

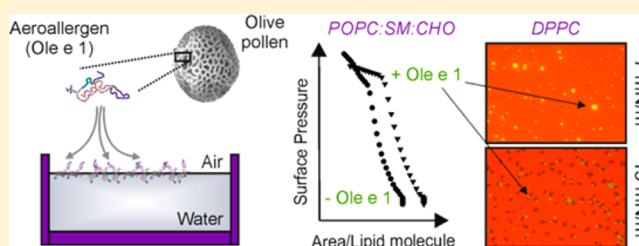
Surface Activity as a Crucial Factor of the Biological Actions of Ole e 1, the Main Aeroallergen of Olive Tree (*Olea europaea*) Pollen

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ABSTRACT: Aeroallergens are airborne substances—mainly proteins—capable of triggering Th2-immune responses in respiratory allergies. They enter into the body through the upper airways, reaching the mucosa afterward. Mucosae lining at the luminal side consists of an epithelial barrier completely covered by mucus and pulmonary surfactant. Both pulmonary surfactant and plasma membrane of the epithelial cells represent two physiological phospholipid-based barriers where allergens first impact before triggering their biological effects. The interaction of allergens with lipids at relevant physiological surfaces could promote structural changes on the molecule, resulting on a potential modification of its allergenic properties. In this work, we have first described the surface and phospholipid interaction capabilities of the clinically relevant aeroallergen Ole e 1, the main allergen of olive tree pollen. By using epifluorescence microscopy of Langmuir transferred films, we observed that lipid-packed ordered domains may function as a preferential location for allergen to accumulate at the air–liquid interface, an effect that is abolished in the presence of cholestenone. The possible implications of phospholipid-interfacial effects in the modification of allergen structural and functional properties will be discussed.



INTRODUCTION

Respiratory allergies constitute a growing health problem that affects approximately 25% of the world population.¹ These inflammatory disorders of the airways involve an exacerbated T helper 2 (Th2)-type immune response against environmental substances, usually harmless to most people, called aeroallergens or airborne allergens.² Aeroallergens—mainly proteins or glycoproteins—enter into the body through the upper airways, generally associated with inhaled particles that are deposited along the airway surface during breathing.³

Upon reaching the mucosal surface, aeroallergens make contact with two anatomical, physiological, and immunological barriers of lipid nature, in addition to the mucus layer: the pulmonary surfactant on the outer side of the mucus and the luminal plasma membrane of the airway epithelial cells.

Pulmonary surfactant is an interfacial lipoprotein complex synthesized by type II pneumocytes, which lines the entire respiratory epithelium. It is composed of approximately 90% of lipid—mainly dipalmitoyl-phosphatidylcholine (DPPC), which accounts for 40–50% of total surfactant mass—and 10% of surfactant proteins (SP): SP-A, SP-B, SP-C, and SP-D. Surfactant is primarily known for its surface-active properties at the alveolar air–liquid interface.⁴ In addition to its biophysical properties, an immunomodulatory character has been shown by the hydrophilic collectins SP-A and SP-D that bind to various glycosylated ligands such as aeroallergens, thus playing a role in the control of airway inflammation including

allergy.^{5–7} However, the role of the hydrophobic proteins SP-B and SP-C in the pulmonary immune system has not yet been well-established.^{8,9}

The airway epithelium consists of a continuous layer composed of different cell types with specific functions. It forms a physical barrier that prevents the invasion of various inhaled particles—including aeroallergens—through the establishment of intercellular junctions, and an active mucociliary clearance.¹⁰ Therefore, there is increasing evidence for an important role played by the airway epithelial cells in orchestrating and influencing the immune responses.¹¹

Surface activity of proteins is a fundamental property that strongly determines its interaction with biological lipid barriers. Protein surface activity is a thermodynamic process influenced by not only its molecular properties such as molecular size, amphipathicity, net charge, conformational flexibility, and stability, but also external factors including temperature and pH.¹² Rheological features of surface-active proteins—such as bovine serum albumin (BSA), β -lactoglobulin, lysozyme, and β -casein—have been widely studied for food industry applications in the formation and stability of food emulsions and foams.^{13,14} However, so far the interfacial behavior of clinically relevant proteins, including allergens, has been poorly characterized.¹⁵

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Our study characterizes in detail, for the first time, the surface activity of the airborne allergen Ole e 1, and its interfacial behavior in the context of phospholipid layers, by using Langmuir devices. Langmuir-derived methods give us the chance to evaluate efficiently the surface activity of proteins and protein–lipid interactions by indirect measurements of surface tension. Due to its abundance and high prevalence, Ole e 1 is considered the main allergen of olive tree (*Olea europaea*) pollen, one of the most important causes of seasonal respiratory allergy in Mediterranean countries and large areas of America, South Africa, and Australia.¹⁶

EXPERIMENTAL SECTION

Materials. 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (POPC), sphingomyelin (SM, porcine brain), cholesterol (CHO), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyle) (Rho-PE), were provided by Avanti Polar Lipids (Alabaster, AL, USA). 4-Cholesten-3-one (CNE) was supplied by Sigma-Aldrich (St. Louis, MO, USA).

Methanol and chloroform were from Scharlab (Barcelona, Spain). Experiments were performed in 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, prepared in double-distilled water.

Methods. Preparation of Lipid Mixtures. Lipid mixtures were obtained by dissolving the appropriate amounts of lipids in chloroform:methanol (2:1, v/v): DPPC, POPC:SM:CHO (2:1:1, molar ratio), POPC:CHO (2:1, molar ratio), SM:CHO (2:1, molar ratio), and POPC:SM:CNE (2:1:1, molar ratio).

Allergen Purification and Fluorescent Labeling. Ole e 1 protein was isolated and purified from olive pollen (IBERPOLEN SL, Jaén, Spain) as previously described.¹⁷ For epifluorescence experiments, Ole e 1 was labeled with Alexa 488 Fluor Succinimidyl Ester (Thermo Fisher Scientific, MA, USA), following manufacturer instructions. Labeled protein concentration and the degree of labeling were determined.

Measurements of Surface Pressure and Interaction with Preformed Monolayers. Ole e 1 adsorption into an air–water interface was determined by means of surface pressure changes ($\Delta\Pi$) over a time interval (Π - t isotherms), after injection of the protein into the buffer subphase (1.8 mL), at a final concentration ranging from 0.05 to 2.78 μM . Studies were carried out using a Langmuir–Wilhemmy Teflon trough from NIMA Technologies (Coventry, UK) at $25 \pm 1^\circ\text{C}$, with constant stirring.

Changes in surface pressure were also monitored after injection of 10 μL Ole e 1 (0.55 μM) underneath preformed lipid monolayers at different initial pressures (Π_i), as described above. Lipid monolayers were formed by depositing small volumes of the different lipid solutions (0.1 mg/mL) on the surface until the required pressure was achieved. After 10 min to allow for solvent evaporation, protein solution was injected into the subphase and $\Delta\Pi$ were monitored. Maximum increase in pressure ($\Delta\Pi_{\text{max}}$) and critical insertion surface (Π_c) pressures were estimated from $\Delta\Pi$ versus Π_i plots for each phospholipid mixture assayed.

Compression Isotherms and Epifluorescence Microscopy Experiments. Lipid monolayers were formed on the top of the subphase using a thermostated Langmuir–Blodgett ribbon trough (NIMA Technologies), as described above. For epifluorescence experiments, Rho-PE (1:100, probe:lipid molar ratio) was included in each lipid mixture, and Alexa 488-labeled Ole e 1 (15 μg) was used. After 10 min to allow for solvent evaporation, monolayers were compressed to 2 mN/m surface pressure. After 5 min of equilibration, the allergen was injected into the subphase and the films were transferred onto a glass slide that was previously immersed in the subphase, to form Langmuir–Blodgett films. Monolayer–protein interaction was monitored as $\Delta\Pi$ for 10 min. For the epifluorescence microscopy experiments, all the monolayers were compressed at the transfer rate of $25\text{ cm}^2/\text{min}$, thus allowing the correct transference without altering its structure.¹⁸ Epifluorescence microscopy observation of the

supported films was performed in a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) by using a Hamamatsu camera (C10600–10B ORCA-R2, Herrsching am Ammersee, Germany). Images were obtained at different positions from the transferred films, and subsequently assigned to the surface pressures obtained during film transfer. Equivalent compression isotherms were subsequently registered by compressing lipid monolayers at $65\text{ cm}^2/\text{min}$ until reaching their collapse pressure.

RESULTS

Characterization of Ole e 1 Allergen Adsorption at the Air–Liquid Interface. Ole e 1 adsorbs at the air–liquid interface, forming a stable protein film as monitored by the increase in surface pressure (Figure 1). Its interfacial adsorption

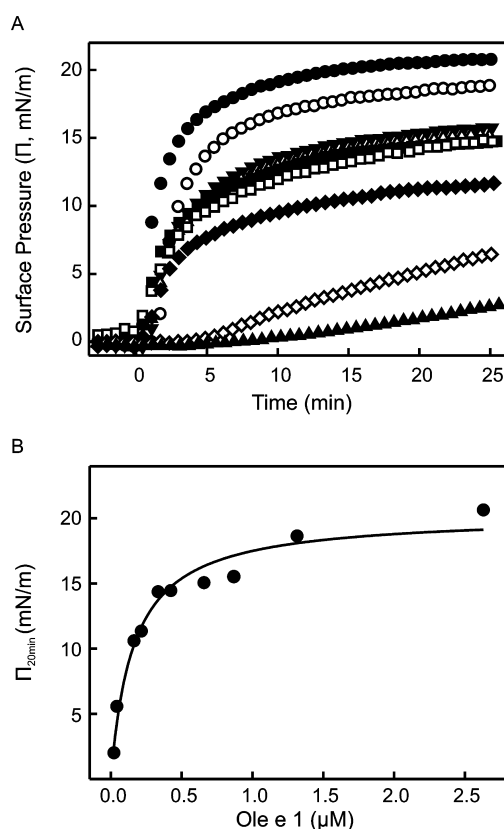


Figure 1. Interfacial adsorption kinetics of Ole e 1, the major olive pollen allergenic protein. (A) Π - t isotherms ($25 \pm 1^\circ\text{C}$) of different amounts of Ole e 1 injected at time = 0 (minutes) in a subphase composed of 5 mM Tris buffer, pH 7, 150 mM NaCl. Protein concentration in the subphase was 2.78 ($\bullet$), 1.36 ($\circ$), 0.89 ($\blacktriangledown$), 0.68 ($\triangle$), 0.42 ($\blacksquare$), 0.37 ($\square$), 0.21 ($\blacklozenge$), 0.16 ($\diamond$), 0.05 ($\blacktriangle$) μM , respectively. (B) Maximum surface pressure achieved at 20 min after injection ($\Pi_{20\text{min}}$, mN/m) versus Ole e 1 (μM). Solid line shows the hyperbolic trend that best fits protein interfacial behavior ($r^2 = 0.97$).

is highly dependent on protein concentration, fitting to a rectangular hyperbola as defined by eq 1

$$\Pi = \frac{\Pi_{\text{max}} \cdot C}{K + C} \quad (1)$$

where Π is the surface pressure value achieved at time = 20 min for each concentration, C is the allergen concentration (μM), Π_{max} depicts the theoretical maximum value of surface pressure that would be achieved at very high protein concentrations, and K represents an invariable value for the protein concentration

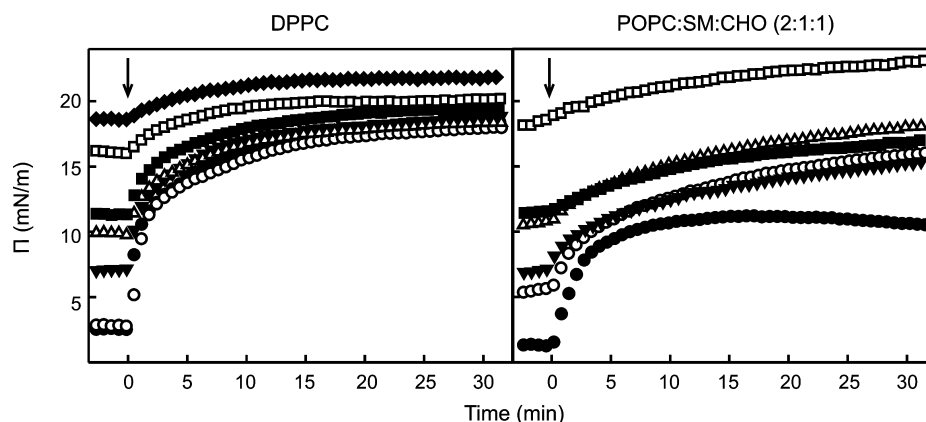


Figure 2. Insertion/adsorption kinetics of Ole e 1 into preformed phospholipid monolayers. Insertion/adsorption kinetics of Ole e 1 in DPPC (left) or POPC:SM:CHO (2:1:1 molar ratio, right) monolayers preformed at different initial surface pressures (symbol lines). Arrows indicate allergen injection ($0.55 \mu\text{M}$) into the subphase. All the assays were carried out at $25 \pm 1^\circ\text{C}$. The subphase was composed of 5 mM Tris buffer, pH 7, 150 mM NaCl.

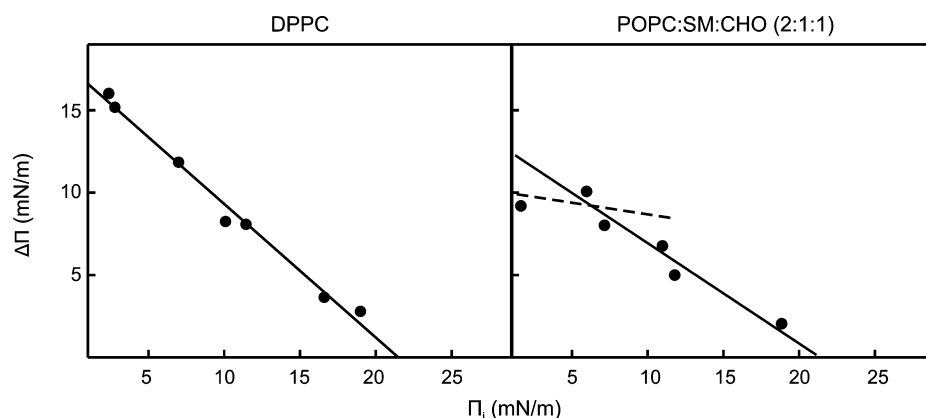


Figure 3. Critical insertion pressure of Ole e 1 into phospholipid monolayers. Increase in surface pressure ($\Delta\Pi$) versus initial pressure (Π_i) of DPPC or POPC:SM:CHO (2:1:1, molar ratio) preformed lipid monolayers upon injection of Ole e 1 at $25 \pm 1^\circ\text{C}$. The subphase and Ole e 1 solution ($0.55 \mu\text{M}$) was composed of a 5 mM Tris buffer, pH 7, 150 mM NaCl. In the left plot, the line represents the linear regression that best fits the experimental data ($r^2 > 0.97$). Each plot shows 1 out of 2 independent experiments. The intersection with the horizontal axis allows estimation of the critical insertion pressure (Π_c) in each of the films. The right panel shows the trends observed when the allergen was injected under POPC:SM:CHO films at low (dashed) and high (solid line) pressure regimes.

that yields half of the Π_{max} value. The values obtained after data fitting were $K = 0.16 \pm 0.03 \mu\text{M}$ and $\Pi_{\text{max}} = 20.3 \pm 0.9 \text{ mN/m}$, with an R^2 correlation value of 0.97. For the following experiments, an allergen concentration of $0.55 \mu\text{M}$ was fixed, because it allowed us to detect an acceptable protein interfacial activity, while avoiding the complete saturation of the liquid surface. Gibbs adsorption eqs 2 and 3 were also used to calculate the protein surface excess concentration (Γ) and surface molecular area (A), respectively

$$\Delta\Pi = \text{Ln}C \cdot R \cdot T \cdot \Gamma \quad (2)$$

$$A = \frac{1}{\Gamma_{\text{max}} \cdot N} (\text{\AA}^2) \quad (3)$$

where $\Delta\Pi$ is the maximum increment in surface pressure reached for each protein concentration, R is the gas constant ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), T is the temperature of the assay (298 K), A is the surface molecular area ($\text{\AA}^2$), Γ_{max} is the surface excess concentration at saturation, and N is Avogadro's constant number ($6.022 \times 10^{23} \text{ mol}^{-1}$). Data obtained for Ole e 1 interfacial activity was $\Gamma_{\text{max}} = (6.94 \pm 0.31) \times 10^{20} \text{ residues/m}^2$ and $A = 0.144 \pm 0.01 \text{ \AA}^2/\text{residue}$.

Interaction of Ole e 1 with POPC:SM:CHO (2:1:1) and DPPC Lipid Surfaces. Next, the interfacial adsorption of Ole e 1 to interfaces that have been previously occupied by DPPC or POPC:SM:CHO (2:1:1, molar ratio) spread monolayers was monitored at different initial pressures (Π_i). These mixtures were chosen due to the different nature of the lipid packaging structures that they produce in the interphase. DPPC is a saturated lipid that organizes in liquid-condensed (LC) phases above its phase transition pressure; on the other hand, the POPC:SM:CHO mixture produces segregation of immiscible liquid-ordered (Lo) and liquid-disordered (Ld) regions because of the presence of cholesterol, even at low surface pressures.¹⁹ Figure 2 shows that Ole e 1 injection into the aqueous subphase causes an increase in surface pressure in both type of films, which never exceed the maximum pressure value reached for the protein, 20 mN/m. Kinetics plots showed that the protein exhibits similar behavior on both films compressed to pressures higher than 10 mN/m, but not for films preformed at low pressures. The protein was unable to cause a pressure change higher than 10 mN/m when injected underneath POPC:SM:CHO monolayers, while it reached much higher values of surface pressure on DPPC experiments.

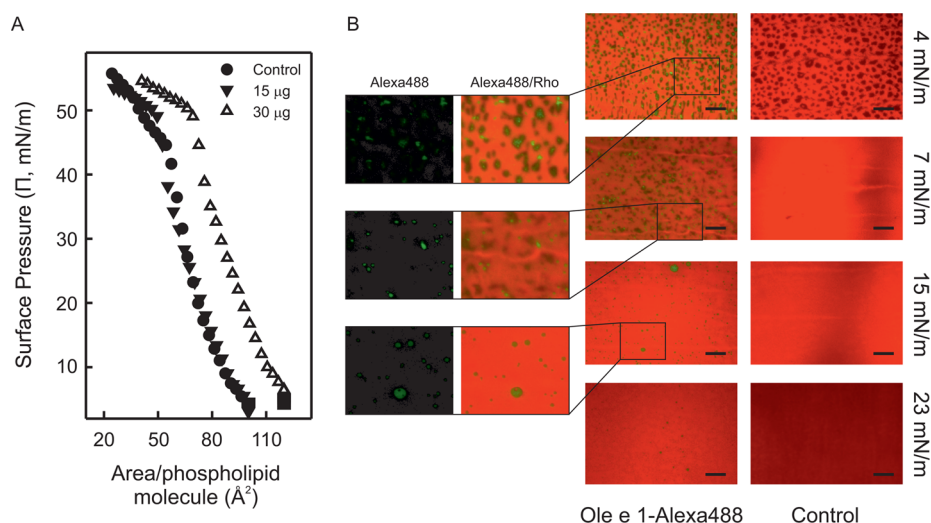


Figure 4. Interaction of Ole e 1 with POPC:SM:CHO (2:1:1 molar ratio) films. Π – A compression isotherms obtained upon injection of different protein amounts (μg) (A) and effects on the organization of POPC:SM:CHO monolayers (B) in the absence (Control) or in the presence of Ole e 1-Alexa488 (green spots). In B, epifluorescence images were taken from transferred films compressed to the indicated pressures. The films contained Rho-PE (red) to allow for observation of the phospholipid distribution at the interface at 25 ± 1 °C. Images with Ole e 1-Alexa488 represent the merge of both channels where a magnification ($\times 3$) of the details is represented (left, Alexa488 channel; right, Alexa488 and Rho channel). Scale bar at the bottom of all the pictures represents $25 \mu\text{m}$. Figure shows representative images taken from 1 out of 3 independent experiments.

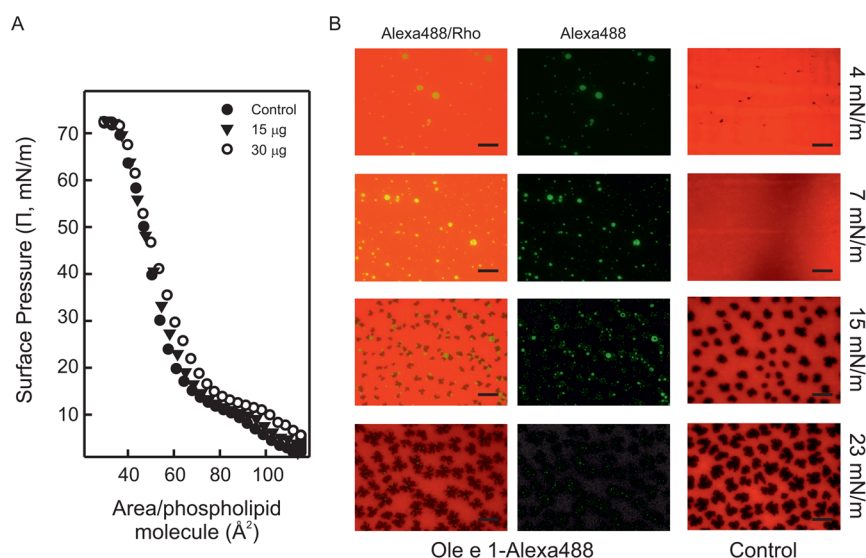


Figure 5. Interaction of Ole e 1 with DPPC films. Π – A compression isotherms obtained upon injection of different protein amounts (μg) (A) and effects on the organization of DPPC monolayers (B) in the absence (Control) or in the presence of Ole e 1-Alexa488 (green spots). In B, epifluorescence images were taken from transferred films compressed to the indicated pressures. The films contained Rho-PE (red) to allow for observation of the phospholipid distribution at the interface at 25 ± 1 °C. Images from films containing the allergen compare fluorescence from the protein (dark, right) and, on the left, the merger of both lipid (red) and protein (green) channels. Scale bar at the bottom of each picture represents $25 \mu\text{m}$. Figure shows representative images taken from 1 out of 3 independent experiments.

We estimated the critical pressure of insertion (Π_c) by plotting the pressure changes caused by the allergen as a function of initial pressure values (Figure 3). This value represents the theoretical maximum initial pressure that allows the insertion of the protein within preformed monolayers. The Π_c values calculated for Ole e 1 insertion in both types of monolayers was the same, 21.0 ± 0.5 mN/m, according to the maximum adsorption values. A linear trend was obtained for the insertion of the allergen into layers of both lipid mixtures compressed to most surface pressures. However, as noticed above, the insertion of the protein into POPC:SM:CHO films exhibits an inflection point at pressures below 5 mN/m,

suggesting a potential reorientation of Ole e 1 upon interaction with compositional or structural motifs generated within this monolayer at those lower surface pressures.

Organization of Lipid and Lipid/Ole e 1 Films: Compression Isotherms and Epifluorescence Analysis.

The interaction of Ole e 1 protein with lipid monolayers at the air–liquid interface and its effect on lipid packing are analyzed in Figures 4 and 5. Figure 4A shows the π – A isotherm of a POPC:SM:CHO (2:1:1) film in the absence (Control) and in the presence of 15 or 30 μg of Ole e 1. Protein injection (30 μg) expanded the isotherm up to 20 $\text{\AA}^2/\text{molecule}$, which indicates that Ole e 1 molecules are able to insert into the

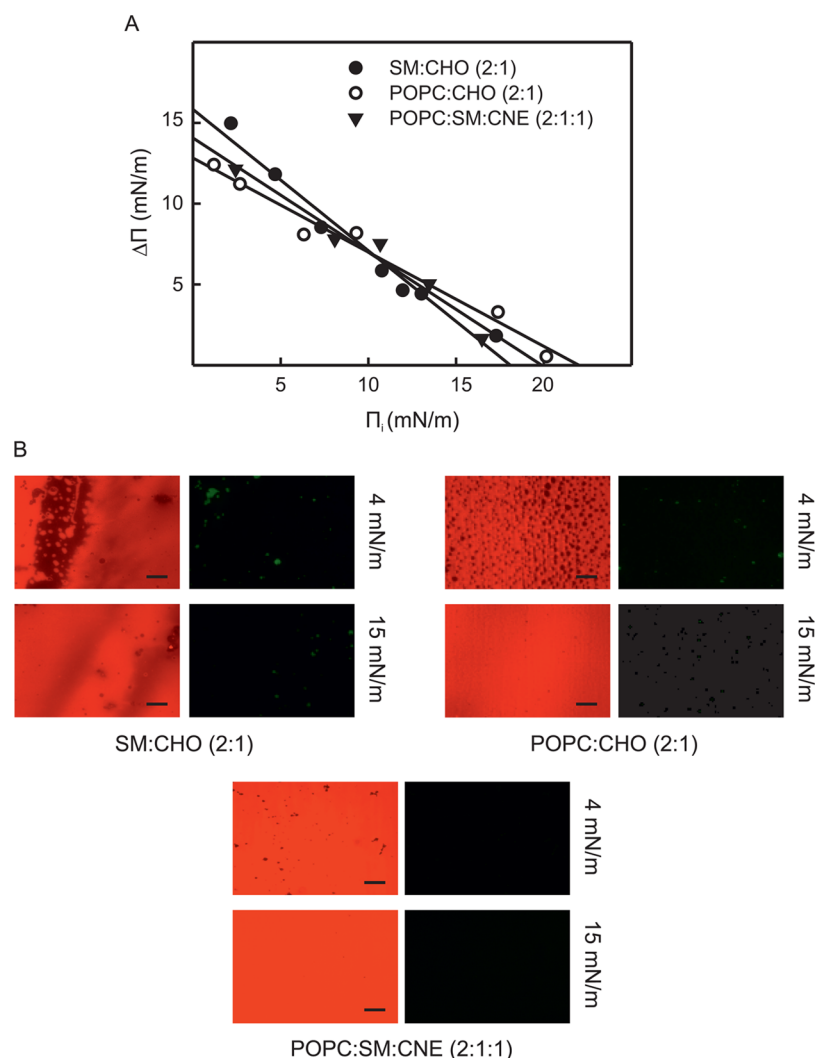


Figure 6. Interaction of Ole e 1 with SM:CHO (2:1), POPC:CHO (2:1), and POPC:SPM:CNE (2:1:1) films. (A) Increase in surface pressure ($\Delta\Pi$) versus initial pressure (Π_i) of SM:CHO (2:1), POPC:CHO (2:1), and POPC:SM:CNE (2:1:1) preformed lipid monolayers upon injection of Ole e 1 at 25 ± 1 °C. The subphase and Ole e 1 solution ($0.55 \mu\text{M}$) was composed of a 5 mM Tris buffer, pH 7, 150 mM NaCl. Lines represent the linear regression that best fits the experimental data ($r^2 > 0.97$) for each of the lipid systems. Figure shows representative data of 1 out of 2 independent experiments. (B) Epifluorescence images of transferred films compressed to the indicated pressures. The films contained Rho-PE (red, 1% mol/mol) to allow for observation of the phospholipid distribution at the interface at 25 ± 1 °C. $15 \mu\text{g}$ of Ole e 1 labeled with Alexa-Fluor488 (green) were used in all the cases. The left image of each condition represents the merger of both channels. Scale bar at the bottom of each picture represents $25 \mu\text{m}$. Figure shows representative images taken from 1 out of 3 independent experiments.

monolayer to occupy an area of the interface, by themselves or upon interaction with the surrounding lipids. All the films, including the ones containing protein, collapse at pressures around 50 mN/m without a clear indication of partial squeeze-out plateaus, indicating that Ole e 1 is able to remain associated with the interface under lateral compression at pressures much higher than its Π_c .

Epifluorescence images of POPC:SM:CHO films transferred at different pressures during compression are illustrated in the Figure 4B. For all the epifluorescence experiments $15 \mu\text{g}$ of Alexa488-labeled Ole e 1 (green fluorescent dots) were used. This amount of protein was judged to be optimal to obtain defined and high-resolution images showing the allergen effect and its localization within the lipid film. To reveal the occurrence of lateral phase separation in the chosen lipid system, Rho-PE—a fluorescent lipid probe preferentially partitioning into Ld phases—was added to the organic lipid solution (red). Compression of POPC:SM:CHO films up to 7

mN/m generated dark regions, likely corresponding to Lo domains enriched in SM and CHO but depleted in Rho-PE probe. These condensed areas were less numerous and they had diffuse edges in lipid/protein films compared to the morphology of domains in protein-free control films. Protein was observed to mainly colocalize within these dark areas, suggesting a relative affinity of the protein for these ordered structures. At pressures higher than 7 mN/m, these dark areas seem to disappear, at least at the microscopic scale observable by epifluorescence, and the protein reordered as large green clusters, coexisting with the remaining lipids at the interface until its apparent complete exclusion at pressures higher than 23 mN/m. These observations support the change of the trend in protein insertion detected at low initial pressures in the adsorption experiments toward POPC:SM:CHO films.

As stated before, the effect of Ole e 1 in DPPC isotherms (Figure 5A) relies on an expansion of $20 \text{ \AA}^2/\text{molecule}$ to larger areas, suggesting the interfacial occupation of the protein. This

effect was more pronounced when injecting 30 μg in the subphase than upon injection of 15 μg of protein. The presence of the protein at the DPPC interface caused a delay in the onset of the liquid-expanded (LE) to LC transition plateau, without apparently causing its alteration or disappearance. Control and lipid/protein curves converge at a surface pressure around 50 mN/m, suggesting that, at this pressure, the protein was totally excluded from the interface and the lipids remained in a highly condensed state. Epifluorescence analysis shows that the interaction of the protein with DPPC films (Figure 5B) was very different than observed in POPC:SM:CHO experiments. At surface pressures below the LE–LC transition plateau (<10 mN/m), the protein appeared as numerous and homogeneously distributed clusters, evenly coexisting with the LE lipid phase. At pressures above 10 mN/m dark LC domains that exclude the Rhodamine-PE probe emerge, as previously observed by Perez-Gil et al.²⁰ These presumably condensed lipid domains were slightly more numerous and smaller in the presence of the protein, which tends to be located at the boundaries as green aggregates. At the highest pressures tested, rosette-shaped domains occupied almost the entire interface and the protein appears as smaller aggregates at their edges. Even at these pressures with the allergen almost excluded, Ole e 1 causes a slight alteration in the shape of the lipid domains, generating greater number of pits and more abrupt edges with respect to the domains nucleated in protein-free control films.

Finally, the interaction of Ole e 1 with other lipid systems was tested by using lipid mixtures in which one of the POPC:SM:CHO components were depleted: SM:CHO (2:1) and POPC:CHO (2:1). As an additional reference, we tested a system, POPC:SM:CNE (2:1:1, molar ratio), in which CHO was substituted by CNE, an sterol that inhibits the formation of Lo domains.²¹ Figure 6A shows the determination of both the maximum change in surface pressure ($\Delta\Pi_{\text{max}}$) and the critical pressure value (Π_c) for these additional mixtures. The values of $\Delta\Pi_{\text{max}}$ were very similar in these systems, being the highest the one obtained for SM:CHO (12.7 mN/m), followed by the insertion in POPC:SM:CNE (11.3 mN/m) and that in POPC:CHO (10.3 mN/m). POPC:CHO achieved the highest value of Π_c (22.1 mN/m), followed not far behind by POPC:SM:CNE (19.8 mN/m) and SM:CHO (18.1 mN/m) films. Epifluorescence images illustrated in Figure 6B support interfacial adsorption results. At 4 mN/m, only POPC:CHO films exhibited segregation of dark regions corresponding to cholesterol-enriched Lo domains. However, the fluorescent-labeled protein seemed not to be located in these domains and its presence therein was much less conspicuous than observed in POPC:SM:CHO films. Noteworthy, cholestenone (CNE) fully prevented the presence of Lo domains, and that was associated with a clearly lower interfacial presence of Ole e 1. At high pressures (15 mN/m), the protein appeared again as green clusters, coexisting with lipid films characterized by having a total absence of ordered regions, at least at a microscopic length scale. In general terms, values, trends, and images obtained for these partially depleted systems seem to be very different from those obtained for POPC:SM:CHO films, suggesting a crucial requirement of the three components—POPC, SM, and CHO—to define maximal affinity in the interaction between Ole e 1 and lipid domains.

DISCUSSION

Ole e 1 Aeroallergen Is Able to Interact Efficiently with Air–Liquid Interfaces. Underneath air–aqueous

interfaces, Ole e 1 allergen has shown an interfacial adsorption capability comparable to those of technologically important surface-active proteins such as BSA, lysozyme, β -lactoglobulin, or β -casein in the same conditions.²²

Ole e 1 allergen is an N-glycosylated protein, showing a high content (40%) in aperiodic conformation determined by circular dichroism.¹⁷ Moreover, predictive analysis of Ole e 1 showed that it is a highly flexible and hydrophobic protein.¹⁷ All these properties support the high surface activity demonstrated here for Ole e 1 at the interface. This is in agreement with the features of other proteins reported to adsorb to air–water interfaces, such as β -casein where a combination of a flexible nonglobular structure and a hydrophilic and charged N-terminal region governs the interfacial adsorption.²³ In this sense, Ole e 1 polar regions may keep exposure to the liquid subphase while hydrophobic ones may be transferred to the airspace, potentially promoting allergen diffusion from the bulk solution toward the interface.

Interfacial activity is associated with conformational changes of the protein, which might induce process of oligomerization and aggregation, as it has been previously shown for β -lactoglobulin,²⁴ latherin,¹⁵ BSA, fibrinogen, and immunoglobulin G.²⁵ In this sense, protein allergenicity is highly influenced by structural rearrangements, oligomerization and aggregation, as it has been reported for the major birch pollen allergen, Bet v 1,²⁶ and other relevant allergenic proteins.^{26,27} Ole e 1 is a protein with a high trend to form oligomers, exhibiting a higher allergenicity.¹⁷ Thus, the relation between Ole e 1 allergenicity and the potential conformational changes induced upon its interfacial adsorption could be key features once the aeroallergen enters the respiratory tract and impacts its wet surface. Potential structural rearrangement of Ole e 1 during its interaction with lipid films could be further explored by using a combination of techniques such as Raman and FTIR spectroscopy.

Ole e 1 Aeroallergen Interacts with Lipid Films Generated at the Air–Liquid Interface. Considering the marked interfacial activity revealed by Ole e 1 aeroallergen, we proceeded to describe its behavior in a lipid film context by using Langmuir–Wilhelmy and Blodgett monolayers. Two physiologically relevant lipid mixtures were used as a model, POPC:SM:CHO (2:1:1, molar ratio), a mixture previously described as potentially mimicking segregation of raft-like domains thought to exist in cellular membranes²⁷ and DPPC, which constitutes the most abundant and surface-active component in pulmonary surfactant.

Ole e 1 adsorption/insertion plot describes a linear tendency with DPPC films, achieving a Π_c value (21 mN/m) comparable to those obtained for the rest of lipid systems assessed. Although this Π_c value did not exceed the threshold of surface pressure value of 30 mN/m, which is commonly considered as indicative of potential ability of a protein to insert into lipid bilayers,²⁸ the Π – A isotherm exhibited an expansion of 20 $\text{\AA}^2$ /molecule that was sustained up to 50 mN/m after Ole e 1 injection into the subphase, confirming allergen coexistence within the lipid film. Epifluorescence images of DPPC Langmuir films at surface pressures higher than 15 mN/m showed LC or bean-shaped domains of DPPC, where the fluorescent-labeled allergen was localized as protein clusters that seem to exclude the lipid. This observation suggests that lipid domains can act as nucleation sites for the allergen, in which new noncovalent interactions could be established between neighboring molecules, due to the interfacial

exposition of hydrophobic residues that are normally hidden in the protein structure.²⁹ The coexistence of protein and DPPC at the interface suggests the existence of several mechanisms that could take place in vivo during the first stages of allergen sensitization. On one hand, Ole e 1 adsorption could impair or abolish normal surface activity of pulmonary surfactant, which is crucial particularly during expiration. This surfactant inhibition process could be explained because of a steric competition, as has been previously reported for the surface-active plasma proteins BSA or hemoglobin.^{30,31} On the other hand, surfactant interfacial spreading properties has been recently proposed as a promising tool for transporting several drugs along the airways and into the body.³² In this context, it is conceivable that pulmonary surfactant could act as a potential carrier or shuttle for a wide variety of airborne compounds and aeroallergens, including Ole e 1, as our results are pointing out. Ole e 1 diffusion along the entire conducting airways (from bronchial to alveolar region) might contribute to the development of allergic responses.

The examination of the $\Delta\Pi$ versus Π_i plot for POPC:SM:CHO (2:1:1) revealed a biphasic trend not observed in the linear tendency obtained with DPPC films (Figure 3), suggesting a different mode of lipid–protein interaction with those monolayers at low surface pressures. It could be confirmed afterward by epifluorescence analysis, whose fluorescent images (Figure 4B) showed the existence of Lo lipid domains (dark areas) at pressures lower than 7 mN/m, where fluorescent-labeled Ole e 1 (green dots) is mainly localized. At pressures higher than 7 mN/m, lateral segregation completely disappeared, and the allergen appeared as homogeneously distributed clusters. The absence of Lo domains at the interface seems to correspond to the linear behavior obtained at the highest pressures of films made of POPC:SM:CHO, DPPC or other lipid mixtures in which the generation of cholesterol-rich domains was prevented (SM:CHO or POPC:SM:CNE). In addition, the presence of all lipid components in the POPC:SM:CHO mixture seems to be required for Ole e 1–domain interaction (Figure 6), as shown with the presence of ordered domains of different nature (POPC:CHO).

In spite of Ole e 1 showing critical interfacial insertion pressures not exceeding 30 mN/m, the allergen was able to remain in the interface under high lateral compression states. Taken together, these results could indicate a mechanism of interaction of Ole e 1 with Lo domains of the epithelial cell membrane at the time of contact. Thus, it has been reported that other allergens interact with structures localized in Lo domains involved in signaling pathways related to the immune response such as Th2-type inflammation. In this sense, Bet v 1 colocalizes with caveolar markers,³³ and house dust mite group-2 allergens interact with the Toll-like receptor 4 (TLR4).³⁴

CONCLUSIONS

In summary, our study has demonstrated the interfacial adsorption capabilities of the aeroallergen Ole e 1. Moreover, Ole e 1 displayed a particular behavior on lipid layers generated at air–liquid interfaces, which show lateral phase separation. The allergen tended to localize associated with liquid-condensed and ordered domains. Collectively, these results support the concept that the interfacial activity of certain allergens—such as Ole e 1—are related to their association with surface lipid films, accumulating preferentially in the coexisting lipid phases. Thus, surface clustering could be a key

feature to define their stable presence at the epithelial barrier, triggering the allergic response.

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Notes

The authors declare no competing financial interest.

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