0.043 ± 0.002	0.046 ± 0.006*	0.048 ± 0.003
4	0.094 ± 0.01	0.078 ± 0.007	0.074 ± 0.0047	nd	nd
20	0.052 ± 0.01	0.076 ± 0.011	0.064 ± 0.002	0.073 ± 0.01*	0.034 ± 0.017
28	0.047 ± 0.002	0.026 ± 0.001*	0.028 ± 0.004	0.048 ± 0.007	nd

[a] Values shown correspond to V_{max} in units of g/l per min determined by a computer aided nonlinerar regression analysis by the least squares method of the experimental data (reaction rate in g/l of starch hydrolized per minute as a funtion of starch concentration in g/l shown in Fig. 5) adjusted to the Michaelis–Menten equation.

* Indicates values statistically different from the control in the absence of lipid ($P<0.05$, LSD post-hoc test; the results from the ANOVA were detailed in the text). Values are the mean ± s.e.m. of two to four experiments each one performed in triplicate determinations. Experimental samples at each PC/protein ratio were compared with a control sample (no lipid) run in parallel experiments.

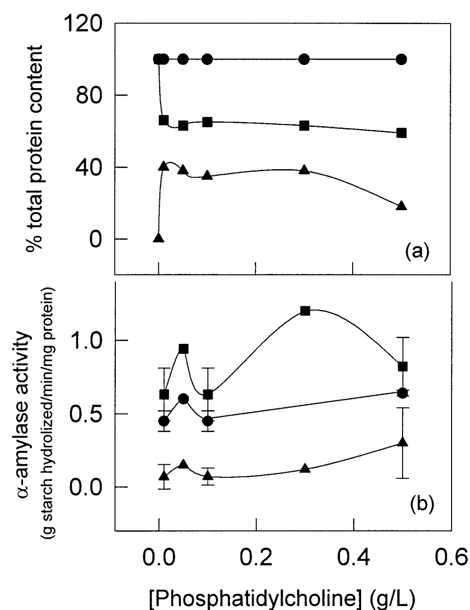

Fig. 6. Effect of PC on α-amylase binding and activity. A dispersion of MLVs containing variable amounts of PC and a fixed protein concentration (285.5 μg/ml) was incubated at room temperature for 20 min and then it was centrifuged at 30 000 × *g* for 40 min and the pellet and the supernatant were separated. Percentage of the total protein in the incubation system (a) and total α-amylase activity (b) recovered in the whole incubation system (●) or in the fractions obtained after centrifugation (supernatant: ■ and pellet: ▲).

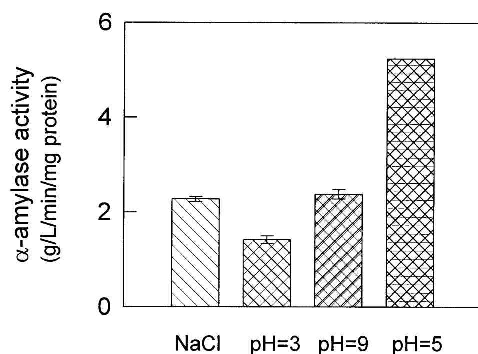

Fig. 7. Effect of pH and saline concentration of α-amylase binding to phosphatidylcholine multilamellar vesicles. Experimental conditions were explained in Section 2. The results shown are the mean ± s.e.m. of triplicate determinations.

absorbance of I_2–starch complex included an effect possibly exerted at the radiation absorbance process which may explain the small decrements in absorbance observed at low PC concentration (Fig. 1(a)). Light scattering is a wavelength dependent phenomenon and in long-wavelength spectrophotometry such as visible usually exerts a background absorbance that lies in a straight line (see Ref. [14]). Light scattering induced by PC vesicles introduced a turbidity becoming significant over 0.1 g/l of PC (Fig. 2) and was corrected for in the experiments that followed.

Detergents are commonly used in order to liberate enzymes entrapped inside liposomes. Knowledge of their effects on the enzymatic kinetics as well as on the colorimetric development of the reaction products was required. From the detergents tested, all the neutral ones (Fig. 1(a) and (b)) interfered with the latter process; SDS affected the affinity of α-amylase for starch (see below, Fig. 5). Consequently, SDS should be discarded and neutral detergents cannot be used with this purpose unless the reaction kinetics is measured using another substrate that do not required I_3^- in high acidic media to be developed.

Both PC and SDS were used at final concentrations above their corresponding critical micellar concentrations (c.m.c.); in this conditions, according to the relative volumes of the hydrophilic and hydrophobic portions of their molecules, PC self-aggregates into bilayers and SDS into spherical micelles [6].

The interaction of α-amylase with lipid-water or detergent-water interfaces did not change the hyperbolic shape of the saturation curve, indicative of a Michaelian behavior (Fig. 5) but induces a decrement of the affinity of the enzyme for the substrate (K_M increases) (Table 1) and of the value of V_{max} (Table 2). These results suggest that there is not a surface activation phenomenon (absence of lag time) usually observed with enzymes that uses surface-organized substrates [15,16] and that α-amylase suffers a conformational change which affects the active site (K_M increases) upon its binding to the lipid or to the detergent. That lipid–protein interaction may also lead to a partial protein inactivation or sequestration (V_{max} decreases).

The efficiency of a protein entrapment in lipid vesicles depends on the size of the vesicle as well as on the size of the protein [17]. For small

unilamellar vesicles (SUVs) it has been shown that the entrapment efficiency of α-amylase (M_W 97000) represents ≅50% of that of lysozyme (M_W 14000) while no difference between both proteins was observed when MLVs were used. In a SUVs with a 8.1 nm length radius, ≈1.3 nm are occupied by a layer of water tightly bound to the polar head groups of the phospholipids in the internal lamella of the bilayer. This does not happen with MLVs due to their longer radius as well as their lower curvature; a similar behavior is expected for GUVs, which are the biggest and have lowest curvature. The probability, P, of a given protein of radius R_p being trapped inside the vesicle is given by:

$$P = [(D - A)/D]^3 \cdot [(D - A - R_p)/(D - A - R_w)]$$

where D, A, R_p and R_w are the radius of the total internal volume of a spherical SUV, the thickness of the bound water layer, the radius of a hydrated protein and water, respectively [17].

We expect the amount of α-amylase entrapped inside MLVs and GUVs to be limited only by the internal volume of the vesicle.

The trap volume of a SUV is 1.4 μl/mg lipid [17]; the entrapped volume of MLVs and GUVs calculated from a molecular area of PC (70 nm^2 at the surface pressure of the bilayer [18]) and the radius of the corresponding vesicle (500 and 1000 nm, respectively) resulted: 98 and 198 μl/mg lipid, respectively. So, in our present experimental conditions, due to the low lipid concentration, the amount of α-amylase entrapped is expected to be 10 and 20%, respectively of total α-amylase in a system containing 1 mg/ml PC; this explains the lack of difference, within the experimental error, between the values of V_{max} measured in the presence and in the absence of low PC and enzyme concentrations (Table 2).

The effect of MLVs in reducing the affinity of the enzyme for starch, was expected to be higher than that of GUVs due to a higher protein adsorbing capacity of the lower molecular packing, higher surface tension and more curved MLVs's surface compared with GUVs's surface. So, our results at PC/protein mass ratio 2 and 20 should be interpreted on the basis of the effect of the somehow aggressive experimental conditions that the enzyme is submitted to, during the entrapment procedure in GUVs. Higher amounts of lipid respect to protein may have increased the probability of protein binding to the liposome surface allowing an increase in K_M and a decrease in V_{max} values that resulted significant from the statistical point of view. It is a common phenomenon that proteins [16] and peptides [19] penetrate lipidic membranes more easily in lower packed membranes (lower lateral surface pressure) as demonstrated by experiments in monomolecular layers at the air-water interface. Moreover, the inactivation of enzymes due to a high penetration in very low packed monolayers has already been described (see Refs [15,16]). The effect of SDS micelles was higher than that of PCs MLVs. If we take into account that the molecular area of a neutral detergent like Triton X-100 has been calculated as 1.48 nm^2, it is expected for that of SDS to be even bigger, due to the electrostatic repulsion between the negative charge in its polar head group. So, the lower molecular packing, which is expected for SDS surface, may explain the higher effect of this detergent on the K_M of α-amylase compared with PC. The presence of the negative charge itself may contribute to the adsorbing capacity of SDS surface through electrostatic interactions because, in our experimental conditions (a pH bellow the isoelectric points of the different isoforms of α-amylase from saliva) the enzyme molecules are positively charged.

The binding of the enzyme to the lipidic surface is reversible and can be modulated by the presence of high salt concentrations or with the pH of the media (Fig. 7). The enzyme is negatively charged at pH 9 and positively charged at pH 3; the zwitterionic surface of PC has a net electrical charge close to zero at $pH > 3$ (pK of the PCs phosphate group is ≅1); consequently, electrostatic repulsion between MLVs and the enzyme cannot explain the lower enzyme activity recovered after its binding at pH 3 or 9. Hydration forces arise whenever water molecules bind to surfaces containing hydrophilic groups, and their strength depends on the energy needed to disrupt the ordered water structure and ultimately dehydrate two surfaces as they approach each other (see Ref. [6]). Hydration forces can be regulated

by exchanging ions of different hydration on surfaces. The results shown in Fig. 6 may be interpreted considering that the presence of net changes in the protein molecule may increase its hydration level respect to the one it may have at a pH close to pI, increasing its hydration repulsion from the zwitterionic surface of the lipid self-assembly; the screening of protein surface charges exerted by NaCl would regulate protein hydration at a value between that found at pH ≅ pI and those at pH 3 and 9 modulating its binding to the lipid–water interface (Fig. 7). Another explanation may be found within the effects of pH on protein folding and the subsequent exposure of certain chemical groups with different affinities for the interacting surface.

In physiological conditions as well as in technological applications, attention should be paid to the possibility of enzyme–surface interactions which may modulate the enzyme activity. The association of a protein to a lipidic phase (micelles, vesicles, etc.) may imply its incorporation to the membrane structure or its adsorption to the membrane surface. This process may induce conformational changes, desolvation of the protein and membrane surfaces put in contact, changes in the structural dynamics of both the membrane and the protein, etc. This mechanism may led to a partial or total decrement of the enzyme activity through the stabilization of low-active or inactive conformations and may explain the results achieved in the present paper.

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