Biophysical and biological impact on the structure and IgE-binding of the interaction of the olive pollen allergen Ole e 7 with lipids

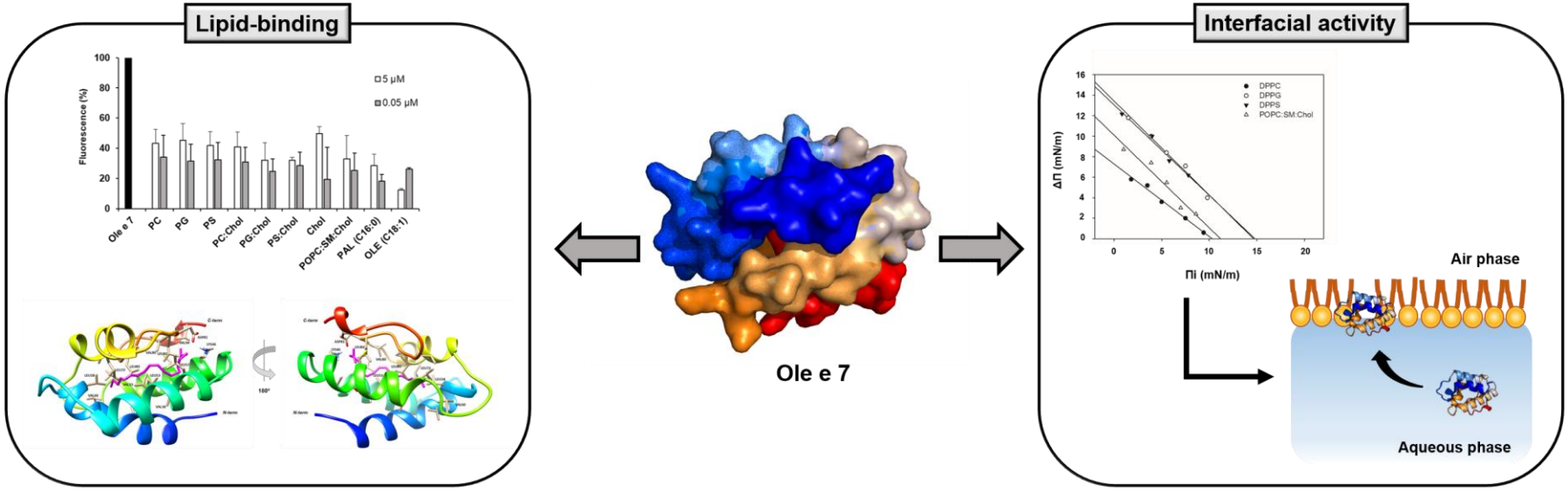
Carmen Oeo-Santos, Juan Carlos López-Rodríguez, Cristina García-Mouton, Pablo San Segundo-Acosta, Aurora Jurado, Carmen Moreno-Aguilar, Begoña García-Álvarez, Jesús Pérez-Gil, Mayte Villalba, Rodrigo Barderas, Antonio Cruz.

Biochim Biophys Acta Biomembr. 2020 Jun 1;1862(6):183258.



https://doi.org/10.1016/j.bbamem.2020.183258

**Graphical Abstract (for review)**



## Highlights

- Ole e 7 mainly interacts with negatively charged phospholipids and oleic acid.
- Aliphatic amino acids are involved in lipid-protein interaction.
- Lipid-binding does not produce structural or immunological changes in Ole e 7.
- Ole e 7 allergen possess interfacial adsorption ability.
- Ole e 7 interacts with and transports lipids from pulmonary surfactant to the interface.

# Biophysical and biological impact on the structure and IgE-binding of the interaction of the olive pollen allergen Ole e 7 with lipids

**Carmen Oeo-Santos, PhD[1], Juan Carlos López-Rodríguez, PhD[1,^], Cristina García-Mouton, BS[2,^], Pablo San Segundo-Acosta, BS[1], Aurora Jurado, MD, PhD[3,4], Carmen Moreno-Aguilar, MD, PhD[3,4], Begoña García-Álvarez, PhD[2], Jesús Pérez-Gil, PhD[2], Mayte Villalba, PhD[1], Rodrigo Barderas, PhD[1,5,*], Antonio Cruz, PhD[2,*].**

*Co-senior authors and co-corresponding authors.
^ Equal contribution.

1. Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain.
2. Departamento Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, and Research Institute "Hospital 12 de Otubre", Universidad Complutense, 28040, Madrid, Spain.
3. Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC)/ Hospital Universitario Reina Sofía/ Universidad de Córdoba, 14004 Córdoba, Spain.
4. Allergy Network ARADyAL. Instituto de Salud Carlos III, Madrid, Spain.
5. Chronic Disease Programme, UFIEC, Instituto de Salud Carlos III, 28220, Majadahonda, Madrid, Spain.

*To whom correspondence should be addressed:
Rodrigo Barderas.
Functional Proteomics Unit, UFIEC, Chronic Disease Programme, Instituto de Salud Carlos III, E-28222 Majadahonda, Madrid, Spain; Tel.: 34-91-8223231; E-mail: r.barderasm@isciii.es

* To whom correspondence should be addressed:
Antonio Cruz.
Departamento Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad Complutense, E-28040, Madrid, Spain; Tel.: +34 913944994 ; E-mail: acruz@ucm.es

## Abstract

Ole e 7 allergen from *Olea europaea* pollen possesses a major clinical relevance because it produces severe symptoms, such as anaphylaxis, in allergic patients exposed to high olive pollen counts. Ole e 7 is a non-specific lipid transfer protein (nsLTP) characterized by the presence of a tunnel-like hydrophobic cavity, which may be suitable for hosting and, thus, transporting lipids -as it has been described for other nsLTPs-. The identification of the primary amino acid sequence of Ole e 7, and its production as a recombinant allergen, allowed characterizing its lipid-binding properties and its effect at air-liquid interfaces. Fluorescence and interferometry experiments were performed using different phospholipid molecular species and free fatty acids to analyse the lipid-binding ability and specificity of the allergen. Molecular modelling of the allergen was used to determine the potential regions involved in lipid interaction. Changes in Ole e 7 structure after lipid interaction were analysed by circular dichroism. Changes in the IgE binding upon ligand interaction were determined by ELISA. Wilhelmy balance measurements and fluorescence surfactant adsorption tests were performed to analyse the surface activity of the allergen. Using these different approaches, we have demonstrated the ability of Ole e 7 to interact and bind to a wide range of lipids, especially negatively charged phospholipids and oleic acid. We have also identified the protein structural regions and the residues potentially involved in that interaction, suggesting how lipid-protein interactions could define the behaviour of the allergen once inhaled at the airways.



## Keywords

Aeroallergen; nsLTP; oleic acid; phospholipids; interfacial activity; pulmonary surfactant.


## Abbreviations

nsLTP: non-specific Lipid Transfer Protein.
Th2: T-helper 2 cell-response.
IgE: immunoglobulin E.
Chol: cholesterol.
PAL: palmitic acid.
OLE: oleic acid.
ANS: 1-anilinonaphthalene-8-sulfonic acid.
SUVs: small unilamellar vesicles
LUVs: large unilamellar vesicles

## 1. Introduction

Prevalence of allergic diseases is increasing worldwide, especially in developing countries [1]. Respiratory allergies constitute a growing health problem that affects around 25% of the population [2]. Most of the allergic disorders are caused by the exposition to foreign and harmless molecules, called allergens. These antigens produce the activation of a T-helper 2 cell-response (Th2), with the subsequent specific IgE production and the onset of clinical symptoms [3]. Although proteins are the main elicitors of allergic responses, they are usually accompanied by other components such as carbohydrates or lipids. Increasing evidences suggest that these components may also promote a Th2 immune response profile, acting as adjuvants [4]. Regarding lipids, they also act as natural ligands of certain allergenic proteins, and thus modify their immunological properties [5-7].

Several allergens of many protein families show lipid binding capacity: i) Bet v 1-like proteins, ii) non-specific lipid transfer proteins (nsLTPs), iii) 2S albumins, iv) secretoglobins, v) lipocalins, vi) oleosins, and vii) mite group 2, 5 and 7 proteins [8-14]. Among them, nsLTPs, belonging to the prolamin protein superfamily [15], are a group of small and soluble ~10 kDa proteins with an ubiquitous distribution in the plant kingdom [16]. nsLTPs are cysteine-rich proteins which have four α-helices stabilized by four conserved disulphide bridges. nsLTPs share common features on their structure, as the presence of a tunnel-like hydrophobic cavity where the lipid-binding site is located [17]. Other physiological roles described for nsLTPs are cell wall organization, membrane stabilization and signal transduction [18]. Ole e 7, the olive pollen nsLTP, is one of the main causes of allergy in the Mediterranean basin [19, 20]. This allergen has been associated to severe symptoms such as anaphylaxis [21]. Despite its clinical

relevance, the influence of lipids on its structural and allergenic features remains to be studied.

Aeroallergens, like Ole e 7, enter into the body through the upper airways, reaching the mucosal surface and making a first contact with two lipid-based barriers: the pulmonary surfactant layers located at the outer side of the *mucus*, and the luminal plasma membrane of the airway epithelial cells. Pulmonary surfactant is a complex mixture of lipids and proteins, which covers the distal respiratory epithelium. It is mainly composed of mostly saturated phospholipids (80% by mass) as phosphatidylcholine (DPPC), which is essential for pulmonary surfactant surface active properties. Pulmonary surfactant also contains proteins (6-8%), including the four surfactant specific entities: SP-A, SP-B, SP-C and SP-D. The main role of pulmonary surfactant is to maintain an operative respiratory surface at the lungs [22]. Additionally, it is also involved in host defence and immune responses in the lung. In fact, there are many evidences that SP-A and SP-D collectins are mediators of allergen binding [23-25], and modulators of effector cell activity [26-28].

In the present study, we aimed at analysing the lipid-binding capabilities of the aeroallergen Ole e 7 and the impact of this interaction on its structure and IgE-binding. In addition, the interfacial behaviour of the allergen was evaluated using models of phospholipid layers. Our data demonstrate that Ole e 7 binds a wide range of lipid ligands, predominantly negatively charged phospholipids, and that the lipid-binding has no effect on its IgE-binding ability.

## 2. Materials and Methods

### *2.1.Patients*

The study was approved by the Ethical Committees of the Reina Sofía University Hospital, Córdoba, Spain (ref. 3033), and the Complutense University and Instituto de Salud Carlos III (CEI P49). Written informed consent was obtained from all patients. All samples were anonymously handled.

Sera from 6 patients recruited at the Immunology and Allergy Department of the Reina Sofía University Hospital (Córdoba, Spain) with a confirmed history of allergy to olive pollen with sensitization to Ole e 7 were included in the study. Clinical evaluation included examination of patient history, SPT and determination of specific IgE (sIgE). The SPT was performed according to the European guidelines [29], using commercial extracts from *Olea europaea* pollen (ALK-Abello, Madrid, Spain). A SPT response was considered positive when the diameter of the wheal was 3 mm greater than that induced by the negative control. The sIgE was measured by ImmunoCAP 250 (Phadia, Uppsala, Sweden) according to the manufacturer recommendations.

### *2.2.Materials*

Native porcine lung surfactant was purified from bronchoalveolar lavage as previously described [30]. Surfactant concentration was measured according to Rouser *et al* [31]. Labelling of native surfactant was performed with the BODIPY-PC (2-(4,4-difluoro-5,7-dimetil-4-bora-3a, 4a-diazo-s-indaceno-3-pentanoil)-hexadeca-noil-sn-glicero-3-phosphocoline) probe (Invitrogen). Briefly, native surfactant (0.5 mg/ml) was resuspended in a buffered solution (5 mM Tris, 150 mM NaCl, pH 7.4) containing BODIPY-PC in a molar ratio 1:100. Then, the mixture was incubated at 37ºC for 1 h, with vigorous shaking every 5 min.

144 Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol
145 (DPPG), dipalmitoylphosphatidylserine (DPPS), egg yolk phosphatidylcholine (PC),
146 egg yolk phosphatidylglycerol (PG), porcine brain phosphatidylserine (PS), porcine
147 brain sphingomyelin (SM), and ovine cholesterol (Chol) were obtained from Avanti
148 Polar Lipids (Alabaster, AL). Palmitic acid (C16:0) (PAL) and oleic acid (C18:1)
149 (OLE) were obtained from Sigma-Aldrich (San Luis, MO).
150

151 ***2.3.Preparation of lipid mixtures and lipid vesicles***

152 Lipids were dissolved or diluted in chloroform:methanol (2:1, v/v): DPPC,
153 DPPC:Chol (2:1), DPPG, DPPG:Chol (2:1), DPPS, DPPS:Chol (2:1), POPC:SM:Chol
154 (2:1:1), PAL (C16:0) and OLE (C18:1).

155 For liposome preparation, each phospholipid mixture was dried under a nitrogen
156 stream. Vacuum was later applied for 2 h to remove any organic solvent trace. Samples
157 were hydrated in a buffer solution (5 mM Tris, 150 mM NaCl, pH 7.4) at 42ºC, prior to
158 use. Hydration was completed after vigorous shaking every 10 min for 1 h to
159 reconstitute multilamellar suspensions. Experiments were performed using the buffer
160 solution described above, unless indicated.

161 SUVs and LUVs were prepared by extrusion of the multilamellar suspensions
162 using polycarbonate filters with a pore size of 50 nm and 100 nm (Nucleopore,
163 Whatman), respectively [32].
164

165 ***2.4.Purification of natural and recombinant olive pollen nsLTP***

166 Natural and recombinant Ole e 7 were obtained as previously described [33]. Both
167 proteins were purified by size-exclusion chromatography (Sephadex-50 medium and
168 superfine) and RP-HPLC, and analysed by 17% SDS-PAGE and by WB with rabbit

antiserum against natural Ole e 7 (1:10000) [33]. All the experiments were carried out with the recombinant allergen, except for the Fluorescence surfactant adsorption test, which was performed comparing natural and recombinant Ole e 7.

### *2.5.Fluorescent ligand displacement assay*

Fluorescent ligand displacement (ANS) assay was performed to detect protein-ligand binding as previously reported in previous works [34-36]. The phospholipids PC, PG and PS, the mixtures PC:Chol (2:1), PG:Chol (2:1) and PS:Chol (2:1), the unsaturated fatty acid, OLE (C18:1), and the saturated fatty acid, PAL (C16:0), were assessed for their abilities to compete with the fluorescent probe ANS (1-anilinonaphthalene-8-sulfonic acid) for the binding to rOle e 7. Each phospholipidic mixture was added as LUVs in the buffer solution, while fatty acids were diluted in a chloroform:methanol (2:1, v/v) solution. The allergen (5 μM) was incubated with each lipid at 1:1 and 1:100 molar ratios (protein:lipid). Ligand binding was analysed by adding 5 μM ANS, measuring the ANS fluorescence emission at 456 nm, upon excitation at 350 nm. All samples were analysed in triplicate. rOle e 7 without ligands was used as positive control of the maximum insertion of ANS into the hydrophobic pocket of the protein, reporting the highest fluorescence levels. Lipid ligands alone were used as negative controls. Fluorescence emission was measured in a FLUOstar OPTIMA microplate reader (BMG-Labtech, Ortenberg, Germany).

### *2.6.Analysis of lipid-protein interaction by interferometry*

Lipid-protein interaction was also analysed by using the BLItz system (*Bio- Layer Interferometry Technology* (FortéBIO, Fremont, CA)), which provides real-time data on protein interactions based on surface interferometry [37]. rOle e 7 interaction analysis

was performed with PC, PG, PS, PC:Chol (2:1), PG:Chol (2:1), PS:Chol (2:1) and POPC:SM:Chol (2:1:1) LUVs, using final concentrations of 1 µg/µl of protein and lipid. An aminopropylsilane (APS) biosensor was used in the measurements because of its ability to adsorb proteins and other molecules through hydrophobic moieties. First, the biosensor was moisturized in buffer solution (5 mM Tris, 150 mM NaCl, pH 7.4). After performing a baseline, the corresponding lipid was immobilized, removing the non-immobilized ligand. Then, the association of rOle e 7 to the biosensor (in the presence of the immobilized ligand) was quantified. Association and dissociation curves were recorded to determine lipid-protein interaction.

### *2.7.Molecular modelling of Ole e 7*

The Ole e 7 allergen model was generated using ExPASy SWISS-MODEL [38] and nsLTP from peach (Pru p 3, PDB: 2b5s.2, 31.52% identity with Ole e 7) as template.

The three-dimensional structure model of Ole e 7 allergen was generated using I-TASSER (Iterative Threading ASSEmbly Refinement) [39]. Among the structural templates used by I-TASSER for modeling, the maize nsLTP (PDB: 1fk5.1, 36.56% identity with Ole e 7) was used, whose structure has been reported bound to OLE (C18:1) [40].

### *2.8.Circular dichroism spectroscopic analyses*

The circular dichroism (CD) spectrum of rOle e 7 in the absence or presence of lipids was recorded in the far UV (190−250 nm wavelength) region in a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using 0.1 cm optical-path quartz cuvettes. CDNN software was used for CD spectra deconvolution [41].

To analyse changes in the secondary structure of the protein as a result of the lipid-protein interaction, PG and PG:Chol SUVs and OLE (C18:1) were used. rOle e 7 and the respective lipid were resuspended in sodium phosphate buffer 50 mM pH 7.5. The allergen (5 μM) was incubated with each lipid at 1:1 molar ratio after optimizing the assay. Incubations before spectroscopic analysis were performed at 4ºC overnight under shaking. The scattering caused by SUVs was corrected from the spectra before carrying out the secondary structure analysis.

### *2.9.IgE-reactivity analysis*

96-well plates (Costar) were coated for 2 h at room temperature with the indicated protein-lipid mixture, previously incubated at 4ºC overnight under shaking. Protein final concentration was 1 μM (equivalent to 1 μg/μl) and lipid concentration 30 μM. Each well was blocked with phosphate-buffered saline pH 7.3 (NaCl 0.8% (p/v), KCl 0.02% (p/v), $KH_2PO_4$ 0.02% (p/v) and $Na_2HPO_4 \cdot 12H_2O$ 0.3% (p/v)) -blocking buffer-, containing 0.5% v/v Tween 20 and 3% w/v calcium fat free milk. Individual sera (n=6) from Ole e 7 allergic patients were diluted (1:10) in blocking buffer and added onto the ELISA plates followed by an incubation at 37ºC for 2 h. Binding of human IgE was detected using a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000) (Southern Biotech, Waltham, MA). Peroxidase reaction was detected by using 50 μl per well of 0.63 mg/ml o-Phenylendiamine in 0.1 M sodium citrate 4% methanol containing 1.6 μl/ml 30% $H_2O_2$. The reaction was stopped with 50 μl 3N $H_2SO_4$ and the corresponding optical density was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

### *2.10. Fluorescence surfactant adsorption test*

Ole e 7 interfacial adsorption ability was evaluated in 96-well microtiter plates (Nunc®, Merck) as previously described [42], using a FLUOstar OPTIMA Microplate Reader (BMG Labtech, Offenburg, Germany). The allergen was added to each well containing 80 µl of a buffer solution (5 mM Tris, 150 mM NaCl, pH 7.4) and the strongly light-absorbing Brilliant Black (BB, 5 mg/ml) agent. Then, native surfactant from porcine lungs (25 µg/µl) labelled with the fluorescent dye Bodipy-PC (2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine) was applied at the bottom of each well at a 1% molar ratio. The plate was incubated under orbital shaking and then, fluorescence intensity measured from above, as due to the adsorption of surfactant to the interface. The plate was analysed for 1 h at 25 °C. Obtained data were represented in relative fluorescence units (RFU) as the mean of two replicate values corrected by background subtraction. A decrease in the fluorescence intensity indicates an inhibition of the adsorption of lung surfactant to the interface due to the presence of the allergen. Fluorescence achieved by the adsorption of natural surfactant in the absence of allergen was used as control of full surfactant adsorption activity. Inhibition by human serum (25 µg/µl) was used as control of full inhibition.

### *2.11. Wilhelmy balance measurements*

Adsorption of the allergen to the air-liquid interface was also determined from the changes in surface pressure (ΔΠ) over a time interval of 35 min (Π-t isotherms). These studies were performed using a Wilhelmy teflon trough (NIMA Technologies, Coventry, UK) thermostated at 25 ± 1 ºC, with constant stirring. First, lipid monolayers were performed by depositing small volumes of the defined lipid solution (0.1 mg/ml) in chloroform:methanol 2:1), until the required surface pressure was reached. After

solvent evaporation, 10 µl of Ole e 7 were injected into the buffer subphase (1.8 ml), at a final concentration of 1 µM, and the ΔΠ was monitored during 35 min. Surface pressure changes were measured upon injection of the allergen against different initial pressures (Πi). Maximum increase in surface pressure ($\Delta\Pi_{max}$) and critical insertion surface pressure (Πc) were estimated for each phospholipid mixture used from ΔΠ versus Πi plots.

## 3. Results

### *3.1.Ole e 7 binds to a wide variety of lipids*

The interaction of rOle e 7 with phospholipid vesicles (LUVs) or fatty acids was studied by a displacement assay using the ANS probe, and by interferometry (Fig 1). Although the highest protein-lipid binding, which corresponds to the lowest fluorescence values, was observed when the protein was incubated with the unsaturated fatty oleic acid (C18:1), we observed that Ole e 7 binds to a greater or lesser extent to all tested lipids (Fig 1A).

Interferometry experiments supported the results obtained by the ANS displacement assay, with Ole e 7 being able to bind to all lipid mixtures with different affinity. We observed that PG affinity to rOle e 7, but not PS, was decreased over time, suggesting that the interaction of rOle e 7 with PG was less stable than with PS (Fig 1B). Moreover, the presence of Chol in the interaction of rOle e 7 with PS seems to stabilize the lipid-protein binding as dissociation constant suggests. In contrast to the obtained results by ANS displacement assay, the interaction between rOle e 7 and PC or PC:Chol (2:1) was almost stealthy.

### *3.2.Lipid interaction does not have a large impact in the secondary structure of the allergen*

Next, to evaluate changes in the secondary structure of the allergen due to lipid binding, CD analyses after lipid-protein interaction with indicated lipids were performed (Fig 2). Oleic acid (C18:1) was used because it showed the highest lipid-binding rates, and PG:Chol (2:1) and PG liposomes were used as models of phospholipid binding.

Slight changes in the secondary structure of Ole e 7 were detected, with the highest observed changes after incubation with oleic acid (C18:1) and PG:Chol (2:1). However, these differences did not exceed 5% regarding the contribution of α-helix to the secondary structure of the protein.

### *3.3.Modelling of the Ole e 7 - oleic acid complex*

Changes in the secondary structure further support that ligand binding might slightly affect the overall 3D structure of the allergen Ole e 7.

To address this question and get further insights into the interaction between Ole e 7 and oleic acid (C18:1), the 3D model of the protein-fatty acid complex was obtained to identify the main amino acids involved in the interaction with oleic acid (C18:1). The 3D model of Ole e 7 showed the traditional conformation of nsLTPs, with a hydrophobic cavity formed between the four packed α-helices. That cavity acts like a deep tunnel where the oleic acid molecule fits between H2, H3 and H4 helixes through interactions with the hydrophobic residues of the protein, while the carboxylate portion is turned towards the solvent. Thus, Ole e 7 was in contact with oleic acid (C18:0) through the aliphatic residues Leu18, Leu57, Leu72, Leu82, Leu85, Val33, Val36 and Val80, and the Asp81 residue (Supporting Figure 1).

### *3.4.Lipid-binding does not modify the recognition of IgE epitopes in rOle e 7*

Next, the potential effect in the IgE-binding ability to Ole e 7 due to protein-lipid interaction was examined by ELISA. No significant IgE-binding variations after lipid interaction in comparison to the ligand-free rOle e 7 were detected in any of the six-olive pollen allergic patients' sera tested (Fig 3).

### *3.5.Ole e 7 shows interfacial adsorption at air-liquid interfaces*

We next evaluated the surface and phospholipid interaction abilities of Ole e 7 as it may be a crucial feature for the allergic response triggered at the airways.

First, the interfacial activity of Ole e 7 was characterized. To this end, we previously determined the optimal allergen concentration to perform the experiments, by following the increase in surface pressure reached by different amounts of allergen in the subphase [32]. Then, for subsequent experiments, the optimal concentration was fixed at 0.5 μM, where a surface pressure of 11 mN/m is observed (Fig 4). Different lipid mixtures were tested according to their high presence in the pulmonary surfactant (DPPC), their charge (DPPS and DPPG) or the packaging that they may produce in cell membranes (POPC:SM:Chol as model of liquid-ordered domains [43]) (Fig 5, Supporting Figure 2).

Injection of Ole e 7 into the subphase produced an increase in surface pressure in all cases, reaching the highest increment (ΔΠ) upon insertion into negatively-charged DPPG and DPPS monolayers, with surface pressure values of 14.4 and 14.8 mN/m, respectively (Supporting Figure 3). These values were 10.4 mN/m for DPPC and 11.2 mN/m for POPC:SM:Chol (2:1:1) (Supporting Figure 3). Conversely, using DPPC as model of study, we detected a slight increment in the surface pressure of insertion of Ole e 7 when Chol was present in the monolayer, resulting in a maximum surface pressure of 17 mN/m (Supporting Figure 3).

In addition, the critical pressure of insertion (Πc), which indicates the theoretical maximum initial pressure that allows the insertion of the protein within a preformed lipid monolayer, was estimated by extrapolating to the abscissa axis the regression lines obtained from the experimental data fitting (Fig 5). Πc values were higher for the insertion of the allergen into DPPS and DPPG monolayers, 14.7 mN/m in both cases,

than in DPPC films (Fig 5A). Moreover, Πc was substantially higher in films of DPPC containing Chol (2:1 w/w) (20.5 mN/m) than in pure DPPC monolayer (10.5 mN/m) (Fig 5B).

### *3.6.Ole e 7 partially inhibits the interfacial adsorption of pulmonary surfactant*

Finally, the ability of Ole e 7 to inhibit the adsorption of pulmonary surfactant was analysed using a fluorescence surfactant adsorption test (SAT). We also tested whether natural or recombinant Ole e 7 showed equivalent interfacial adsorption behaviour to confirm the feasibility of using the recombinant isoform instead natural Ole e 7 in functional experiments, as previously reported [33]. Both natural and recombinant allergens displayed an equivalent capability to reduce the rate of adsorption of pulmonary surfactant to the air-liquid interface, especially at short periods of time (Fig 6). Next, the Ole e 7 interfacial activity was compared to that of another allergen from olive pollen with marked interfacial activity, Ole e 1 [32]. In contrast to Ole e 7, Ole e 1 inhibited completely the adsorption and spreading of native surfactant into the air-liquid interface at the highest concentrations (50 and 25 µg/µl) tested, in a comparable manner to BSA, which also possess a marked interfacial activity [44-46] (Fig 6). Interestingly, in a mixture of Ole e 1 and Ole e 7, we observed that Ole e 7 abolishes the inhibitory action of the highest concentrations of Ole e 1 against pulmonary surfactant (Fig 6).

## 4. Discussion

Non-specific lipid transfer proteins (nsLTPs) are relevant panallergens present in fruits, vegetables, nuts, pollen and latex [16]. These allergens present a hydrophobic cavity with the ability to accommodate a wide variety of lipids, from phospholipids to fatty acids, interacting with apolar amino acids [47, 48]. Although it is known that the ligand specificity depends on the protein family member [40, 49-54], the potential lipid ligands of most nsLTPs and the clinical effects produced by this interaction remain unclear. Related to the affinity of nsLTPs to bind hydrophobic ligands, to clarify the potential interfacial properties of aeroallergens may facilitate the understanding of their behaviour once they reach the airway mucosa. However, few works have been focused on the study of the interfacial activity of clinically relevant allergens [32, 55]. In this sense, the completion of the primary amino acid sequence of Ole e 7 by proteomics, and its recombinant production [33], have made possible to deepen into these processes.

In this study, we have investigated the lipid-binding ability of Ole e 7, the specificity of that binding and their effect on its structure and immunological properties. Furthermore, we have checked whether Ole e 7 is able to interact with air-liquid interfaces efficiently, which may be linked to its potential to develop an allergic response.

Binding assays demonstrated that Ole e 7 interacts with a wide range of lipid ligands, as it has been described for other nsLTPs [56]. According to the literature, the lack of specificity of nsLTPs relies on the high plasticity and flexibility showed by their lipid-binding cavity, which influence the protein-lipid interaction [57, 58]. Interestingly, it was a fatty acid, oleic acid, which showed the highest binding to Ole e 7, which agrees with other related studies with nsLTPs, such as maize, peach, apple, hazelnut, sun flower seeds and walnut [34-36, 40]. Moreover, we built a 3D structural model of

Ole e 7 interacting with the oleic acid by using the maize nsLTP as template. Despite its scarce identity sequence with Ole e 7, this nsLTP showed the highest score after the analysis by the I-TASSER software, which provides accurate structural and function template predictions using state-of-the-art algorithms. Furthermore, lipid-protein interaction takes place in the hydrophobic pocket of the allergen, which is a consistent and highly conserved feature of nsLTPs. Hence, the location of this cavity, and most of the amino acids forming it, are highly conserved in the maize nsLTP and Ole e 7, as well as in all nsLTPs described so far. Thereby, the amino acids potentially involved in the lipid-protein interaction were determined (Supporting Fig 1). It was confirmed that most of them were aliphatic residues (Leu18, Leu37, Leu72, Leu82, Leu85, Val33, Val36 and Val80) as previously described for Jug r 3, the nsLTP from nut [36]. Oleic acid is an unsaturated fatty acid with a long C18 chain, and a double bond that imposes a specific conformation, both optimal features to form stable complexes, which are not present in saturated fatty acids such as palmitic acid (C16:0) [36, 57]. Moreover, Han *et al.* [40] suggest that oleic acid has a similar affinity to other unsaturated fatty acids. Consequently, the interaction of Ole e 7 with other unsaturated fatty acids cannot be excluded. Regarding phospholipids, allergen interaction with PG and PS vesicles was reported, with or without the presence of Chol. The analysis of the interaction of the protein with lipid vesicles by interferometry allowed us to monitor the real-time interaction with lipid vesicles, which also determines their binding stability. Hence, we observed that Ole e 7-PG or Ole e 7-PG:Chol (2:1) interaction was less stable than the interaction observed with PS or PS:Chol (2:1) vesicles. Furthermore, a high interaction of Ole e 7 with POPC:SM:Chol (2:1:1) LUVs was also observed, suggesting that Ole e 7 could potentially interact with eukaryotic plasma membranes [59, 60].

No significant variations were detected in the secondary structure of the allergen upon ligand binding, with changes in the proportions of secondary structure elements below 5%. In this regard, the use of the recombinant protein purified by HPLC makes it possible to ensure the absence of lipids previously attached to the cavity that could prevent the detection of these changes caused by the binding of the lipids with respect to their free form. However, slight changes in the secondary structure may produce the exposition of epitopes which would be hidden in the native conformation of the protein, modifying the immunological properties of the allergen, as it has been previously suggested for other nsLTPs [34, 35]. Hence, we performed the *in vitro* analysis of the IgE-binding capability of Ole e 7, in the absence or presence of lipid ligands. Our findings indicate that no additional IgE epitopes are exposed after lipid-binding, although modifications in the IgE recognition have been reported by other authors in other nsLTPs [34, 36]. Therefore, since there are no studies related to the IgE-epitope mapping of Ole e 7, it could also be speculated that the major epitopes (or some of them) may overlap with certain amino acids relevant for the interaction with lipids, avoiding any change in the IgE reactivity. Further studies *ex vivo* by basophil activation test and *in vitro* using the RBL-2H3 mast cell model should confirm whether an effect of lipid-binding on Ole e 7 allergenic properties or not might be observed.

Regarding the interfacial activity of the allergen, Wilhelmy plate experiments demonstrated that Ole e 7 was able to effectively adsorb onto air-liquid interfaces. This behaviour is consistent with the hydropathy calculated for the allergen (Supporting Fig 3) [61]. We estimated the *Grand average of hydropathicity* (GRAVY) value in comparison to Ole e 1 [61], an olive pollen aeroallergen exhibiting a remarkable interfacial activity [32]. Ole e 7 and Ole e 1 reached a GRAVY values of -0.105 and -0.473 respectively, which indicate that Ole e 7 exhibits a higher hydrophobicity than

Ole e 1 (Supporting Fig 3), most likely due to the presence in Ole e 7 of the characteristic hydrophobic pocket of LTPs. Despite GRAVY theoretical values, Ole e 1 showed experimentally higher interfacial activity than Ole e 7, suggesting that Ole e 7 might convey additional effects at the air-liquid interface related with its lipid-transfer ability.

Once the interfacial capabilities of Ole e 7 were confirmed, this feature was studied in the context of other surface-active lipid-based complexes of physiological relevance, such as the pulmonary surfactant. Ole e 7 inhibited the adsorption of surfactant to the interface, as well as Ole e 1, although surfactant was able to spread against the allergen at the air-liquid interface at long term, indicative of a competitive inhibition of the allergen to rapidly occupy the air-liquid interface instead of pulmonary surfactant. Differences observed between Ole e 7 and Ole e 1 could be explained by the allergen size and its steric effect at occupying the interface. Ole e 1 is a protein with different glycoforms of 20 and 22 kDa and shows a high trend to form oligomers [62], rising higher molecular masses in comparison to the 9.8 kDa monomer of Ole e 7 [63]. Thus, at similar molar ratios, Ole e 7 would provide more space at the interface for other surfactant components while the size and disposition of Ole e 1 would difficult the spreading of those components to the air-liquid interface. Another explanation would imply the role played by nsLTPs [64-66]. These proteins are characterized for transferring lipids between membranes, mainly phospholipids such as phosphatidylcholines (PC), phosphatidylinositols (PI), or phosphatidylglycerols (PG) [67-69], and thus, in this context, Ole e 7 could be effectively transferring surfactant lipids to the interface.

On the other hand, the interaction of Ole e 7 with a preformed lipid film at the interface seems to be determined by the charge of the lipid component, being higher

when lipids are negatively charged. This is in agreement with the results derived from the binding assays, where a higher interaction affinity was achieved with PG and PS. In addition, a model of Ole e 7 was obtained taking as a model the structure of the nsLTP Pru p 3 from peach (Supporting Fig 4) [54]. It was relevant to observe that positively charged amino acids of the allergen are accumulated in α-helical segments, which may be involved in the direct interaction with biological membranes and lipid binding [70, 71]. Interestingly, those regions overlapped with the most hydrophobic regions of Ole e 7 according to the Kyte-Doolittle diagram showed in Supporting Fig 4, which supported the involvement of these α-helix structures in the interaction of Ole e 7 with lipids. The spontaneous interaction/adsorption of aeroallergens such as Ole e 7 with lipid layers such as those formed by pulmonary surfactant at the respiratory surface, or with the outer membranes of underlying epithelial cells, could be then a major determinant to initiate their distribution at the airways to further induce its pathophysiological effects.

## 5. Conclusions

In summary, we have here performed a multidisciplinary study of the Ole e 7 aeroallergen which provides, for the first time, information about its lipid-binding capacity. Moreover, the Ole e 7 structure model in the presence of oleic acid, the ligand with the highest binding affinity for the allergen, showed that the amino acids involved in the interaction are aliphatic residues. Furthermore, we have determined the adsorption of Ole e 7 into air-liquid interfaces free or occupied by phospholipids, which can be relevant to define the fate of the allergen after entering the airway mucosa and its potential to trigger the allergic response in the lung. Further studies should be performed to clarify the effects of Ole e 7 once transferred from the interface into the epithelium and on the epithelial barrier integrity.

## Acknowledgements

This work was supported by SAF2014-53209-R and SAF2017-86483-R grants from the Ministerio de Economía y Competitividad to R.B. and M.V., and to M.V., respectively, RTI2018-094564-B-I00 from the Spanish Ministry of Science and Universities and P2018/NMT4389 from the Regional Government of Madrid to J.P-G. and A.C., and RIRAAF Network RD12/0013/0015 grant and ARADyAL Network RD16/0006 grant from the Instituto de Salud Carlos III (ISCIII) co-founded by Fondo Europeo de Desarrollo Regional -FEDER- for the Thematic Networks and Co-operative Research Centres. A.J. and C.M. acknowledge PI-01119-2016 from the Consejería de Salud (Junta de Andalucía) and the Alergosur Foundation. JC.L-R., P.SS-A. and C.G-M. are recipients of a FPU fellowship from the Ministerio de Educación, Cultura y Deporte. We also gratefully acknowledge the financial support from the AES-ISCIII for the PI17CIII/00045 grant to R.B. We also thank the excellent technical support of Sara Abián.

## Supporting Information

Supporting information to this article can be found online.

## References


[1] R. Pawankar, Allergic diseases and asthma: a global public health concern and a call to action, World Allergy Organ J, 7 (2014) 12.

[2] K.D. Petersen, C. Kronborg, D. Gyrd-Hansen, R. Dahl, J.N. Larsen, A. Linneberg, Characteristics of patients receiving allergy vaccination: to which extent do socio-economic factors play a role?, Eur J Public Health, 21 (2011) 323-328.

[3] C.A. Akdis, M. Akdis, Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens, World Allergy Organ J, 8 (2015) 17.

[4] M.G. del Moral, E. Martínez-Naves, The Role of Lipids in Development of Allergic Responses, Immune Netw, 17 (2017) 133-143.

[5] S. Scheurer, M. Toda, S. Vieths, What makes an allergen?, Clin Exp Allergy, 45 (2015) 1150-1161.

[6] C. Gomez-Casado, A. Diaz-Perales, Allergen-Associated Immunomodulators: Modifying Allergy Outcome, Arch Immunol Ther Exp (Warsz), 64 (2016) 339-347.

[7] U. Jappe, C. Schwager, A.B. Schromm, N. Gonzalez Roldan, K. Stein, H. Heine, K.A. Duda, Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy, Front Immunol, 10 (2019) 122.

[8] M.E. Bashir, J.H. Lui, R. Palnivelu, R.M. Naclerio, D. Preuss, Pollen lipidomics: lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response, PLoS One, 8 (2013) e57566.

[9] J.E. Mogensen, R. Wimmer, J.N. Larsen, M.D. Spangfort, D.E. Otzen, The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands, J Biol Chem, 277 (2002) 23684-23692.

[10] A. Bonura, S. Corinti, E. Schiavi, D. Giacomazza, F. Gianguzza, G. Di Felice, P. Colombo, The major allergen of the Parietaria pollen contains an LPS-binding region with immuno-modulatory activity, Allergy, 68 (2013) 297-303.

[11] M. Onaderra, R.I. Monsalve, J.M. Mancheno, M. Villalba, A. Martinez del Pozo, J.G. Gavilanes, R. Rodriguez, Food mustard allergen interaction with phospholipid vesicles, Eur J Biochem, 225 (1994) 609-615.

[12] A. Bossios, M. Theodoropoulou, L. Mondoulet, N.M. Rigby, N.G. Papadopoulos, H. Bernard, K. Adel-Patient, J.M. Wal, C.E. Mills, P. Papageorgiou, Effect of simulated gastro-duodenal digestion on the allergenic reactivity of beta-lactoglobulin, Clin Transl Allergy, 1 (2011) 6.

[13] J.H. Akkerdaas, F. Schocker, S. Vieths, S. Versteeg, L. Zuidmeer, S.L. Hefle, R.C. Aalberse, K. Richter, F. Ferreira, R. van Ree, Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library, Mol Nutr Food Res, 50 (2006) 18-23.

[14] G.A. Mueller, R.A. Gosavi, J.M. Krahn, L.L. Edwards, M.J. Cuneo, J. Glesner, A. Pomes, M.D. Chapman, R.E. London, L.C. Pedersen, Der p 5 crystal structure provides insight into the group 5 dust mite allergens, J Biol Chem, 285 (2010) 25394-25401.

[15] S. El-Gebali, J. Mistry, A. Bateman, S.R. Eddy, A. Luciani, S.C. Potter, M. Qureshi, L.J. Richardson, G.A. Salazar, A. Smart, E.L.L. Sonnhammer, L. Hirsh, L. Paladin, D. Piovesan, S.C.E. Tosatto, R.D. Finn, The Pfam protein families database in 2019, Nucleic Acids Res, 47 (2019) D427-D432.

[16] M. Hauser, A. Roulias, F. Ferreira, M. Egger, Panallergens and their impact on the allergic patient, Allergy Asthma Clin Immunol, 6 (2010) 1.

[17] T.A. Salminen, K. Blomqvist, J. Edqvist, Lipid transfer proteins: classification, nomenclature, structure, and function, Planta, 244 (2016) 971-997.

[18] F. Liu, X. Zhang, C. Lu, X. Zeng, Y. Li, D. Fu, G. Wu, Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis, J Exp Bot, 66 (2015) 5663-5681.
[19] G. Salcedo, R. Sanchez-Monge, D. Barber, A. Diaz-Perales, Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy, Biochim Biophys Acta, 1771 (2007) 781-791.
[20] M. Villalba, R. Rodriguez, E. Batanero, The spectrum of olive pollen allergens. From structures to diagnosis and treatment, Methods, 66 (2014) 44-54.
[21] J.F. Florido Lopez, J. Quiralte Enriquez, J.M. Arias de Saavedra Alias, B. Saenz de San Pedro, E. Martin Casanez, An allergen from Olea europaea pollen (Ole e 7) is associated with plant-derived food anaphylaxis, Allergy, 57 Suppl 71 (2002) 53-59.
[22] C. Autilio, J. Perez-Gil, Understanding the principle biophysics concepts of pulmonary surfactant in health and disease, Arch Dis Child Fetal Neonatal Ed, (2018).
[23] J.Y. Wang, U. Kishore, B.L. Lim, P. Strong, K.B. Reid, Interaction of human lung surfactant proteins A and D with mite (Dermatophagoides pteronyssinus) allergens, Clin Exp Immunol, 106 (1996) 367-373.
[24] D.C. Malherbe, V.J. Erpenbeck, S.N. Abraham, E.C. Crouch, J.M. Hohlfeld, J.R. Wright, Surfactant protein D decreases pollen-induced IgE-dependent mast cell degranulation, Am J Physiol Lung Cell Mol Physiol, 289 (2005) L856-866.
[25] V.J. Erpenbeck, D.C. Malherbe, S. Sommer, A. Schmiedl, W. Steinhilber, A.J. Ghio, N. Krug, J.R. Wright, J.M. Hohlfeld, Surfactant protein D increases phagocytosis and aggregation of pollen-allergen starch granules, Am J Physiol Lung Cell Mol Physiol, 288 (2005) L692-698.
[26] K.G. Brinker, H. Garner, J.R. Wright, Surfactant protein A modulates the differentiation of murine bone marrow-derived dendritic cells, Am J Physiol Lung Cell Mol Physiol, 284 (2003) L232-241.
[27] L. Hortobagyi, S. Kierstein, K. Krytska, X. Zhu, A.M. Das, F. Poulain, A. Haczku, Surfactant protein D inhibits TNF-alpha production by macrophages and dendritic cells in mice, J Allergy Clin Immunol, 122 (2008) 521-528.
[28] C.M. Minutti, L.H. Jackson-Jones, B. Garcia-Fojeda, J.A. Knipper, T.E. Sutherland, N. Logan, E. Ringqvist, R. Guillamat-Prats, D.A. Ferenbach, A. Artigas, C. Stamme, Z.C. Chroneos, D.M. Zaiss, C. Casals, J.E. Allen, Local amplifiers of IL-4Ralpha-mediated macrophage activation promote repair in lung and liver, Science, 356 (2017) 1076-1080.
[29] S. Dreborg, A. Frew, Position paper: Allergen standardization and skin tests. The European Academy of Allergology and Clinical Immunology, Allergy, 48 (1993) 48-82.
[30] H.W. Taeusch, J. Bernardino de la Serna, J. Perez-Gil, C. Alonso, J.A. Zasadzinski, Inactivation of pulmonary surfactant due to serum-inhibited adsorption and reversal by hydrophilic polymers: experimental, Biophys J, 89 (2005) 1769-1779.
[31] G. Rouser, A.N. Siakotos, S. Fleischer, Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots, Lipids, 1 (1966) 85-86.
[32] J.C. Lopez-Rodriguez, R. Barderas, M. Echaide, J. Perez-Gil, M. Villalba, E. Batanero, A. Cruz, Surface Activity as a Crucial Factor of the Biological Actions of Ole e 1, the Main Aeroallergen of Olive Tree (Olea europaea) Pollen, Langmuir, 32 (2016) 11055-11062.
[33] C. Oeo-Santos, S. Mas, S. Benede, M. Lopez-Lucendo, J. Quiralte, M. Blanca, C. Mayorga, M. Villalba, R. Barderas, A recombinant isoform of the Ole e 7 olive pollen allergen assembled by *de novo* mass spectrometry retains the allergenic ability of the natural allergen, J Proteomics, 187 (2018) 39-46.

[34] R. Aina, P. Dubiela, S. Geiselhart, M. Bublin, M. Bruschi, C. Radauer, C. Nagl, P. Humeniuk, R. Asero, C.G. Mortz, C. Hafner, K. Hoffmann-Sommergruber, T. Borowski, Distinct Lipid Transfer Proteins display different IgE-binding activities that are affected by fatty acid binding, Allergy, 74 (2019) 827-831.
[35] P. Dubiela, R. Aina, D. Polak, S. Geiselhart, P. Humeniuk, B. Bohle, S. Alessandri, R. Del Conte, F. Cantini, T. Borowski, M. Bublin, K. Hoffmann-Sommergruber, Enhanced Pru p 3 IgE-binding activity by selective free fatty acid-interaction, J Allergy Clin Immunol, 140 (2017) 1728-1731 e1710.
[36] P. Dubiela, R. Del Conte, F. Cantini, T. Borowski, R. Aina, C. Radauer, M. Bublin, K. Hoffmann-Sommergruber, S. Alessandri, Impact of lipid binding on the tertiary structure and allergenic potential of Jug r 3, the non-specific lipid transfer protein from walnut, Sci Rep, 9 (2019) 2007.
[37] A. Sultana, J.E. Lee, Measuring protein-protein and protein-nucleic Acid interactions by biolayer interferometry, Curr Protoc Protein Sci, 79 (2015) 19 25 11-26.
[38] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F.T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res, 46 (2018) W296-W303.
[39] A. Roy, A. Kucukural, Y. Zhang, I-TASSER: a unified platform for automated protein structure and function prediction, Nat Protoc, 5 (2010) 725-738.
[40] G.W. Han, J.Y. Lee, H.K. Song, C. Chang, K. Min, J. Moon, D.H. Shin, M.L. Kopka, M.R. Sawaya, H.S. Yuan, T.D. Kim, J. Choe, D. Lim, H.J. Moon, S.W. Suh, Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography, J Mol Biol, 308 (2001) 263-278.
[41] G. Böhm, CDNN, CD Spectra Deconvolution (Univ. of HalleWittenberg, Halle, Germany), Version 2.1, (1997).
[42] A. Ravasio, A. Cruz, J. Perez-Gil, T. Haller, High-throughput evaluation of pulmonary surfactant adsorption and surface film formation, J Lipid Res, 49 (2008) 2479-2488.
[43] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat Rev Mol Cell Biol, 1 (2000) 31-39.
[44] M.A. Bos, T. van Vliet, Interfacial rheological properties of adsorbed protein layers and surfactants: a review, Adv Colloid Interface Sci, 91 (2001) 437-471.
[45] S.V. Vaidya, A.R. Narvaez, Understanding interactions between immunoassay excipient proteins and surfactants at air-aqueous interface, Colloids Surf B Biointerfaces, 113 (2014) 285-294.
[46] V. Mitropoulos, A. Mutze, P. Fischer, Mechanical properties of protein adsorption layers at the air/water and oil/water interface: a comparison in light of the thermodynamical stability of proteins, Adv Colloid Interface Sci, 206 (2014) 195-206.
[47] R. Kalla, K. Shimamoto, R. Potter, P.S. Nielsen, C. Linnestad, O.A. Olsen, The promoter of the barley aleurone-specific gene encoding a putative 7 kDa lipid transfer protein confers aleurone cell-specific expression in transgenic rice, Plant J, 6 (1994) 849-860.
[48] L.R. Offermann, M. Bublin, M.L. Perdue, S. Pfeifer, P. Dubiela, T. Borowski, M. Chruszcz, K. Hoffmann-Sommergruber, Structural and Functional Characterization of the Hazelnut Allergen Cor a 8, J Agric Food Chem, 63 (2015) 9150-9158.
[49] E. Gincel, J.P. Simorre, A. Caille, D. Marion, M. Ptak, F. Vovelle, Three-dimensional structure in solution of a wheat lipid-transfer protein from multidimensional 1H-NMR data. A new folding for lipid carriers, Eur J Biochem, 226 (1994) 413-422.

[50] M.H. Lerche, F.M. Poulsen, Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins, Protein Sci, 7 (1998) 2490-2498.
[51] H.C. Cheng, P.T. Cheng, P. Peng, P.C. Lyu, Y.J. Sun, Lipid binding in rice nonspecific lipid transfer protein-1 complexes from Oryza sativa, Protein Sci, 13 (2004) 2304-2315.
[52] P. Da Silva, C. Landon, B. Industri, A. Marais, D. Marion, M. Ponchet, F. Vovelle, Solution structure of a tobacco lipid transfer protein exhibiting new biophysical and biological features, Proteins, 59 (2005) 356-367.
[53] K.F. Lin, Y.N. Liu, S.T. Hsu, D. Samuel, C.S. Cheng, A.M. Bonvin, P.C. Lyu, Characterization and structural analyses of nonspecific lipid transfer protein 1 from mung bean, Biochemistry, 44 (2005) 5703-5712.
[54] N. Pasquato, R. Berni, C. Folli, S. Folloni, M. Cianci, S. Pantano, J.R. Helliwell, G. Zanotti, Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specific lipid transfer protein pan-allergens, J Mol Biol, 356 (2006) 684-694.
[55] R.E. McDonald, R.I. Fleming, J.G. Beeley, D.L. Bovell, J.R. Lu, X. Zhao, A. Cooper, M.W. Kennedy, Latherin: a surfactant protein of horse sweat and saliva, PLoS One, 4 (2009) e5726.
[56] S. Scheurer, S. Schulke, Interaction of Non-Specific Lipid-Transfer Proteins With Plant-Derived Lipids and Its Impact on Allergic Sensitization, Front Immunol, 9 (2018) 1389.
[57] S.U. Abdullah, Y. Alexeev, P.E. Johnson, N.M. Rigby, A.R. Mackie, B. Dhaliwal, E.N. Mills, Ligand binding to an Allergenic Lipid Transfer Protein Enhances Conformational Flexibility resulting in an Increase in Susceptibility to Gastroduodenal Proteolysis, Sci Rep, 6 (2016) 30279.
[58] E.I. Finkina, D.N. Melnikova, I.V. Bogdanov, T.V. Ovchinnikova, Lipid Transfer Proteins As Components of the Plant Innate Immune System: Structure, Functions, and Applications, Acta Naturae, 8 (2016) 47-61.
[59] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys J, 80 (2001) 1417-1428.
[60] E.J. Dufourc, Sterols and membrane dynamics, J Chem Biol, 1 (2008) 63-77.
[61] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J Mol Biol, 157 (1982) 105-132.
[62] M. Villalba, E. Batanero, C. Lopez-Otin, L.M. Sanchez, R.I. Monsalve, M.A. Gonzalez de la Pena, C. Lahoz, R. Rodriguez, The amino acid sequence of Ole e I, the major allergen from olive tree (Olea europaea) pollen, Eur J Biochem, 216 (1993) 863-869.
[63] M.L. Tejera, M. Villalba, E. Batanero, R. Rodriguez, Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen, J Allergy Clin Immunol, 104 (1999) 797-802.
[64] J.C. Kader, Proteins and the intracellular exchange of lipids. I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber, Biochim Biophys Acta, 380 (1975) 31-44.
[65] K.J. Bourgis F., Lipid-transfer proteins: Tools for manipulating membrane lipids, Physiologia Plantarum, 100 (2006) 78-84.
[66] M. Egger, M. Hauser, A. Mari, F. Ferreira, G. Gadermaier, The role of lipid transfer proteins in allergic diseases, Curr Allergy Asthma Rep, 10 (2010) 326-335.

[67] J. Ostergaard, C. Vergnolle, F. Schoentgen, J.C. Kader, Acyl-binding/lipid-transfer proteins from rape seedlings, a novel category of proteins interacting with lipids, Biochim Biophys Acta, 1170 (1993) 109-117.
[68] F. Guerbette, M. Grosbois, A. Jolliot-Croquin, J.C. Kader, A. Zachowski, Comparison of lipid binding and transfer properties of two lipid transfer proteins from plants, Biochemistry, 38 (1999) 14131-14137.
[69] F. Guerbette, M. Grosbois, A. Jolliot-Croquin, J.C. Kader, A. Zachowski, Lipid-transfer proteins from plants: structure and binding properties, Mol Cell Biochem, 192 (1999) 157-161.
[70] A.G. Lee, Lipid-protein interactions in biological membranes: a structural perspective, Biochim Biophys Acta, 1612 (2003) 1-40.
[71] L. Malinina, D.J. Patel, R.E. Brown, How alpha-Helical Motifs Form Functionally Diverse Lipid-Binding Compartments, Annu Rev Biochem, 86 (2017) 609-636.
[72] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera--a visualization system for exploratory research and analysis, J Comput Chem, 25 (2004) 1605-1612.

## Legend to the Figures

**Figure 1. Binding assays by ANS displacement and interferometry analysis of Ole e 7 with indicated lipids.** (A) Fluorescence changes induced by the incubation of Ole e 7 with phospholipids liposomes and fatty acids at two different molar ratios (protein:ligand 1:1 = 5 µM ligand, or 1:100 = 500 µM ligand). Fluorescence of Ole e 7 without lipid incubation was considered as negative control of lipid binding. (B) Ole e 7 biolayer interferometry sensor after incubation with phospholipids. A fixed concentration of 1 µg/µl of protein and lipid mixtures was used. Association and dissociation kinetics were analysed during 300 sec. Chol, colesterol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; POPC, palmitoyloleoylphosphatidylcholine; SM, sphingomyelin; PAL (C16:0), palmitic acid; OLE (18:1), oleic acid. All the experiments were performed in duplicate.

**Figure 2. Analysis by CD of changes in the secondary structure of Ole e 7.** CD spectra of recombinant Ole e 7 after incubating with lipids at a fix molar ratio (protein:lipid 1:1= 5 µM) in 20 mM sodium phosphate buffer 50mM pH 7.5 at 20 °C. The experiments were performed in duplicate.

**Figure 3. Analysis of the IgE-binding to recombinant Ole e 7 in presence or absence of indicated lipidic ligands**. IgE-binding from six Ole e 7-allergic patients was evaluated in duplicate with recombinant Ole e 7 alone and after protein incubating with different phospholipids liposomes and fatty acids. 1 µM (or 1 µg/µl) protein concentration and 30 µM lipid concentration were used. The response of two non-allergic patients to Ole e 7 was used as negative control of the IgE-binding.

745

746 **Figure 4. Interfacial adsorption kinetics of Ole e 7.** (A) Π-t isotherms (25 ± 1 ºC) of
747 Ole e 7 at different concentrations (mg/ml) 3 (●), 1.5 (○), 0.75 (▼), 0.375 (△) and
748 0.1875 (■)- injected in buffer solution (5 mM Tris, 150 mM NaCl, pH 7.4) at time = 0
749 (min). (B) Maximum surface pressure after 30 min after allergen injection ($\Pi_{30min}$,
750 mN/m). Hyperbolic trend for the protein interfacial behavior was shown. Solid line
751 shows the fits protein interfacial behavior (r = 0.9031). Each concentration was tested in
752 duplicate.

753

754 **Figure 5. Critical insertion pressure of Ole e 7 into preformed phospholipid**
755 **monolayers.** (A) Critical insertion of the allergen in DPPC, DPPG, DPPS, and
756 POPC:SM:Chol (2:1:1) monolayers. (B) Critical insertion of the allergen in presence of
757 Chol, using DPPC:Chol monolayer as model. The intersection with the horizontal axis
758 allows estimation of the critical insertion pressure (Πc) in each film. All the assays were
759 performed at 25 ± 1 ºC. The subphase was composed of 5 mM Tris, 150 mM NaCl, pH
760 7.4. All the experiments were carried out in duplicate.

761

762 **Figure 6. Effect of Ole e 7 on the interfacial adsorption kinetics of pulmonary**
763 **surfactant.** Interfacial adsorption of the recombinant Ole e 7 allergen was compared
764 with the natural Ole e 7 allergen, Ole e 1 allergen and bovine serum albumin (BSA) at
765 five different concentrations of protein (50 mg/ml to 0.1 mg/ml). Data are expressed as
766 fluorescence intensity in relative fluorescence units (RFU) and represent the mean of 2
767 independent experiments. Native surfactant (NS) was consider as positive control. Sera

was considered as negative control of fluorescence. Ole e 1 and BSA were considered as model of proteins which reach the air-liquid interface [30].

## Legend to the Supporting Figures

**Supporting Figure 1. Model of the three-dimensional structure of Ole e 7 based on non-specific lipid binding in maize lipid-transfer protein complexes with oleic acid.** (A) Ribbon diagram of Ole 7 e model complexed with oleic acid. The figure was drawn with the Chimera 1.8.1 [72]. The model contains four α-helixes H1 (dark blue), H2 (light blue), H3 (green), H4 (yellow) and an oleic acid molecule (magenta). Amino acids involved in the lipid-protein interaction are shown.

**Supporting Figure 2. Interaction of Ole e 7 with DPPC, DPPG, DPPS, DPPC:Chol (2:1), and POPC:SM:Chol (2:1:1) films.** Insertion/adsorption kinetics of Ole e 7 into preformed phospholipid monolayers at different initial surface pressures (symbol lines). Arrows indicate the injection of Ole e 7 (0.5 μM or 0.5 μg/μl) into the subphase. All the assays were performed at 25 ± 1 ºC. The subphase was composed of 5 mM Tris, 150 mM NaCl, pH 7.4. All the experiments were carried out in duplicate. Each concentration was analysed in duplicate.

**Supporting Figure 3. Hydrophobicity of Ole e 7 in comparison to Ole e 1 allergen.** Hydrophobicity profile and GRAVY value of the allergenic proteins was calculated according to [54]. The window size employed was 9 amino acids. Graph was made through simulation at Expasy ProScale web server tool (http://web.expasy.org/protscale/).

**Supporting Figure 4. Molecular modelling of Ole e 7.** The nsLTP structure from peach, Pru p 3, was used as model. (A) Amino acid sequence of Ole e 7. Positive charged amino acids -Lys, K- were marked in bold and negative charged amino acids

794 -Asp, D- were underlined. Rows indicate Leu11, Val33, Ser55 and Val80, the amino
795 acids with high hydrophobic character according to values calculated by using
796 simulation at Expasy ProScale web server tool (http://web.expasy.org/protscale/); (B)
797 Ribbon diagram of unligated Ole e 7. Amino acids Lys10, Lys35, Ser55 and Val80 are
798 indicated. Red, (C-D) Hydrophobic residues on the structure (C) and surface (D) are
799 shown in white, polar residues are shown in yellow, negative charged residues are
800 shown in red and positive charged residues are shown in blue.

Figure 1

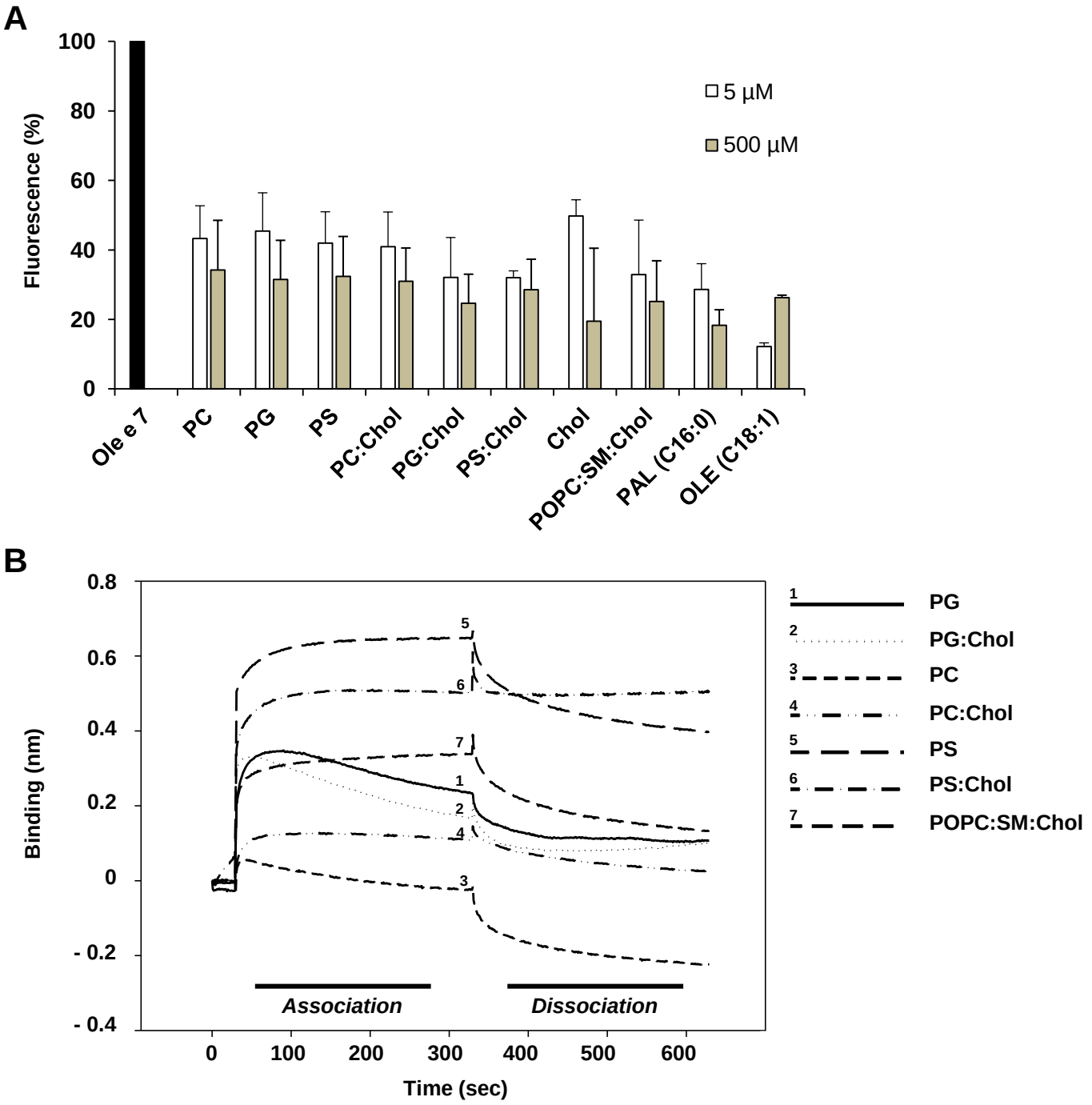



**Figure 1**

Figure 2

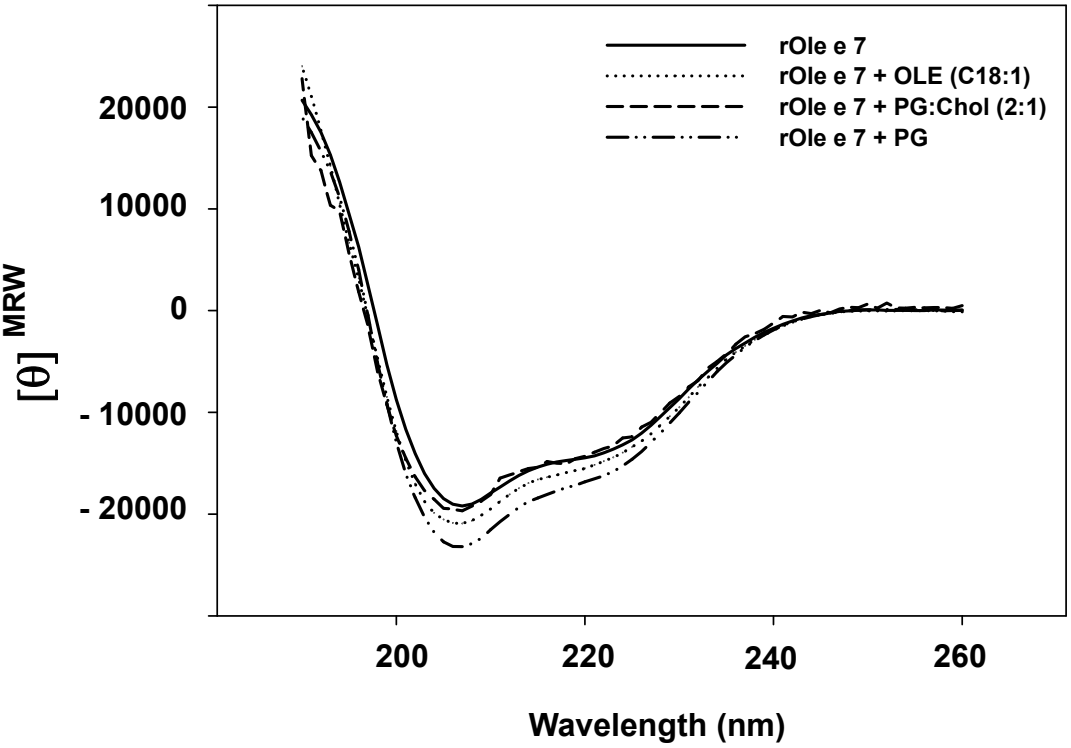



**Figure 2**

Figure 3

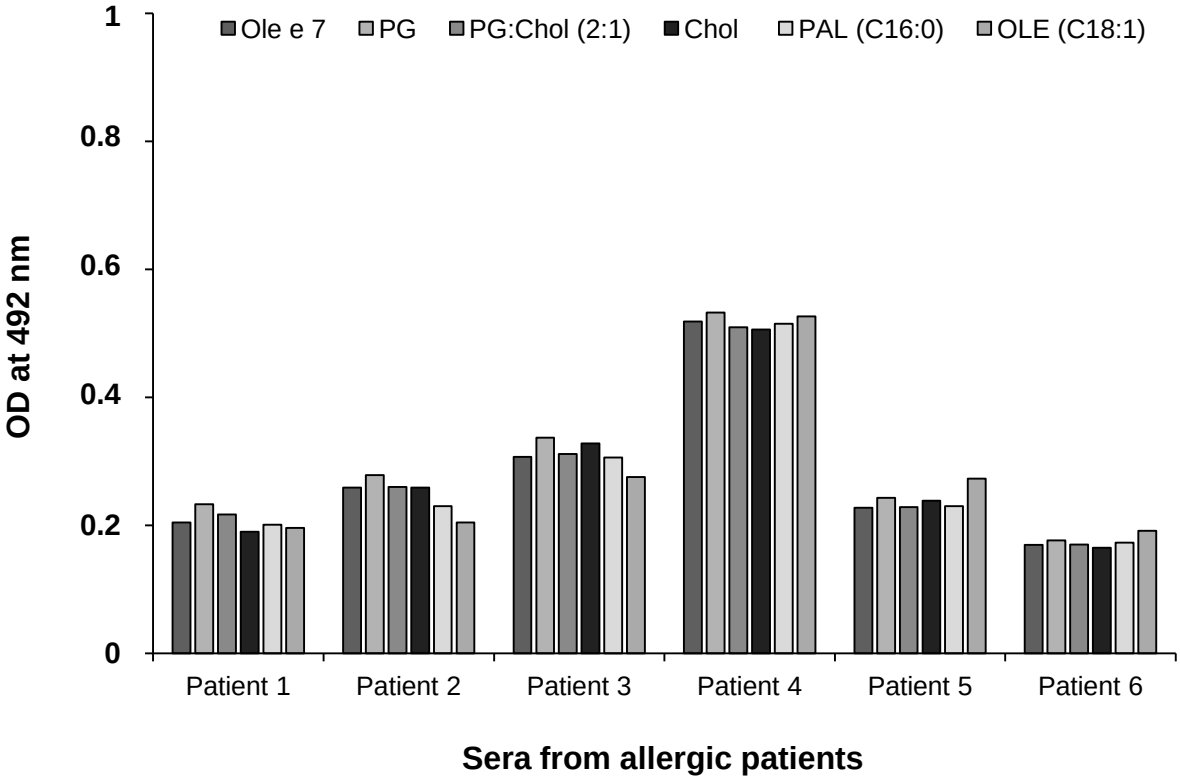



**Figure 3**

Figure 4

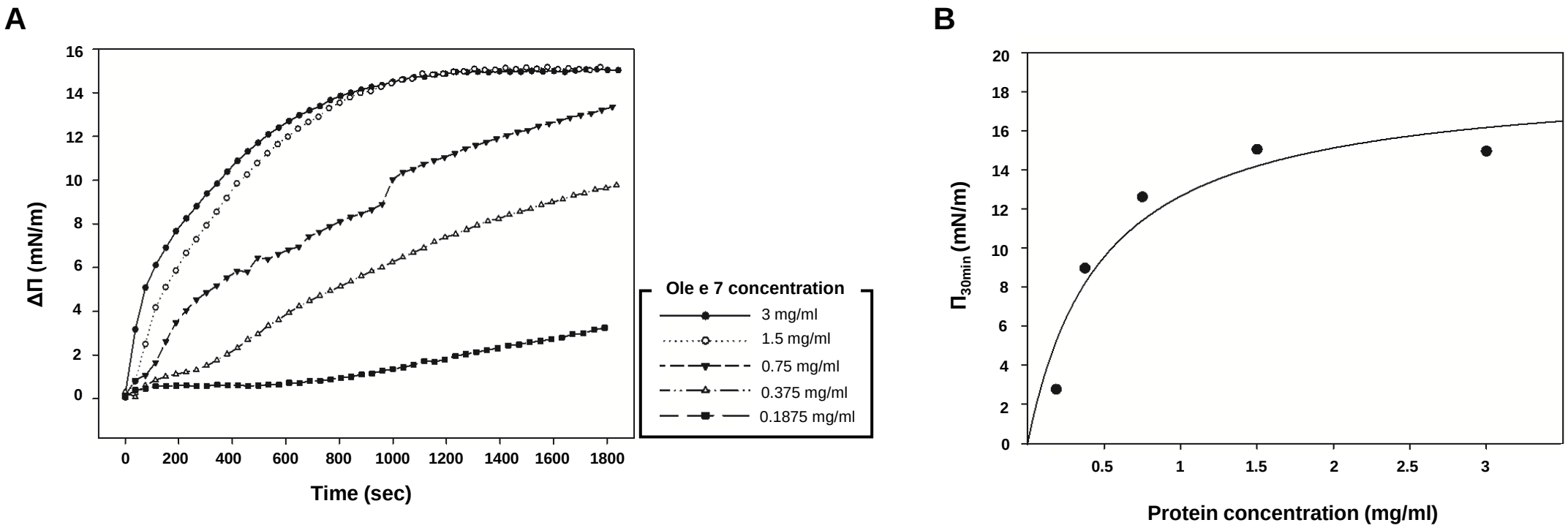



Figure 4

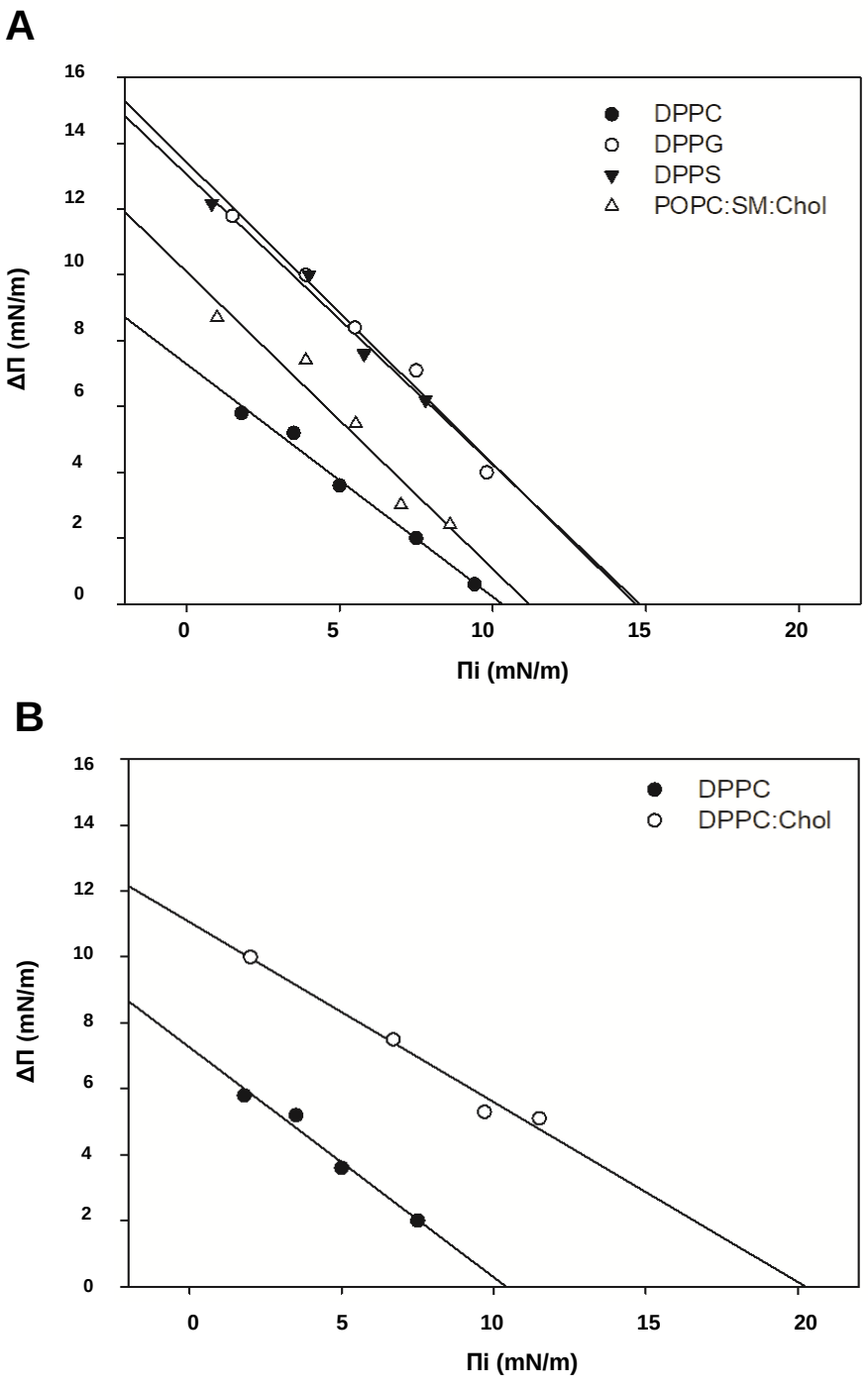

A
16
14
12
10
8
6
4
2
0
ΔΠ (mN/m)
0
5
10
15
20
Πi (mN/m)
DPPC
DPPG
DPPS
POPC:SM:Chol
B
16
14
12
10
8
6
4
2
0
ΔΠ (mN/m)
0
5
10
15
20
Πi (mN/m)
DPPC
DPPC:Chol

**Figure 5**

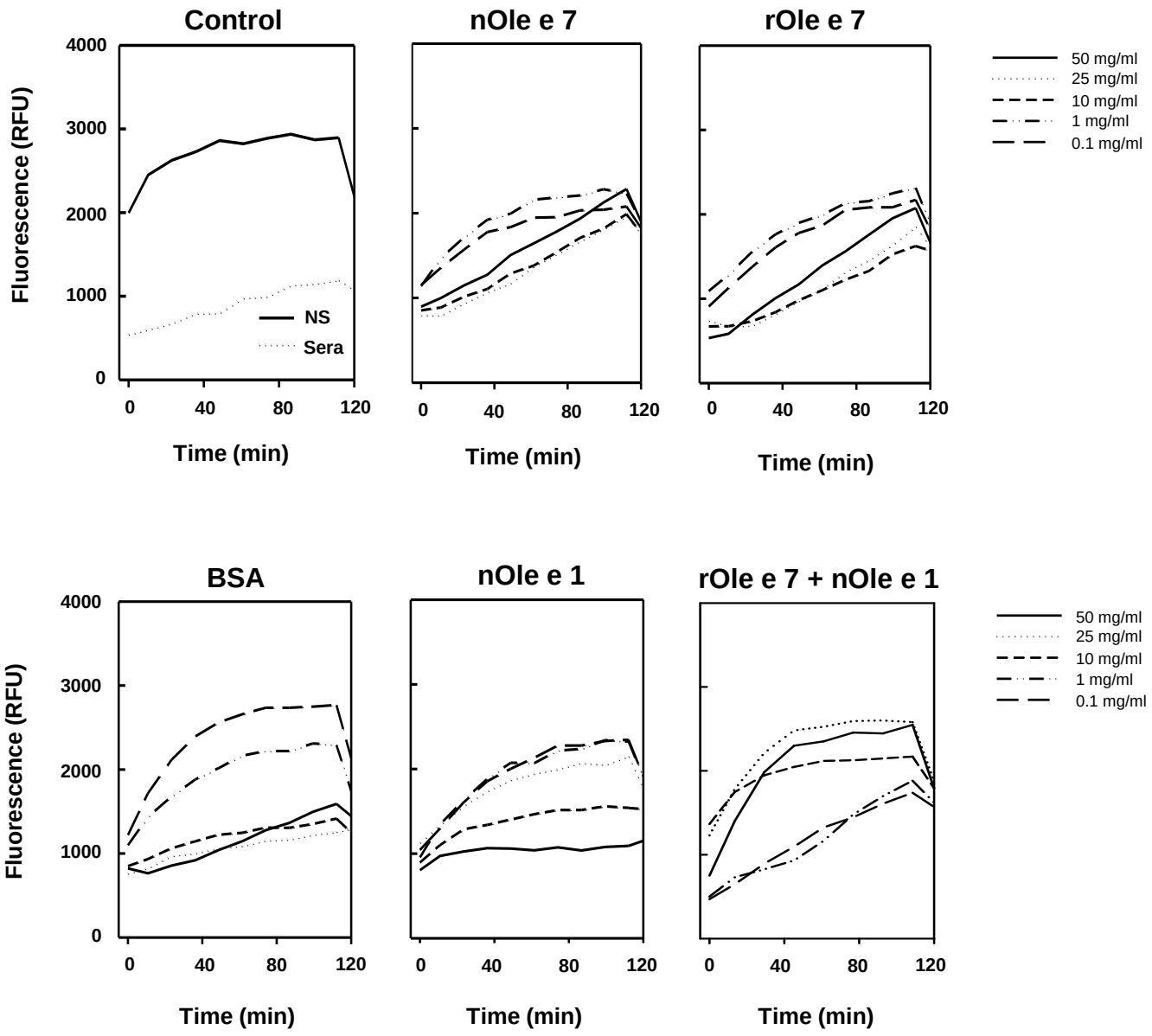
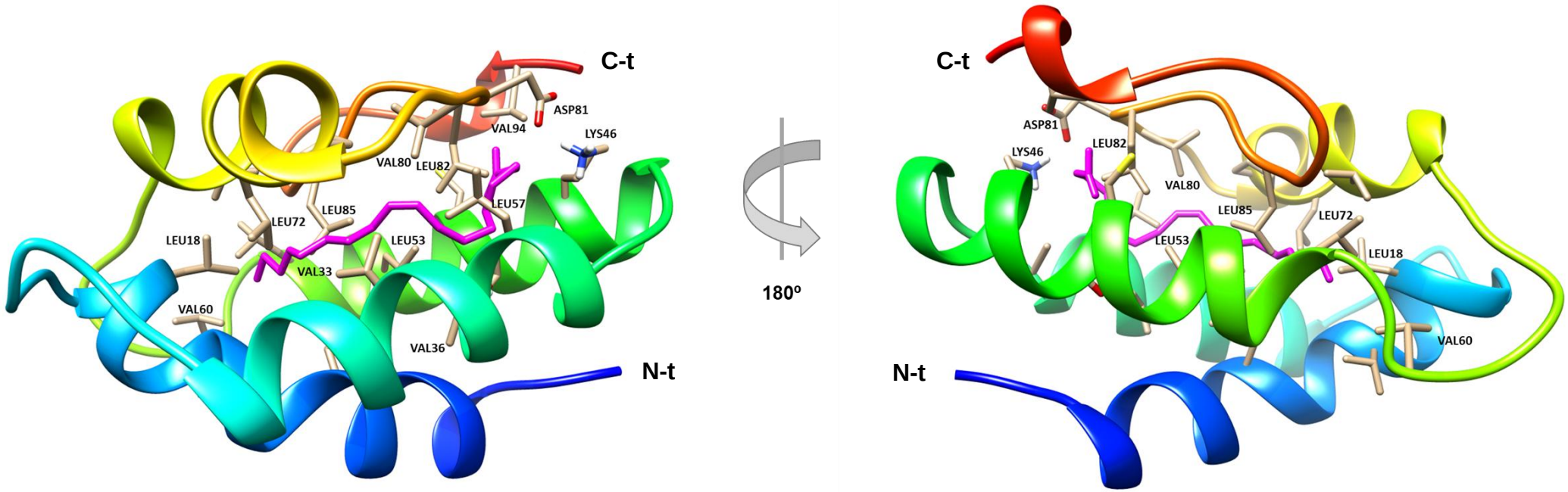

Control
nOle e 7
rOle e 7
Fluorescence (RFU)
4000
3000
2000
1000
0
NS
Sera
0
40
80
120
Time (min)
50 mg/ml
25 mg/ml
10 mg/ml
1 mg/ml
0.1 mg/ml
BSA
nOle e 1
rOle e 7 + nOle e 1

Figure 6

**Supporting Figure 1**

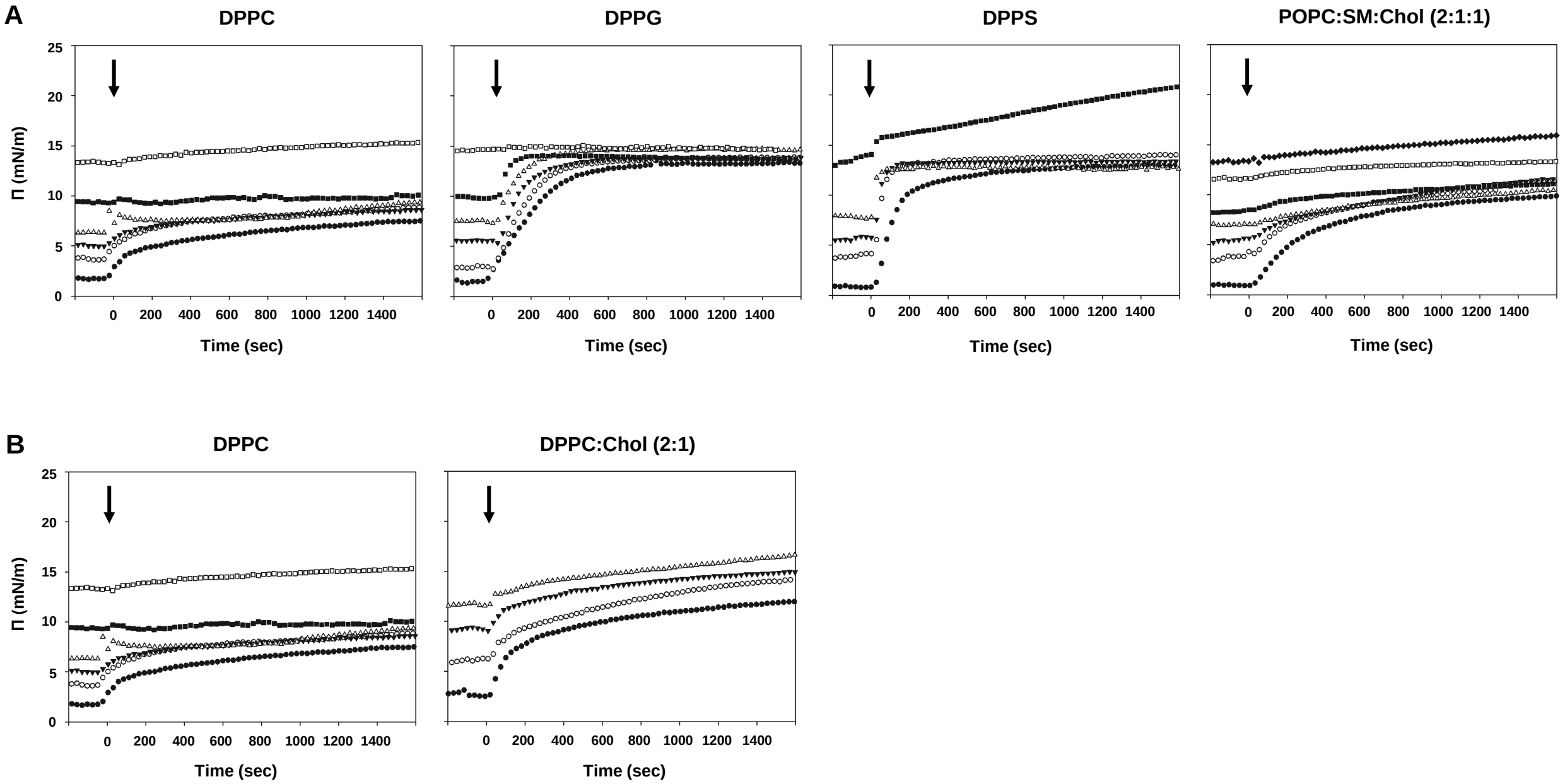
Click here to download Supplementary Material (for online publication): Supporting Figure 1.pptx




# Supporting Figure 1

Supporting Figure 2

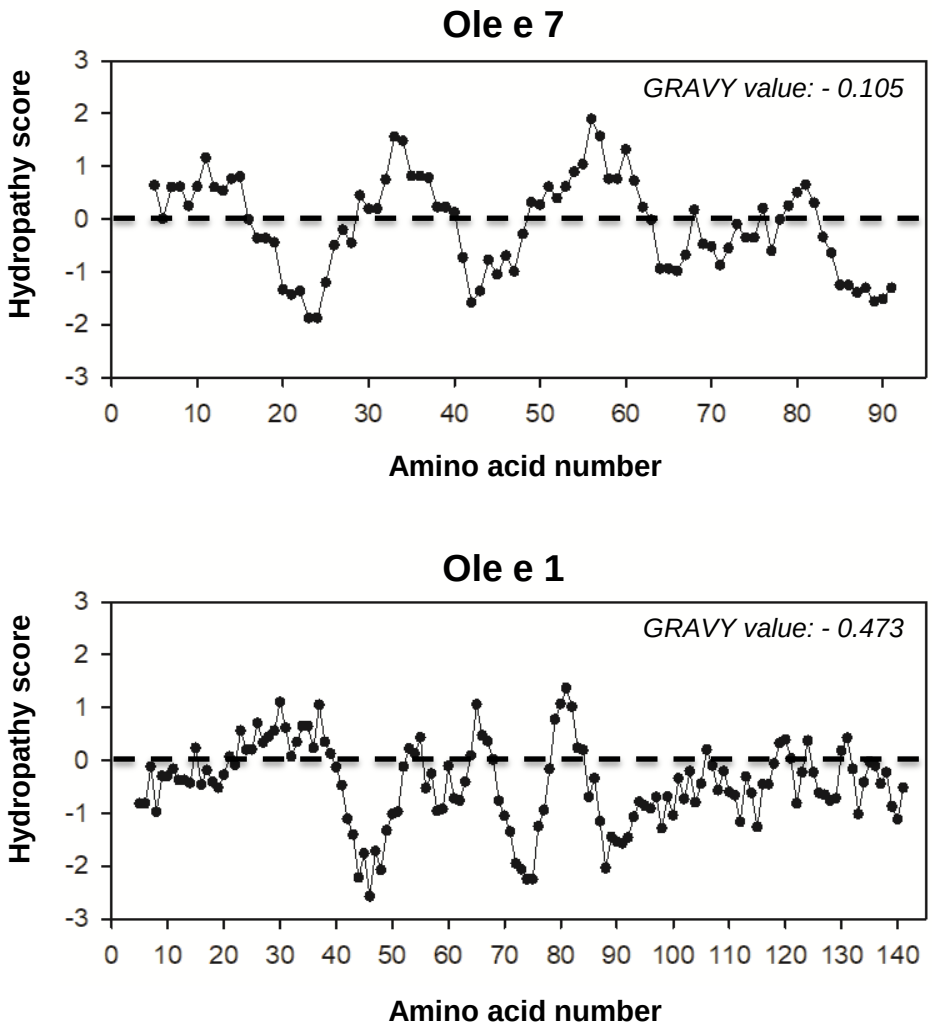
Click here to download Supplementary Material (for online publication): Supporting Figure 2.pptx




# Supporting Figure 2

**Supporting Figure 3**

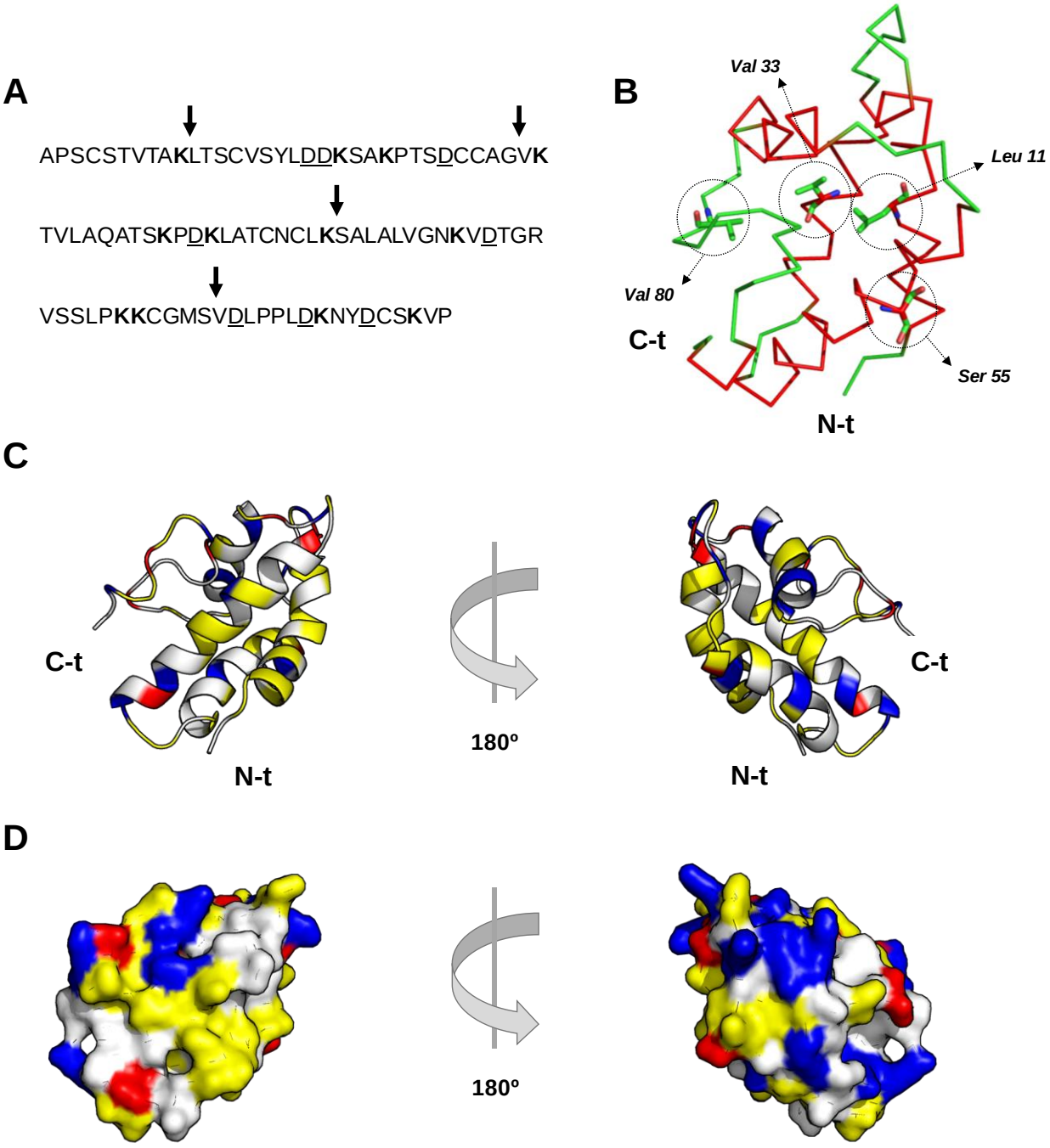
**Click here to download Supplementary Material (for online publication): Supporting Figure 3.pptx**




# Supporting Figure 3

**Supporting Figure 4**

Click here to download Supplementary Material (for online publication): Supporting Figure 4.pptx




# Supporting Figure 4