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Interaction of an acid protease with positively charged phosphatidylcholine bilayers

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Abstract

Positively charged bilayers composed of phosphatidylcholine (PC) and stearylamine (SA) in a 4:1 ratio reduce the effectiveness of a protease from Mucor miehei to produce milk clotting. This is related to the adsorption of the protein, which at pH 7 is negatively charged, by electrostatic forces. However, an increase in SA, which increases the membrane packing parallel to the increase in the surface charge density, counteracts the protein membrane association. This is in agreement with the fact that the protease can also adsorb on pure phosphadylcholine bilayers in the fluid state but not in the gel state. In addition, the presence of phosphatidylethanolamine also inhibits protease adsorption. It is concluded that the protein affects the membrane interface of fluid PC membranes because the electrostatic charges pull the protein to the bilayer interface causing changes in hydration and area per molecule. The adsorption is only at the level of the polar head groups since no effects were observed in the hydrocarbon core region.

Keywords: Lipid membranes; Stearylamine; Phosphatidylcholine; Acid protease; Surface potential; Interfacial changes; Protein penetration

1. Introduction

The study of enzyme activity in amphiphilic self-organizing systems has been a matter of interest in the last years, both in basic and biotechnological research. Several systems have attracted attention in regard to enzyme immobilization: solid supports, occlusion in polymers, reverse micelles and liposomes [1–5]. Reverse micelles and liposomes are the experimental model systems in which the environment of the proteins can be better mimicked [2,6]. Reverse micelles are suitable to study the influence of the water organized at the lipid interface on the protein behavior with a negligible contribution of bulk water [7,8]. However, contributions of cooperativity and topology relative to a lipid bilayer cannot be evaluated. Liposomes or unilamellar vesicles are more appropriate in this sense, although they may have

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the disadvantage that measurements are done in the presence of an excess of water.

In particular, proteolytic enzymes such as chymotrypsin and trypsin have been studied in reverse micelles of phosphatidylcholines with the aim to relate the activity of these enzymes to water activity [9]. These studies have shown that enzyme stability and activity in these systems are comparable or better than that found in bulk water. In contrast, studies using vesicles or liposomes as model systems are lacking.

The physical chemistry of the relationship between the structure of the lipid aggregates used as model systems and the enzyme activity is not yet completely understood. Although it is known that the presence of phospholipids greatly alters the steady state kinetics of the proteolytic reactions [10], it is not clear which is the role of the membrane in these activation processes. Systematic studies of the membrane–protein interaction in relation to the surface properties have been reported by many authors. These investigations have shown the effects of surface active agents [11], salts [12], and lipid composition [13], on the extent of association. However, these studies do not imply that the eventual changes in enzyme activity are due solely to electrostatic interactions. Other physical properties of the surface, such as hydration and the phase state of the lipids can affect association and, in addition, affect the protein activity.

In regard to proteases, several processes seemingly involve membrane microenvironment: the degradative role of acid proteinases is related to the acid pH in their surroundings [14] and, proteolytic activity in several microorganisms appears related to protease exchanged or adhered to cell walls [10]. Considering these possibilities, it appears of importance for biotechnological purposes to take advantage of these features to modulate the extent of proteolytic activity. For this reason, more details about the interactions of these enzymes with liposome membranes are required.

In this paper, we analyze some aspects of the binding of a soluble protease of Mucor miehei, which belongs to the group of the aspartyl-proteases with a similar structure than the bovine chymosine [15], to lipid membranes of different composition and phase state. The purpose of this study is to determine the conditions of the membrane on the activity of proteases.

2. Materials and methods

2.1. Lipids and enzymes

Egg yolk phosphatidylcholine (ePC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), 1 - palmitoyl - 2 - oleoylphosphatidylcholine (POPC) and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Dicetylphosphate (DCP), stearylamine (SA) and Trizma were from Sigma (Saint Louis, MO). Lipids were checked by thin layer chromatography using a chloroform: water methanol (65:25:25, vlvlv) mixture. Single spots after exposure to iodine vapours were obtained. Therefore, lipids were used without further purification. Protease (Rennet from Mucor miehei; MW: 34000) were obtained from Sigma. The purity of the enzyme was checked by SDS polyacrylamide gel electrophoresis. A solution of the protein was dialyzed against MilliQ water in order to eliminate the salt. Fresh water was replaced three times before using protease solution in the experiments. Merocyanine 540 (MC), diphenyl hexatriene (DPH), and dansylhexadecylamine (DA) were obtained from Molecular Probes, Eugene (OR) and used as received. All other chemicals were of analytical grade and water twice distilled in a standard MilliQ equipment.

2.2. Multilamellarand unilamellar vesicle preparation

Lipids in a chloroform solution were dried in a round bottom flask under vacuum. Lipid mixtures were prepared by co-dissolving in chloroform the appropriate proportions, expressed as mol/mol ratios. Multilamellar liposomes (MLVs) were prepared by dispersing the dry lipid film in

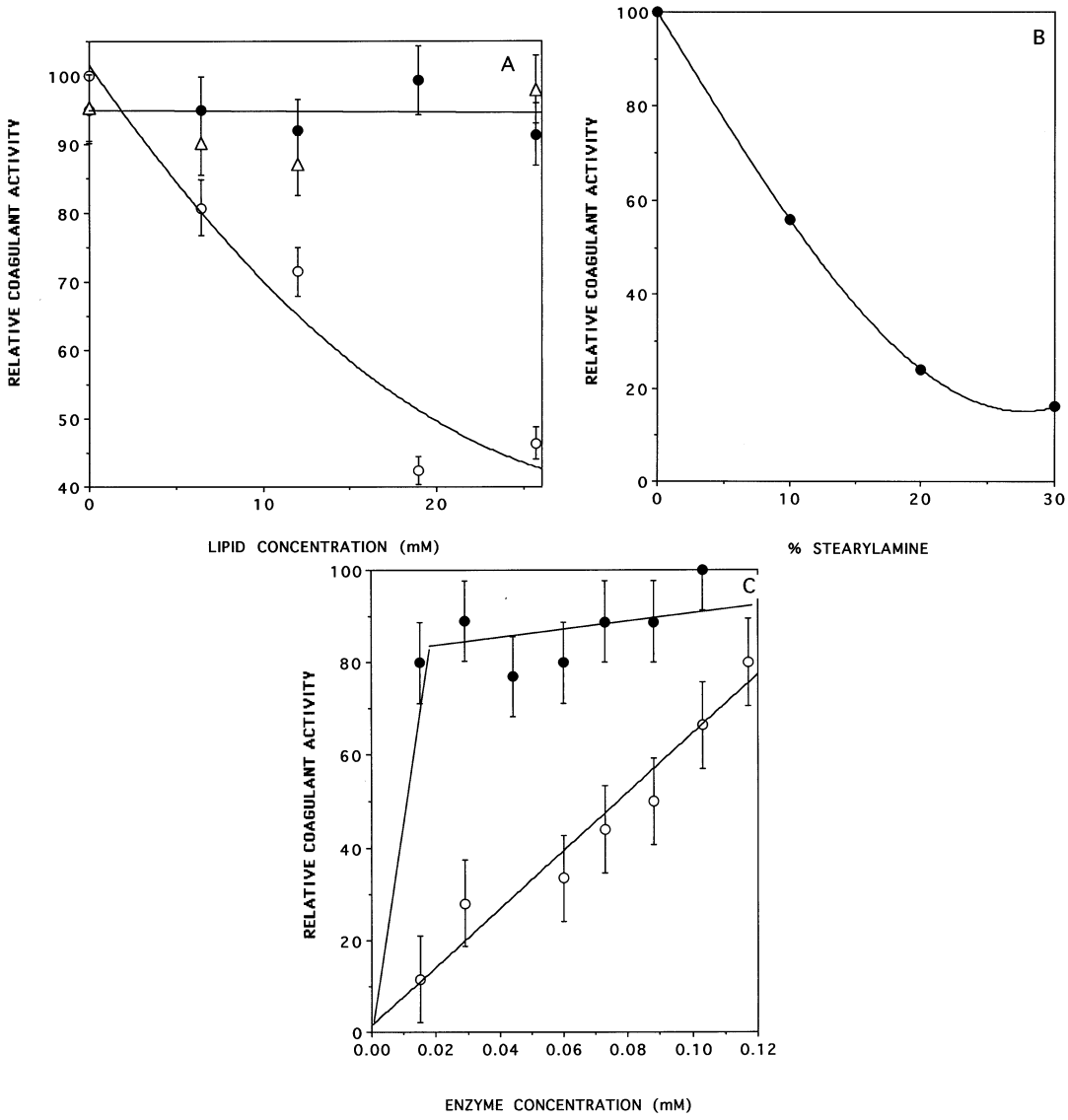

Fig. 1. Clotting activity of protease in the presence of different types of liposomes. (A) Clotting activity in the presence of (○) POPC/SA(4:1), (Δ) POPC/DCP(4:1) and (●) POPC liposomes. Enzyme concentration: 0.02 mM. (B) Clotting activity as a function of the SA ratio in POPC membranes, measured at 48 h for 12 mM lipid concentration. Enzyme concentration: 0.02 mM. (C) Clotting activity induced by protease in the presence of liposomes of POPC/SA (4:1 ratio) (○) and in pure water (●). The relative clotting activity was calculated considering the 100% of enzyme activity in water for each type of assay.

MilliQ water, 0.01 M KCI or in Tris 10 mM pH 7.4 buffered solutions, depending on the type of assay.

Large unilamellar vesicles (LUVs) were prepared from the MLVs suspension by extrusion through polycarbonate membranes of 100 nm diameter using a Liposofast equipment (Vancouver, Canada) at temperatures above those corresponding to the main transition temperature of the lipids (45°C).

2.3. Clotting activity

Milk clotting activity determinations were done following the procedure of Arima et al. [16]. Briefly, this method is useful to determine the enzymatic activity of proteases using casein or milk as a substrate.

The clotting time was determined by the visual detection of the appearance of clots. For this purpose, 100 μl of a milk dispersion, were mixed with 10 μl of the assayed sample and incubated at 35°C. The unit milk clotting activity (CAU) is defined as the enzyme amount capable of clotting 1 ml of sample in 40 min at 35°C. A 10% solution of skim milk powder in 10 mM $CaCl_2$ was used as substrate.

The different conditions were done with the same batch of substrate to enhance reproducibility. In order to compare the different conditions assayed, the data are reported as relative activity units taken as reference the 100% obtained in water.

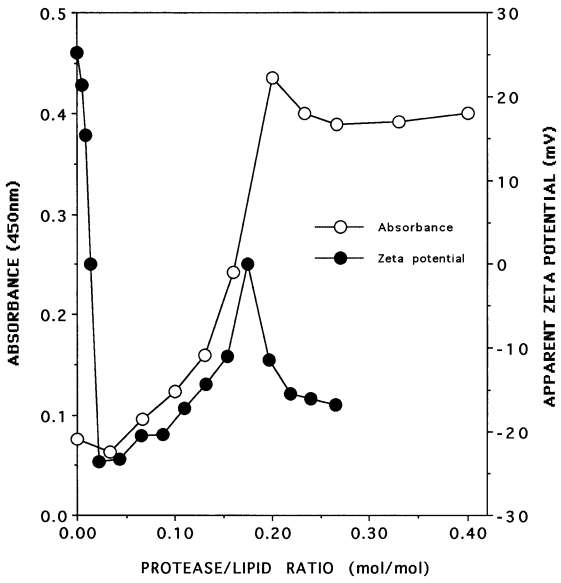

Fig. 2. Liposome aggregation induced by protease at high lipid concentration. Turbidity (○) and surface potential changes (●) upon titration of a lipid concentration of 5×10^4 M with protease, at pH 7.

2.4. Aggregation index

The aggregation index was measured by turbidity at 450 nm of MLV suspensions at 25°C in a Hitachi 100-60. The procedure consisted in fixing the zero of absorbance with both, sample and reference cuvettes filled with the same lipid concentration of a dispersion of the same batch of liposomes. After the addition of each protease aliquot to the sample cuvette, the mixtures were incubated until a constant absorbance was obtained. The changes produced in the sample turbidity upon the addition of the protease were monitored after vigorous stirring. The aggregation is reported as the absorbance difference (A − Ao) between the sample absorbance obtained in the presence (A) and in the absence of protease (Ao).

Aggregates were observed by electron microscopy. For this purpose, an aliquot of each MLV sample was placed in a 400 MESH grid covered with carbon during 15–20 s at room temperature. The sample was washed with two drops of 1% uranyl acetate at pH 7.2, and dried at constant temperature during 10–15 min interval. The grids were examined in a JEM 1000 microscope and the microphotographies obtained with a magnification between 15000 and 75000 ×.

2.5. Surface potential (electrophoretic mobility)

The electrophoretic mobility (μ) of multilamellar liposomes dispersed in 0.01 M KCl were determined in a capillary cell in which two black Pt electrodes were connected to a DC source. Total lipid concentration was in all cases 2×10^{-4} M. The voltage was fixed at 40 V and temperature at 20°C. A total of 10 measurements (five in each direction) were done focusing on a single particle. Data reported are the average of the measurements done, for each condition, with three different batches of liposomes (mean standard deviation: ± 0.5 mV).

The zeta potential (ζ) was calculated from the mobility using the Helmholtz–Smoluchowski equation $\zeta = \eta\mu/\varepsilon\varepsilon o$ where ε, εo and η are the dielectric permittivity of the aqueous solution, the permittivity of the free space and the dynamic viscosity of the suspension, respectively [17].

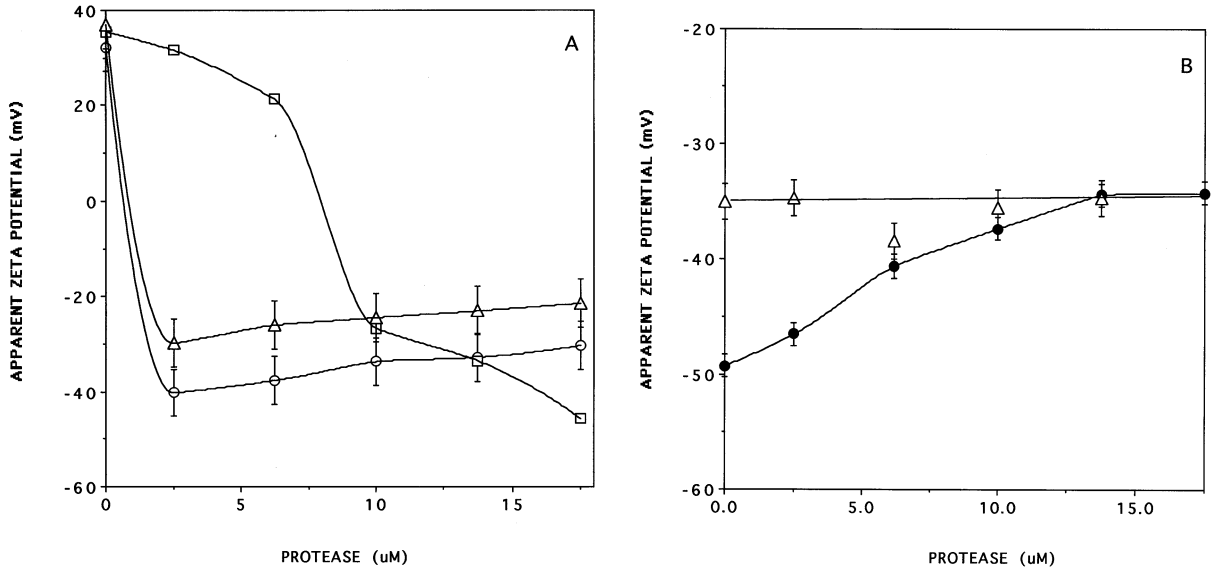

Fig. 3. Protease adsorption on neutral and positively charged liposomes as measured by the electrokinetic potential. (A) (○) DOPC/SA (4:1 ratio); (□) DOPC/SA (3:2 ratio); (△) DSPC/SA (4:1 ratio); (B) DOPC (●) and DSPC (△). All measurements were done at 20 ± 5°C, in 0.01 M KCl. Total lipid concentration was in all cases 1×10^{-4} M.

2.6. Fluorescence anisotropy and optical assays with Merocyanine 540

DPH and DA probes were incorporated into the bilayer of LUVs by co-dissolving the probes with the lipids in the chloroform solution used to prepare the films. The probe was added in the proper amount in order to reach a probe/lipid ratio between 1:400 and 1:300. All measurements were done at 25°C using unilamellar vesicles of 100 nm diameter in water milliQ solutions.

Fluorescence anisotropy was measured in a Kontrom SFM-25 spectrofluorometer equipped with excitation and emission polarizers.

Steady state anisotropy was calculated using the equation

$$\langle r \rangle = (I\mathrm{vv} - GI\mathrm{vh})/(I\mathrm{vv} + 2GI\mathrm{vh})$$

where Ivv and Ivh denote the intensities obtained with vertical and horizontal orientations of the excitation and emission polarizers. The factor $G = I\mathrm{hh}/I\mathrm{hv}$ is a correction factor that accounts for the polarization bias in the detection system. Excitation and emission wavelengths for DPH were 350–420 nm, respectively. In the case of dansyldihexadecylamine, the maximum excitation wavelength of each sample was found at 337 ± 2 nm. This excitation wavelength was used in all cases, being the emission detected at the maximum emission wavelength which varied with the lipid compositions of each sample assayed.

The Stokes shift was calculated as:

$$S(\mathrm{cm}^{-1}) = 1/\lambda_{\max\ \mathrm{exc}} - 1/\lambda_{\max\ \mathrm{em}}$$

where $\lambda_{\max}$ is the maximum emission or excitation wavelengths expressed in cm.

2.7. Merocyanine absorption spectra

The absorption spectra of MC were obtained in a double beam Hitachi 100-60 spectrophotometer. An aliquot of MC from a stock solution in water was added to the liposome dispersion to yield a final concentration of 10^{-5} M of the dye. A sample of an equal concentration of liposomes without the dye was used as reference in order to discount the lipid spectra. The surface changes were studied following the variation in the intensity of the peak at 570 nm which denotes the ability of the dye monomer to partitioning into the membrane [18].

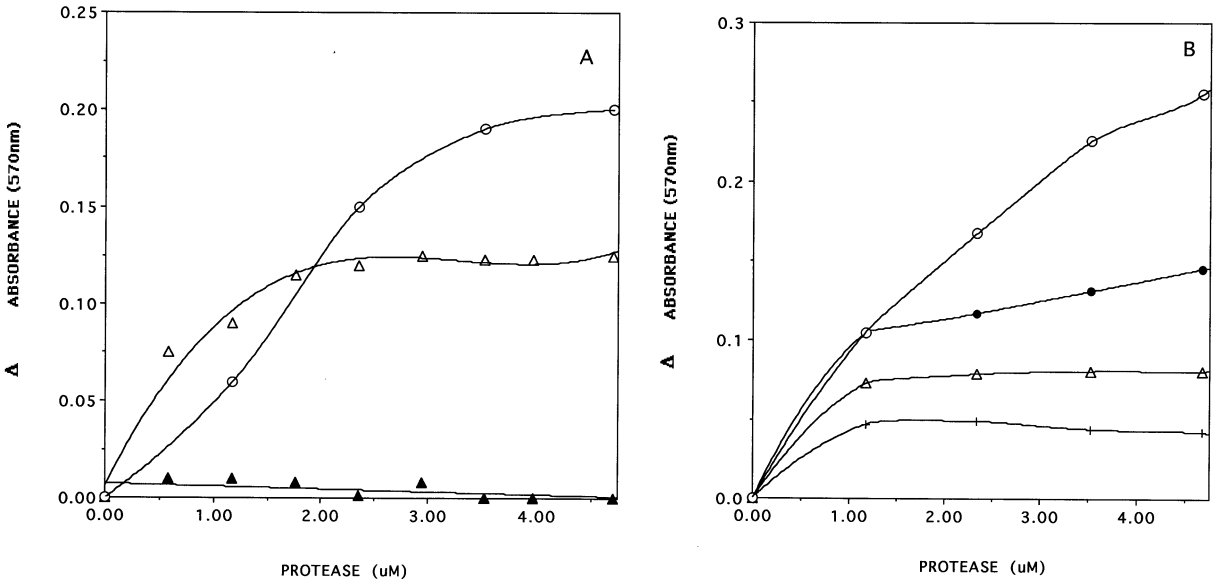

Fig. 4. Effect of protease adsorption of different lipid composition as measured by Merocyanine 540. (A) (▲) DPPC at 25°C; (△) DPPC at 45°C; (○) POPC/SA4:1. at 25°C. (B) Liposomes containing 0 (○); 0.2 (●); 0.4 (△) and 0.5 (+) egg phosphatidylethanolamine/POPC molar ratios. Lipid concentration: 1×10^{-4} M. Total dye concentration: 10^{-5} M. The values at 570 nm were obtained using as reference a lipid dispersion of the same turbidity without protease.

2.8. Monolayer experiments

Monolayers were prepared using a Teflon laboratory-built Langmuir trough. This trough (15 cm × 30 cm × 1 cm), was filled with MilliQ water, pH 5.5 or with NaCl 150 mM solution. The surface pressure was measured using a displacement force transducer (Kaman Sciences, Colorado Springs, CO). An electronic device enabled to keep the monolayer pressure constant by monitoring the displacement of the barrier. This system was used during penetration experiments.

Lipids from a 10^{-3} M concentration in 1:1 ethanol/chloroform, were spread at the air–water interface. First, the compression isotherm was recorded without protease, then the film was decompressed to 5 mN m^{-1} and maintained at that pressure. In this condition, an aqueous solution of protease (final concentration: 3×10^{-5} M) was injected with a microsyringe at numerous points underneath the monolayer interface and stirred. The area variation (ΔA) was recorded, and the monolayer compressed again.

3. Results

The clotting activity of protease decreases as a function of the concentration of liposomes composed by lecithin and 4% stearylamine. This effect is not observed in the presence of similar concentrations of negative or neutral lipids (Fig. 1A). At a fixed lipid concentration, the coagulant activity decreases with the increase of the SA ratio in the membrane (Fig. 1B). A comparison of the enzyme activity in water and in the presence of PC: SA liposomes is shown in Fig. 1C. The activity in water reaches the highest value at 0.02 mM. In the presence of PC: SA liposomes, for the same enzyme concentration, the activity is eight times lower. The final enzyme/lipid ratio at this point is 0.002. The clotting activity in the presence of liposomes equals to that obtained in pure water when the enzyme concentration is above 0.12 mM.

How this result is a consequence of the adsorption of the protein to the membrane surface is studied next. Addition of protease to DOPC:SA liposome dispersions, in the absence of the en-

Table 1
Effects of protease on the surface and hydrocarbon chain region properties of lipid bilayers composed with DOPC and stearylamine[a]

	S (cm^{-1})	r (DA)	r (DPH)
DOPC	9300	0.08	0.080
DOPC/SA 4:1	8700	0.15	0.095
DOPC/SA 3:2	8000	0.20	0.095
DOPC/SA 4:1 (+0.005 protease/lipid)	8000	0.23	0.095

[a] S, Stokes shift in cm^{-1}. $\langle r \rangle$ (DA), fluorescence anisotropy measured with dansylhexadecylamine. $\langle r \rangle$ (DPH), fluorescence anisotropy measured with diphenylhexatriene.

zyme substrate casein, causes a shift of the surface potential from +30 to −25 mV at a 0.03 protease/lipid ratio (Fig. 2). Above this point, an increase in turbidity parallel to the displacement of the surface potential towards zero values, is observed with further addition of the enzyme. The turbidity increase corresponds to the formation of vesicle aggregates as shown by electron microscopy (data not shown). After the isoelectric point corresponding to a 0.18 protein/lipid ratio, a decrease in turbidity is observed denoting the flocculation of the aggregates.

With the interest to determine the features of the enzyme association to different types of membranes in the absence of aggregation, the surface potential of liposomes was measured at low lipid concentrations (Fig. 3A). The protease/lipid ratio in these determinations was between 0 and 0.03 protease/lipid ratio, i.e. the range at which the turbidity remains unchanged according to Fig. 2. The adsorption of Mucor miehei protease neutralizes the positively charged membranes, as shown in Fig. 3A. The addition of the protein produces a sharp decrease in the zeta potential for the DOPC:SA and DSPC: SA (4:1 ratio mixtures) from +30/35 mV to −20/40 mV, respectively, at 2.5 μM protease concentration. The potential for the DOPC:SA 3:2 liposomes remains positive during a wider range of protease concentration and drops to −30 mV at 10 μM.

Further addition of protease to DOPC:SA and DSPC: SA (4:1 ratio mixtures) seemingly induces a slight upshift of 10–20 mV from the minimum at −20 and −40 mV, respectively. This might be indicating that enzyme adsorption may be produced further onto liposomes in which the membrane charges have been neutralized. However, the large scattering in the data does not allow to conclude if this is the case. To get a further insight of the interaction of protease with neutral phospholipids DOPC liposomes were titrated in the same range of protein concentration, at 20°C. The results, show a shift of the surface potential from −50 to −34 mV, (Fig. 3B). In contrast, the zeta potential value for pure DSPC bilayers, which are in the gel state at 25°C, does not change upon the addition of protease. This denotes that the protein can adsorb onto neutral bilayers provided they are in the fluid state.

Focusing on the enzyme concentration at which the major differences in surface potential were observed in Fig. 3B (0–5 μM), the effect of protease on fluid and gel membranes was studied with Merocyanine 540 (Fig. 4A). Increases in the absorbance at 570 nm of MC540 were observed in this range of protease concentration for DPPC in the fluid state but not in the gel state. The effect of protease on PC: SA liposomes, as measured with merocyanine, denotes a higher change in the absorbance at 570 nm. The effect of the protease on the membrane surface is also related to the phospholipid species. The increase of PE in a membrane of phosphatidylcholines in the fluid state also decreases the protease effect on the membrane surface (Fig. 4B).

Thus, the protease effect is partially due to the presence of PC in the fluid state, although the major effects take place on fluid membranes containing SA. With the aim to know how this interaction affects the hydrocarbon core and the interfacial regions, the effect of the protease on the order parameter at the acyl chain level and the polar group region was measured with DPH and dansyl probes, respectively.

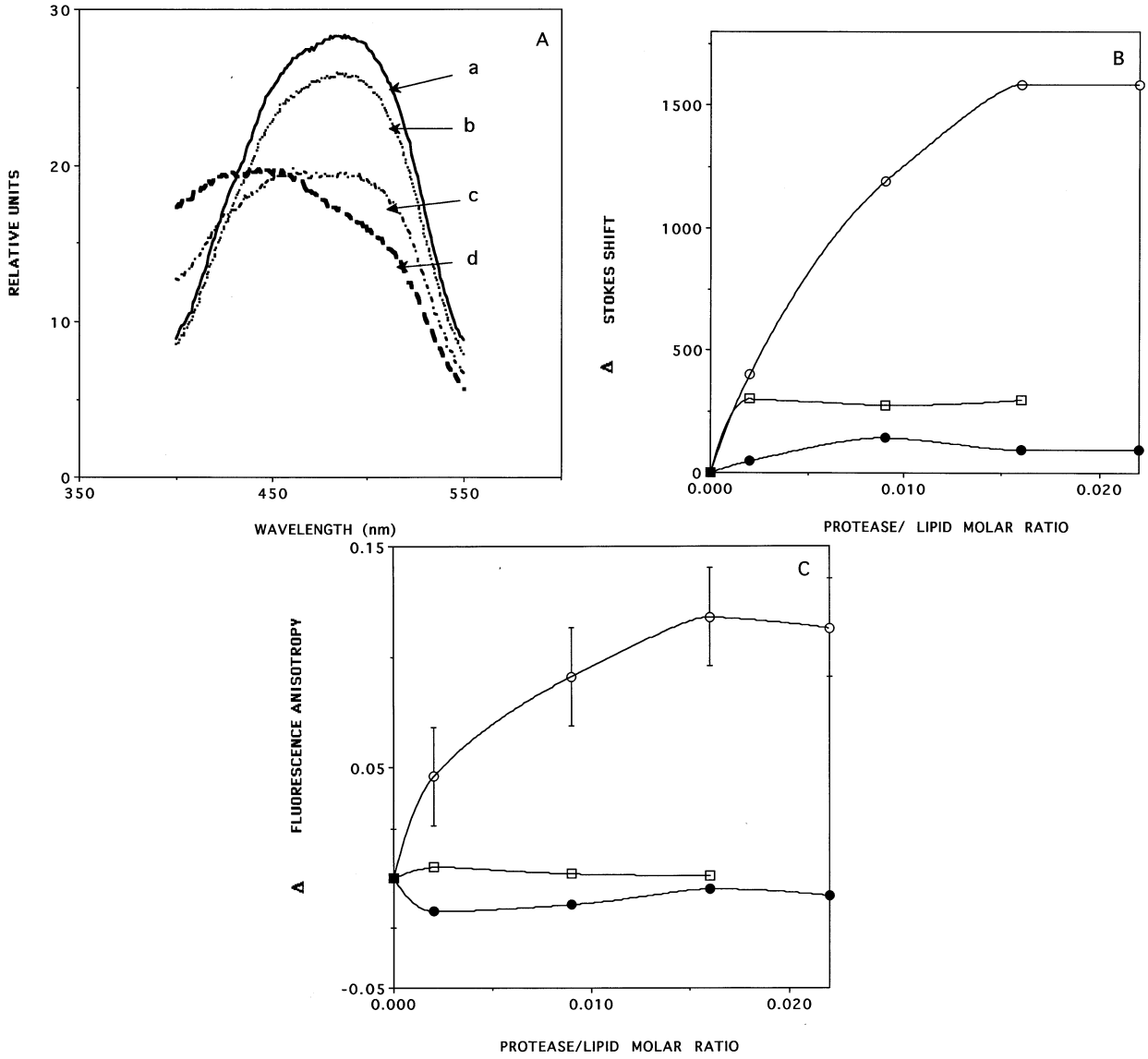

Fig. 5. Effect of protease association on charged and neutral lipid bilayers as measured with dansyl probes. (A) Emission spectra of dansylhexylamine in DOPC bilayers without (a) and with (b) 0.016 protein/lipid ratio; and in DOPC: SA 4:1 molar ratio bilayers without (c) and with (d) 0.016 protein/lipid ratio. (B)Protease-induced Stokes shifts changes on neutral and positively charged lipid vesicles. Results are expressed as the absolute value of the Stokes shift difference (IASI) between ΔS without and with protease (○) DOPC/SA (4:1 ratio); (●) DOPC and (□) DOPC/SA (3:2 ratio). (C) Fluorescence anisotropy measured at the maximum emission wavelength as a function of protein concentration; (○) DOPC: SA (4:1 ratio); (●) DOPC and (□) DOPC/SA (3:2 ratio).

Fluorescence anisotropy measured with DPH remains unchanged in all the membranes when protease is added (Table 1). In contrast, a significant blue shift of DA, located at the interface, and in the fluorescence anisotropy are observed in DOPC: SA (4:1 ratio) membranes. This effect is observed at the protein/lipid ratio at which a shift from positive to negative values of the zeta potential were found (see Fig. 2). No effects of protease on the Stokes shift and the fluorescent anisotropy

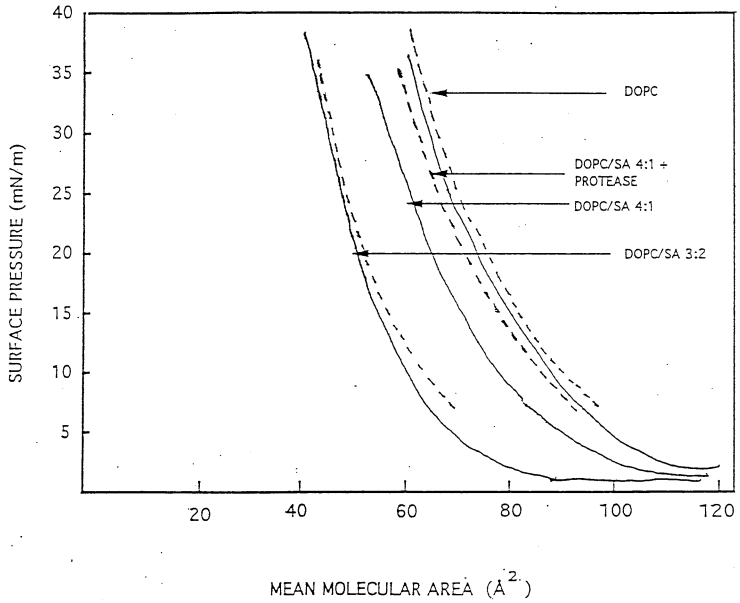

Fig. 6. Surface pressure versus molecular area isotherms of monolayers of DOPC and DOPC/SA with and without protease. Isotherms without protease (full lines) and with 3×10^{-5} M protease (broken lines) corresponding to DOPC: SA (3:2 ratio); DOPC: SA (4: 1 ratio); and pure DOPC monolayers from left to right. Isotherms were obtained at 20°C.

of DA were observed in pure DOPC membranes (Fig. 5 B and C). In addition, no effect was also observed with DA on DOPC:SA (3:2 ratio) membranes, that showed a slight decrease of the positive surface potential with protease (Fig. 3A).

Table 2
Variation of the area per molecule of lipid monolayers in the presence of protease[a]

Lipid	Area/molecule at $P = 5$ mN m^{-1}	ΔA (Å^2)
Liquid expanded monolayers		
DOPC	98	4
DOPC/SA 4:1	88	10
DOPC/SA4:1 NaCl 0.1 M	92	1
DOPC/SA 3:2	67	8
Liquid condensed monolayers		
DSPC	39	0
DSPC/SA 4:1	30	0
DSPC/SA 3:2	31	3

[a] Temperature was fixed at 21°C. The second columm is the area per molecule of the lipids; ΔA is the increase produced in the area per lipid when protease is added to the subphase.

In order to have an insight on the correlation between protease effects on the neutral regions with the amount of surface charges, surface pressure/area isotherms of DOPC and DOPC: SA monolayers with protease were analyzed. At 21°C, pure DOPC monolayers are in a liquid expanded phase. Addition of protease produces a very slight increase in area (Fig. 6 and Table 2). More significant changes in the π versus A curves are produced when the protein is added to the subphase of a 4:1 DOPC:SA monolayers. In this case, the area expansion is 10 (Table 2). However, when protease is added to a DOPC:SA (3:2 ratio) the area increase is again low.

It must be also observed that the area of monolayers without protease follows the order DOPC > DOPC: SA (4:1) > DOPC: SA (3:2). Thus, the presence of increasing SA increases the membrane packing. In condensed monolayers of DSPC or DSPC:SA (4:1 ratio) no change in area was observed. However, a slight change was observed at the DSPC: SA (3:2 ratio) probably due to charge repulsion.

4. Discussion

The decrease in the enzymatic activity is parallel to the displacement of the surface potential from positive to negative values in the lower range of protein concentrations. Since the active site of the protease has two aspartic acid residues, that at pH 7 are negatively charged [19], the binding of protease observed in the electrophoretic experiments of Fig. 3 may be related to a direct interaction of these groups with the stearylamine present in the membrane. Thus, the decrease in the clotting activity in the presence of PC: SA liposomes could be ascribed to electrostatic protein–membrane interactions that affects the enzyme activity.

The studies in the concentration range corresponding to the higher activity decrease (20 μM) and, in which aggregation is absent according to Fig. 1C and Fig. 2, suggests that the protease adsorbs to the interface interacting with the non-charged membranes. The displacement of zeta potential of DOPC:SA (4:1 ratio) liposomes to more positive values when, after the shift of potential from positive to negative values the protease concentration is increased above 2.5 μM, is similar to that observed when protease is added to pure DOPC membranes (Fig. 3). However, the adsorption by non-electrostatic interactions does not affect enzyme activity, since it remains unchanged in the presence of pure DOPC membranes (Fig. 1A). Although the increase after the minimum, observed in DOPC:SA liposomes, is not conclusive due to the data scattering, the protease seems to interact with pure DOPC This effect may be explained by a rearrangement of the adsorbed protein at high concentrations.

The increase in the absorbance peak at 570 nm, observed in Fig. 4, corresponds to an increase in the monomer penetration suggesting an expansion of the membrane interface upon protease adsorption. The changes in MC absorbance and of DA fluorescence indicate that the binding of the protein to the lipid bilayer affects the membrane at the polar head groups level. This suggests that other interactions, besides the electrostatic forces, take place in the enzyme–membrane association. The finding that this increase is found only in PC bilayers in the fluid state, (namely DOPC, POPC or DPPC), but not in fluid PEs denotes that the non-electrostatic interactions are related to the area per molecule and probably hydration of the interface. Therefore, the area per molecule and the hydration of the lipid species could be important factors to modulate protease activity, in addition to the positive surface charge density.

In regard to hydration, the strong blue shift of dansyl fluorescent emission promoted by protease in DOPC:SA 4:1 vesicles, would indicate that the polarity of the probe environment has decreased, if the emission corresponds to a completely relaxed excited state [21]. The dansyl probe (DA) is located at the glycerol backbone level in the interface, and is sensitive to the solvent relaxation behavior of its environment [20,21]. However, a blue shift could also be obtained if the probe environment becomes more rigid (as in a gel state) and the emission is due to an unrelaxed excited state [21]. This last possibility seems to be more likely in this case, since the steady state fluorescence anisotropy measured at the maximum emission wavelength increases significantly in the presence of protease. In contrast, measurements of anisotropy with DPH do not reveal effects of protease at the hydrocarbon level (Table 1). This result would be indicative that the protein does not perturb significantly the hydrophobic core, probably because its action remains at the surface. The monolayer experiments discussed below seems to conform this hypothesis.

The absence of effects of protease on the Stokes shift in pure DOPC vesicles indicates that the changes in polarity and surface anisotropy are driven by the electrostatic attraction between the protein and the membrane containing SA. Thus, the electrostriction would cause the protein to perturb the PC surface. The changes in area per molecule denotes a higher increase when protease is added to DOPC:SA (4:1 ratio) membranes in comparison to pure DOPC membranes (Table 2 and Fig. 6). The electrostatic interaction may pull the enzyme into the lipid interface provided this is fluid. However, an increase in charges has no further effect as observed in DOPC: SA (3:2 ratio) vesicles. Thus, the protease interaction depends either on a specific surface charge density or, the higher proportion of SA promotes a compression

of the interface [22], hindering the interaction of the protein with the membrane. At higher SA ratio, the increase in surface charges is counteracted by an increase in the packing, as denoted by the decrease in the area per molecule of the DOPC:SA (3:2) membranes (Table 2). Thus, in this case, protease would remain adsorbed in an outer plane of the membrane surface.

This action can probably be due to a condensing effect of SA, as suggested by Korner et al. [22], by the acyl chains that prevents further changes in the membrane surface area. This interpretation is congruent with the fact that protease does not adsorb on membranes in the gel state (Fig. 4A). Finally, the area change in monolayers in the fluid state is not as drastic as to infer a protein penetration. Therefore, it seems unlikely that hydrophobic forces are driving the protein adsorption. However, the participation of non-electrostatic forces such as hydrogen bonds and dipolar interactions should not be discarded. Moreover, the decrease in the interaction observed when the membrane contains PE suggests a certain degree of specificity. This point is the subject of future investigations.

In conclusion, at least three conditions can be accounted for attenuating the protease activity in solution with the presence of liposomes: a positive surface charge density below 20%, the inclusion of phosphatidylethanolamines, and the solidification of the PC membranes.

This could be an important tool in biotechnology since protease usually are exported from the inner media to the outer solution across the cell membrane and the level of protease determines the flavour and texture of several milk products. Also, this might be indicative of properties related to regulatory processes in more complex systems. Further investigations should be done in this direction.

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