UNIVERSIDAD POLITÉCNICA DE MADRID

Escuela Técnica Superior de Ingenieros Agrónomos

***“Estudio de la alergenicidad en Alt a 1, una proteína única
de hongos”***

Tesis Doctoral

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ABBREVIATIONS

3D	Three-dimensional
ALI	Air-liquid interface
APBS	Adaptive Poisson-Boltzmann solver
APC	Antigen presenting cell
ASL	Airway liquid surface
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBA	Diphenylboric acid 2-aminoethyl ester
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fc ϵ RI	High affinity IgE receptor
GM-CSF	Granulocyte macrophage colony stimulating factor
HDM	House dust mite
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HST	Host-selective toxin
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
LCN	Lipocalin
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption ionization
MD	Molecular dynamics
MHC-II	Major histocompatibility complex II
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil gelatinase-associated lipocalin
NHBE	Normal human bronchial epithelial

OD	Optical Density
PAR	Proteinase-activated receptor
PB	Poisson-Boltzmann
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PFA	Paraformaldehyde
PISA	Proteins, interfaces, structures, and assemblies
PR	Pathogenesis-related
PTGL	Protein topology graph library
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI	Stimulation index
TEER	Transepithelial resistance
Th2	Type-2 helper lymphocytes
TLC	Thin layer chromatography
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoietin

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RESUMEN

La alergia es un problema social que supone una grave disminución de la calidad de vida del paciente, estando considerada como la mayor enfermedad crónica en Europa en la actualidad. Desde mediados del siglo XX, se ha producido un incremento en la prevalencia de estas enfermedades. Sin embargo, el origen de este incremento y el factor que desencadena las alergias son desconocidos. El conocimiento de este factor sería de gran ayuda para poder prevenir y tratar este tipo de enfermedades.

El objetivo principal de esta Tesis ha sido estudiar los mecanismos moleculares que están implicados en los procesos de sensibilización alérgica. Para ello, la investigación se ha centrado en Alt a 1, alérgeno principal del hongo *Alternaria alternata* que ha sido caracterizado desde los puntos de vista estructural, fisiológico e inmunológico. La proteína Alt a 1 está relacionada con asma crónica y patogénesis en plantas, aunque su función biológica es desconocida. Sin embargo, en esta Tesis se ha podido caracterizar a este alérgeno como efector, siendo secretado por la espora del hongo en el momento en que se encuentra en la superficie de la planta, e inhibiendo a proteínas de defensa como PR5.

Por otro lado, a partir de la estructura tridimensional de Alt a 1 determinada mediante cristalografía de rayos X, en esta Tesis se ha estudiado *in silico* el comportamiento, estabilidad y propiedades de varios estados de agregación de este singular alérgeno. Ese estudio, realizado en solución acuosa salina mediante cálculos de Dinámica Molecular, ha logrado dilucidar la evolución dinámica de la estabilidad, propiedades e interacciones de los diferentes estados de oligomerización de Alt a 1 que resultan depender de la presencia de ligandos y de cambios en el pH del medio. Combinando ensayos experimentales *in vitro* con procedimientos computacionales de análisis *in silico* (cálculos de *docking* y modelado molecular), en esta Tesis también se ha identificado la presencia de un ligando unido a Alt a 1, relacionado con la familia de los flavonoides. Aunque no ha sido posible identificar con total precisión el compuesto químico de que se trata, las evidencias experimentales que aquí se presentan, han permitido proponer una estructura plausible con un soporte flavona.

Finalmente, se ha abordado la respuesta inmunológica producida por Alt a 1 en el epitelio bronquial. De forma análoga a lo que sucede cuando *Alternaria* entra en contacto con una planta, se ha observado que la presencia de esporas produce un incremento en la secreción de proteínas de defensa con las que Alt a 1 es capaz de interaccionar. Los hechos que desencadenan este reconocimiento cursan con la liberación del ligando y favorecen la formación de la estructura

dimérica de Alt a 1. Esta forma dimérica es la que eventualmente puede ser reconocida por las células presentadoras de antígeno desencadenando la respuesta alérgica.

Resumiendo, los datos expuestos en esta Tesis revelan que el conocimiento de la actividad biológica de un alérgeno aporta una información absolutamente esencial para comprender su intervención en los mecanismos que provocan el desencadenamiento de una reacción alérgica.

ABSTRACT

Allergy is a social problem that represents a serious decrease in quality of life of the patients, being considered the biggest chronic disease in Europe today. Since mid-twentieth century, a large increase in the prevalence of these diseases has occurred. However, the underlying causes for this increase and the factors that trigger allergy are still unknown. The knowledge of these factors would be very valuable to prevent and treat allergy disease.

The main aim of this doctoral thesis was to study the molecular mechanisms involved in the allergic sensitization processes. With this purpose, the research was focused on Alt a 1, the major allergen from the fungus *Alternaria alternata*, which has been characterized from structural, physiological, and immunological standpoints. Alt a 1 protein is related to chronic asthma and pathogenesis in plants, although its biological function still remains unknown. However, this allergen has been characterized in this doctoral thesis as an effector which is released when the fungal spores reach the plant surface and is able to inhibit plant defense proteins such as PR5.

On the other side, based upon the three-dimensional structure of Alt a 1 obtained in X-ray crystallography, this doctoral thesis also presents an *in silico* study of the behavior, stability, and properties of several aggregation states of this singular allergen. This study, conducted on salt aqueous solution by means of Molecular Dynamics simulations, has managed to elucidate the dynamical evolution of stabilities, properties, and interactions of the different oligomerization states of Alt a 1 that happened to depend on the presence of ligands and on the pH of the medium. By combining experimental assays *in vitro* and computational procedures for analyses *in silico*, the presence of a flavonoid-type ligand bound to Alt a 1 has been also identified in this doctoral thesis. Although it has not been possible to elucidate the precise chemical nature of this ligand, the experimental evidence reported has allowed to propose a plausible structure based on a flavone-like moiety.

Finally, the immune response triggered by Alt a 1 in bronchial epithelium was studied. In a similar manner to what happens when *Alternaria* contacts plants, it has been observed that the presence of fungal spores produces an increase in secretion of defense proteins. Moreover, it has been also observed that Alt a 1 is able to interact with those proteins. The sequence of events that lead to this molecular recognition occur with release of the ligand and promote dimerization of Alt a 1. This dimeric structure is that which is ultimately recognized by antigen presenting cells, thus triggering the allergic response.

Summarizing, data presented in this doctoral thesis demonstrate that the knowledge of the biological activity of an allergen provide essential information to better understand its participation in the complex mechanisms that lead to the allergic process.

1.INTRODUCTION

1.1 ALLERGIES AS A PUBLIC HEALTH PROBLEM

The prevalence of allergies is increasing in Western societies and it is estimated that by 2050 over 50% of population will suffer from allergy (1–4). This increase can be only explained by changes in gene expression that predispose new generations to allergic sensitization and related disease. The most dramatic increase is being seen in childhood, where over 40% of children under age 14 suffer from some allergic disease and 20% suffer from asthma, a condition that seriously impairs their quality of life (5–9).

The annual cost of allergic diseases for national health systems is as high as for seasonal flu and it has been estimated that the annual cost per patient with food allergy is over 1000 euros in Europe (10–14). Much effort has focused on research into potential causes of this increase in the prevalence of allergy, but unfortunately we are still far from understanding this trend.

Type I allergy is a clinical disorder characterized by the production of high levels of specific IgE and primarily affect mucosa of skin, airways, and gut (15). Two phases are distinguished which differ at the time of contact with the allergen.

- *Sensitization phase*: It is characterized by the specific production of IgE. From the immunological point of view, allergen is uptaken by antigen-presenting cells (APC), processed and presented by the major histocompatibility complex II (MHC-II) as a small peptide, inducing differentiation into a Th2 subset (16–18). Th2 cells, with the necessary cytokine environment, interact with activated B cells, which change to plasmatic cells, producers of specific IgE against the allergen. These specific IgEs are released into blood, binding to the high affinity receptor (FcεRI) onto the surface of mast cells (tissue resident cells) and basophils (circulating cells) (19).
- *Effector phase*: It is a symptomatic phase. After a new exposition, the allergen interacts with two IgE molecules bound to FcεR on the surface of mast cells and basophils. This induces crosslinking and subsequent degranulation, releasing a wide range of inflammatory mediators such as histamine (19, 20). The action of these mediators results in the typical symptoms associated with allergic rhinitis, conjunctivitis and asthma. If this phase is prolonged on time, the *late phase reactions* could take place. During this, the allergen is presented to memory T cells resulting in IL5 release and highly eosinophils infiltration.

1.2 AIRWAY MUCOSA

Airway epithelial cells are considered as an inert barrier between inhaled environmental bioparticles and inner lung tissues (21). However, recent studies have shown that the epithelium plays a pivotal role in many airway functions. Factors present in the environment interact with receptors of the epithelial surface leading to activation and triggering the production of different mediators that result in an inflammatory process (22). Bronchial asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome represent a broad range of conditions involving pulmonary inflammation (21).

The epithelium produces diverse lipid mediators such as growth factors and bronchoconstricting peptides as well as chemokines and cytokines such as arachidonic acid metabolites and nitric oxide. These act as chemoattractants and recruit inflammatory cells like neutrophils, macrophages, mast cells, eosinophils, and Th-2 cells that further exacerbate the intensity of inflammation. Other substances like protease enzymes can disrupt the epithelium barrier and gain entry to immune cells of the body leading to their activation.

1.2.1 Anatomy of the airway epithelium

Epithelial cells in human respiratory epithelium may be classified into three categories based on ultrastructural, functional and biochemical criteria: Basal, ciliated and secretory cells (23). Eight morphologically different epithelial cell types are present, being the most representative:

Columnar ciliated epithelial cells: Ciliated epithelial cells are the predominant cell type within the airways, accounting for over 50% of all epithelial cells (23). Ciliated epithelial cells arise from either basal or secretory cells and until recently were believed to be terminally differentiated (24).

Mucous cells (goblet cells): Mucous cells are characterized by membrane-bound electron-lucent acidic–mucin granules, secreted to trap foreign objects in the airway lumen (25, 26). Production of the correct amount of mucus and its own viscoelasticity are important for efficient mucociliary clearance. In chronic airway inflammatory diseases such as chronic bronchitis and asthma, mucous cell hyperplasia and metaplasia are a common pathological finding thought to contribute to productive cough associated with these diseases (27). These cells are believed to be capable of self-renewal and may also differentiate into ciliated epithelial cells (28) .

Basal cells: Basal cells are ubiquitous in the conducting epithelium, although their number decreases with airway size (28, 29). There is a direct correlation between epithelium thickness and

the number of basal cells as well as percentage of columnar cell attachment to the basement membrane via the basal cell. The basal cell has a sparse electron-dense cytoplasm that contains bundles of low molecular weight cytokeratin (30). Within the epithelium, basal cells are the only ones that are firmly attached to the basement membrane (28, 29, 31) and as such, play a role in the attachment of more superficial cells to the basement membrane via hemidesmosomal complexes (32).

Clara cells: In humans, Clara cells are located in large (bronchial) and small (bronchiolar) airways. These cells contain electron-dense granules thought to produce bronchiolar surfactant and are also characterized by a granular endoplasmic reticulum in the apical cytoplasm and granular endoplasmic reticulum basally (33). More recent evidence suggests that Clara cells play an important stem cell role, serving as a progenitor for both ciliated and mucus secreting cells (34).

1.2.2 Interaction of airway epithelial cells with allergens

Inhaled particles from the environment can damage airway epithelial cells. A greatest damage of airway epithelium has been observed in patients with chronic obstructive pulmonary disease and rhinitis. The allergens disrupt the barrier of epithelial cells by altering the occluding proteins of tight junctions because of their proteolytic activity and gain access to the inner tissues through paracellular pathway (35, 36).

Der p 1, a cysteine protease from house dust mite (HDM), has been shown to cleave tight junction proteins, thereby facilitating allergen penetration at epithelial surfaces. Induction of Th2 immunity by HDM allergens depends on dendritic cells (DCs) such as antigen-presenting cells (APCs) (37). Serine proteases in extracts from cockroach and from HDM (Der p 3 and Der p 9), activate PAR-2 in epithelial cells producing an increase in the expression of thymic stromal lymphopoietin (TSLP) and DCs activation towards Th2 phenotype, providing thus a mechanism for the allergenicity of serine proteases (38).

By contrast, there are many allergens without protease activity. Lipid binding seems to be a quite frequent property of allergens including group 2 and 7 mite allergens (39). Der p 2 can bind LPS and substitute the homologous human MD-2 protein thereby promoting Th2 responses in mice via TLR4 signaling. LPS exposure seems to play a variable role in both promoting and inhibiting allergic sensitization (40). Der p 7 shows structural similarities to the LPS-binding protein and bind weakly the bacterial lipopeptide polymyxin B (41). Thus, Der p 7 might promote Th2 immunity via co-stimulation of TLR2 pathways.

In the case of Alt a 1, the major allergen of *Alternaria alternata*, the protein has no protease activity but it induces the production of proteinase-dependent IL-6 and CXCL8 release from alveolar (42) and bronchial epithelial cells (43). Furthermore, a significant acute airway eosinophil response is triggered on the epithelium (44) with release of GM-CSF, TSLP, IL-33 and IL-25.

In summary, the current knowledge supports the view that intrinsic features of certain allergenic proteins seem to be intimately connected to their recognition by innate immune receptors expressed by DCs and epithelial cells (38, 45).

1.2.3 Receptors on epithelial cells playing crucial role in inflammation

Protease activated receptors (PARs)

Among the allergens with enzymatic function, protease activity is the most common. House dust mite and pollen allergens are included in this category. These allergens activate airway epithelial cells by interacting with PARs (46). PARs are a family of G protein coupled receptors that are activated on protease cleavage within the extracellular N-terminal region to form a tethered ligand that binds intramolecularly and activates the receptor. Four PARs have been identified (47, 48) being PAR1-3 activated by trypsin-like-enzymes.

No studies have been carried out to elucidate the presence of PAR3 and PAR4 on airway epithelium and the presence of PAR1 has been related only to airway epithelial cells but not to gastrointestinal and pancreatic epithelium (49, 50). The activation of PAR receptor stimulate human respiratory epithelial cells to secrete proinflammatory cytokines like IL6 and IL8 (46).

Toll like receptors (TLRs)

Airway epithelium is known to express TLRs. These pattern recognition receptors bind to their partners at an extracellular domain and transmit the signal via activation of adaptor proteins which in turn leads to activation of nuclear factor NF- κ B or IFN regulatory factor 3 and 7 (IRF 3/7). This finally gives rise to the expression of proteins such as IFN, antimicrobials, chemokines, and proinflammatory cytokines. It has been proposed that TLR expression and activation on airway structural cells is necessary for the production of TSLP, IL25, IL33, and GMCSF after exposure to HDM extracts (51).

Lipocalin LCN2 or NGAL

Lipocalin 2 (LCN2) or neutrophil gelatinase-associated lipocalin (NGAL) is a small glycoprotein that belongs to the lipocalin superfamily. These proteins share a common fold, the ability to bind small molecules such as siderophores, and the capacity to modulate the immune system. Lipocalins have a wide range of functions that include transport of lipophilic substances (52), iron cell homeostasis, antimicrobial effects (53) and an important role in cell regulation (54).

Despite having frequently sequence identities as low as 20%, lipocalins share a same well-conserved three-dimensional structure. Human LCN2 protein is composed of eight anti-parallel β -strands that form a β -barrel with a relatively narrow pocket at one end able to bind low molecular weight ligands and a wider cup-like binding site (the "calyx" that gives name to the family) at the opposite end able to accommodate larger molecules (55).

Lipocalins bind siderophores with high affinity (56–59), and LCN2 in particular can transport iron by stabilizing considerably the binding to iron/siderophore complexes (60, 61). This ability enables the lipocalin to act as an antibacterial agent by chelating bacterial siderophores. A higher LCN2 expression allows cells to tolerate increases in the physiological iron concentrations (62–64).

In its relationship with the immune system, LCN2 is considered as an acute phase protein (65–69), upregulated in inflammatory conditions and involved in autocrine apoptosis of pro-B cells (70), and tumourigenesis (71–74). However, the overall impact of LCN2 is variable, showing pro- and anti-apoptotic activities so that the final effect is a balance between the different signals (75).

1.2.4 Airway Epithelium cells model.

Traditionally, studies on protein transport across the mucosa barrier have been performed using cell lines cultured as a monolayer. One of the major advantages of these models is the relatively high yield at which transported proteins can be studied in high throughput setting. Therefore, respiratory epithelial cell lines are used extensively as representative models because the experimental conditions are more reproducible and easier to control. Several cell lines deriving from bronchial epithelial cells, such as Calu-3, BEAS-2B, 16HBE, NHBE (76–78) have been shown to be suitable models for studies of airway epithelium. H441 and A549 cell lines are derived from alveolar epithelial cells and have been characterized to present alveolar epithelial phenotype.

In traditional cell culture models, adherent cells are usually immersed in a medium. However, this does not reflect the physiological condition of lung epithelial cells exposed to air. In studies with lung epithelial cells an air-liquid interface is established, growing the cells on microporous membranes in a two chamber system to mimic the physiological conditions and drive differentiation (Figure 1, (76)).

Figure 1: Diffusion chamber used with airway epithelial cells in order to get the right cell differentiation.

In our study, we used normal human bronchial epithelial cells (NHBE), isolated from epithelial lining of airways above bifurcation of the lungs. Cell culture was performed on a commercial diffusion chamber (Transwell ®) and only when cultured at an air-liquid interface (ALI), cells formed a pseudo-stratified, fully-differentiated culture of muco-ciliary phenotype.

The pseudo-stratified bronchial epithelium-like tissue displayed the morphological characteristics of the four main cell types that constitute human lung epithelium: ciliated and nonciliated cells, goblet cells located at the apical side, and p63-positive basal cells.

Secreted airway surface liquid (ASL) is visible from the first week after ALI. ASL is a protective liquid film necessary for muco-ciliary clearance; however, the correlation between ASL volume and composition and the control mechanism of mucin secretion is still poorly understood (79). ASL contains a mucus component, antibacterial agents such as lysozyme and lactotransferrin, cell debris shed during differentiation, ectoenzymes, cytokines and signaling molecules.

During the experiment, the ‘tightness’ of the cell layer is determined by the tight junctions between cells, and can be assessed by measurement of trans-epithelial electrical resistance (TEER) or the permeability of compounds transported across cell layers exclusively by the paracellular route.

1.3 ALTERNARIA

Alternaria spp. are ubiquitous, saprophytic endophytic fungi, highly distributed, especially in warm regions (80), and they are capable of survival on low nutrient media. This genus includes a huge number of species which has been described based on morphology and host-specificity (81).

Alternaria infects plants as a saprophyte of senescence plants, so in early stages it does not induce symptoms in their hosts. The fungus is able to infect a wide variety of plants including cereals, fruits, and vegetables (82, 83) and these infections usually occur on leaves and stems of the host plant. The disease produces brown lesions and destroys leaves so that this saprotrophic lifestyle causes considerable agricultural yield losses.

Endophytic fungi show the ability to produce two important families of metabolites:

A) *Mycotoxins*: usually non-host selective and implicated in the infection of crops (84).

B) *Host-dependent metabolites*: fungi produce similar bioactive molecules as the host-plant (85) and act in defense, growth and reproduction processes (86). Some species, such as *Alternaria alternata*, are able to produce host-selective toxin (HSTs) (87) that help the fungus penetrate plant tissue and are essential for fungal pathogenesis (88). These bioactive compounds could be classified as alkaloids, terpenoids, flavonoids, steroids, quinones, lignans, phenols and lactones (89).

Alternaria spp. not only affects plants, but also produces different types of infections and diseases in humans, being *Alternaria alternata* highly associated with human airways disorders such as allergy, asthma and sinusitis (90–94). In Southern Europe, over 20% of patients with a history of respiratory allergy are sensitized to *A. alternata* (95).

1.3.1 The principal allergen of *Alternaria alternata*: Alt a 1

Alt a 1 (AAM90320.1, NCBI Protein Database) is a protein detected in the cytoplasm of mold spores of *Alternaria alternata* (96) and its release is highly increased during the spores germination process (97).

The biological function of Alt a 1 remains unknown. A homolog gene expression related with infection process of *Arabidopsis thaliana* has been reported (98). The ability to interact with pathogenesis-related proteins (PR5) inhibiting its activity in the case of kiwi-fruit has also been

published (99). These data suggest a clear role in the fungal infection process although the mechanism by which this action is performed has not yet been fully elucidated.

On the other hand and with regard to the relationship of Alt a 1 with humans, the protein has been referred to as a major allergen of *A. alternata* (100–102) and strongly associated with chronic asthma (103). Alt a 1 is the responsible for the allergenicity in over 90% of patients with *Alternaria spp.* respiratory allergy (104). Fungal spores would reach the respiratory tract and release large amounts of the allergen (97, 105) thus facilitating Alt a 1 contact with the airways.

Natural Alt a 1 has been described as a 30 kDa homodimer in a “butterfly-like” shape (Figure 1A) presumably linked by an intermolecular disulfide bond between two cysteines at different chains that dissociates under reducing conditions in two subunits of 16.4 and 15.3 kDa (106). The high resolution X-ray crystal structure of monomeric Alt a 1 reveals a unique β -barrel fold made of eleven β -strands which has been claimed not to have equivalent in the Protein Data Bank (107). Although no binding site is found in the monomeric structure of Alt a 1 (106) one might speculate about the possibility to bind some kinds of ligands, in analogy with other β -barrel proteins that frequently act as transporters of small molecules.

On the other hand, *Kurup et al.* (108) identified IgE binding linear epitopes which are located on the protein surface and happen to be properly oriented in the dimer (Figure 1B) to permit crosslinking with IgE thus driving the allergic reaction.

Figure 1: Alt a 1 dimer: **A)** Structure of the Alt a 1 homodimer shown in cartoon representation. N-terminal Cys residues presumably involved in an intermolecular disulfide bridge are shown as sticks at the top. **B)** Solvent accessible surface. Subunits are shown in light green and deep cyan. Epitope regions are shown in pink.

1.4 FLAVONOIDS

Flavonoids are one of the largest groups of plant secondary metabolites with more than 900 different compounds. They are found in all plant tissues and organs (109) and are involved in very diverse biological processes (110–112). The interest in their study has been largely increased in last years because of their anticancer and antioxidant properties (113).

Free radicals such as reactive oxygen species (ROS) can be produced by UV radiation, electron transport chains or by different pollutants in the environment. An increased amount of these radicals can result in pathological conditions such as inflammation, carcinogenesis or neurological diseases (114, 115). Antioxidants are responsible for neutralizing these substances and preventing their effects (116), being flavonoids and particularly flavonols one of the most potent free radical scavengers in nature.

Flavonols are hydroxylated phenolic compounds synthesized by plants in response to microbial infection. Their activities depend essentially on their chemical structure being the functional hydroxyl groups the responsible for their antioxidant effects (117–119). Flavonols can also regulate gene expression and biological activity of plant growth factors such as auxin (120).

Flavonoid levels in plants are modulated in response to environmental changes or development and growth signals (121), and their synthesis is induced in response to light properties (quality or intensity) (122, 123), phytohormones such as abscisic acid, jasmonic acid, auxin or ethylene (112, 124–126), oxidative stress (127, 128), temperature (129), drought (128, 130) or nitrogen deficiency (131), among other factors.

In the deeply studied defense processes involved in plant-fungi interactions, it is known that plants produce flavonoids with antifungal activity (132). However, very little is known about the role of those compounds produced by fungi although the presence of enzymes implicated in flavonoid biosynthesis has been reported (133). Until now, only a small number of fungal flavonoids have been identified such as chloroflavin and a dihydrochalcone from *Aspergillus candidus* and *Phallus impudicus* (134, 135), two C-methyl-flavonols reported from *Colletotrichum dematium f.sp* (136) and kaempferol 3-O- α -L-rhamnoside as a result of a biotransformation from *Alternaria tagetica* (137)

1.5 COMPUTATIONAL BIOLOGY APPLIED TO PROTEIN STRUCTURAL STUDY.

Computation has become essential in biological research and we can currently use it to understand biological systems. Computational methods not only allow us to represent and simulate biological systems but they also provide interpretation of experimental data and, on occasion, are even able to predict experiments. Moreover, computational modeling can be used to predict the response of a biological system to external changes or different environments, thus providing key information about its stability.

Given that the function of proteins is directly related to their three-dimensional (3D) structure, a first step to address the unknown function of a given protein is to search for structural relationships with other proteins of known structure. At the most basic level, this search involves protein topology. Methods such as those implemented in the Protein Topology Graph Library (138) are able to perform topological comparisons and find fundamental relationships. The next search level involves protein architecture, that is to say, trying to identify structural resemblances in the 3D space. There are many structural alignment methods that implement a large variety of comparison algorithms depending on the different criteria used to select subsets of residues included in the geometrical search. These structural relationships are very often highly successful in inferring protein function.

In silico analyses permit the study not only of the structural characteristics of a protein that determine its interactions but also the nature of binding sites, stability and properties of possible protein-ligand complexes, or the ability to show distinct oligomerization states. Moreover, computational methods also allow us to explore the influence of environment variables (all particularly pH-dependence) on all those features of a protein. It is thus possible to obtain *in silico* a valuable picture on the biological function of a protein. This computational research may be detailed as follows.

1. To study aggregation states we can predict protein oligomers of X-ray crystal structures using PISA software (139, 140), the approach customarily followed by the Protein Data Bank.
2. To study pH-dependences pKa values of residues with ionizable side chains can be computed. The main information provided by these calculations is the detailed protonation states of all the residues at a given pH. Empirical predictors such as Propka 3.1 (141,142) calculate the effect on pKa values of spatial proximity of titratable groups by computing free energy changes arising from the corresponding interactions. It is thus possible to

determine *in silico* the protonation states in the different assemblies of the protein at media with different pH values.

3. To study the charge distribution at entire proteins (monomers or multimers), local regions (binding sites or protein-protein and protein-ligand interfaces), and ligands as well, the Poisson-Boltzmann (PB) electrostatic potential (141) is a primary property. 3D grids with the electrostatic potential value at every point surrounding the molecule can be obtained by solving numerically the PB equation for the molecular system. Either mapped onto the molecular surface or plotted as isopotential 3D maps, those PB electrostatic potential grids reveal spatial features of the charge distribution in protein systems. This way, sources of electrostatic interactions in different regions in all the oligomerization states of the protein at different protonation states of ionizable residues can be identified to study protein-ligand and protein-protein interactions.
4. If a putative binding site has been identified, docking calculations allow the prediction of geometries for protein-ligand complexes. This binding site is used as reference to set a search grid in order to perform docking calculations with approaches such as AutoDock Vina (142, 143), a highly reliable method.

The stability of protein-ligand complexes and the different oligomeric states of a protein as well as the dynamical evolution of their structures and properties can be investigated by means of Molecular Dynamics (MD) calculations (144–146). The trajectories calculated by integrating the equations of motion for a molecular system in aqueous media provide essential information to address not only the own stability of the system but also the dynamical change of virtually any of its properties. Upon preparing the structures of multimeric states and their complexes at different pH's by immersing them in water at salt solution in proper solvation boxes, a cycle of optimization-equilibration-simulation calculations over representative simulation times (50 – 100 ns) can be used for this dynamical study. However, the great numbers of atoms and the large number of steps (more than 50 million for 100-ns simulations) usually needed for the MD study of oligomers at different pH's demand computational resources so huge that resorting to supercomputers able to perform large-scale parallel calculations is necessary. The information obtained with these MD simulations allows to understand in depth the reasons for the stability of different oligomeric states, the influence of the absence or presence of ligands and the effect on structures and properties arising from different pH's.

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2. OBJECTIVES

OBJECTIVES

The principal aim of this PhD project was to understand the molecular mechanisms involved in the Alt a 1 sensitization by epithelium bronchial mucosa. With this purpose, the following objectives were established:

1. To study the role of Alt a 1 in fruit infection using kiwi as model, and the allergic co-sensitization phenomenon with a thaumatin-like protein from kiwi (Act d 2).
2. To study the stability in saline solution (pH 7) of different oligomer structures of major allergens, analyzing the structural flexibility of ligand binding sites and the dynamical stability of epitope regions by means of Molecular Dynamics simulations (20 ns).
3. To study *in silico* the oligomerization capacity of Alt a 1 and its dependence on the pH of the medium and on the presence of the flavonoid quercetin by means of computational analyses and Molecular Dynamics calculations.
4. To characterize the ligand carried by Alt a 1 and its biological activity.
5. To study structural relationships between Alt a 1 and other allergens.
6. To study the molecular basis of the interactions between Alt a 1 together with its ligand and bronchial epithelium as well as to analyze the capacity to induce an inflammatory response.

3. RESULTS

3.1 Alt a 1 from *Alternaria* interacts with PR5 thaumatin-like proteins

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Doctoral candidate's contribution to this work:

Isolation of *Alternaria* spores in kiwi fruit

Controlled infection assays

Purification of Alt a 1

Determination of endo- β -1,3-glucanase activity

Alt a 1 from *Alternaria* interacts with PR5 thaumatin-like proteins

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ABSTRACT

Alt a 1 is a protein found in *Alternaria alternata* spores related to virulence and pathogenicity and considered to be responsible for chronic asthma in children. We found that spores of *Alternaria* inoculated on the outer surface of kiwifruits did not develop hyphae. Nevertheless, the expression of Alt a 1 gene was upregulated, and the protein was detected in the pulp where it co-localized with kiwi PR5. Pull-down assays demonstrated experimentally that the two proteins interact in such a way that Alt a 1 inhibits the enzymatic activity of PR5. These results are relevant not only for plant defense, but also for human health as patients with chronic asthma could suffer from an allergic reaction when they eat fruit contaminated with *Alternaria*.

Structured summary of protein interactions:

Alt a 1 binds to PR5-thaumatin-like protein by pull down (1, 2) **Alt a 1 binds to peach PR5** by pull down (View interaction) **Alt a 1 binds to banana PR5** by pull down (View interaction) **Alt a 1 physically interacts with PR5-thaumatin-like protein** by pull down (View interaction).

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1. Introduction

The fungal genus *Alternaria* includes many saprobic and endophytic species, but is well known for containing many notoriously destructive plant pathogens [1,2]. *Alternaria* species have been classified and identified based on conidial characters and molecular-genetic data and comprise more than 280 species [3]. With the exception of studies regarding pathogen-derived host-specific phytochemicals, the physiological and molecular mechanisms underlying the interactions between saprobic and endophytic fungi and their respective host plants remain largely unexplored [4]. It is known that insidious fungal infections of postharvest mould remain quiescent as biotrophs during fruit growth and harvest, but actively develop and transform into saprobes during ripening and senescence [1]. Exposure of unripe hosts to the fungus quickly initiates defensive signal-transduction cascades that limit fungal growth and development, but exposure to the same fungus during ripening activates a very different signalling cascade that facilitates fungal colonization [5].

Strains of *Alternaria alternata* are ubiquitous in nature and exist predominately as saprobes. They are frequently found on fruits, vegetables and cereals. They can produce mycotoxins and other signals described as pollutants of plant-flavoured products such as juices, sauces and preservatives, thereby entering the human food chain without any fungal development having been observed [6].

A. alternata is considered to be one of the most prolific producers of fungal allergens. In particular, Alt a 1 (AAM90320.1, NCBI Protein Database), its principal allergen, has been associated with asthma, and sensitivity to this allergen was recently shown to be a risk factor for life-threatening asthma [7–9]. Alt a 1 is a heat-stable, 28 kd dimer, which dissociates into 14.5- and 16-kd subunits under reducing conditions [10]. Until now, the function of Alt a 1 in fungal metabolism or ecology is unknown [11–13]. Recently, its homologue in *Alternaria brassicicola* was found in *Arabidopsis thaliana* to be highly expressed during the infection process of *A. thaliana*, suggesting that the protein may be involved in plant pathogenicity [7,14]. It is possible that *Alternaria* spores are present in foods/fruits without developing hyphae, with the spores producing components involved in allergenicity, such as Alt a 1 protein.

Thus, we studied the behaviour of *Alternaria* spores in a non-host fruit—kiwifruit. Our results suggested that *A. alternata* in kiwi

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behaved as a saprobic fungus. Alt a 1 was detected, despite the fact that hyphae development was not observed after 14 days post-inoculations (dpi). In order to search for a kiwi receptor for Alt a 1, pull-down assays were performed. Alt a 1 interacted with the kiwi PR 5-TLP, and both proteins co-localized in the kiwi pulp by immunofluorescence in confocal microscopy. The interaction resulted in the inhibition of the PR5 enzymatic activity. This effect was not limited to kiwi PR5; PR5 from other fruits, such as peach and banana, also interacted with Alt a 1. Thus, Alt a 1 was characterized as an enzymatic inhibitor of the PR5 family.

2. Materials and methods

2.1. Plant material and fungus growing conditions

Alternaria spores were isolated from kiwifruits by scraping the surface and cultured on PDA medium (potato dextrose agar; Difco™ Becton Dickinson and Company, Sparks, MD, USA) with cefotaxime (200 µg/ml) (Calbiochem®, Merck KGaA, Darmstadt, Germany). After one week, isolated fungi were re-cultured separately on PDA. Isolated fungi were identified by ITS rDNA sequencing. For this purpose, genomic DNA was extracted using a Plant DNA Preparation Kit (Jena Bioscience GmbH, Jena, Germany). The region of interest was amplified with oligonucleotides ITS1F 5'-C TTGGTCATTTAGAGGAAGTAA-3' and ITS4 5'-TCCTCCGCTTATTGAT ATGC-3' [15]. The amplified sequences were then sent for identification by alignment/comparison with the NCBI database. After 8 days, identified *Alternaria* spores were recovered with sterile water and stored at –80 °C in 20% glycerol.

2.2. Controlled infection of kiwifruits with *Alternaria* spores

Kiwifruits (*Actinidia deliciosa*, commercial variety Hayward), washed with 20% bleach solution for 10 min, were infected by placing 1×10^6 *A. alternata* spores in one drop (20 µl) onto the outer surface. The fruit was covered with plastic film and incubated at 24 °C. To quantify Alt a 1 expression by Real Time PCR, samples were collected at days 1–7 post-infection, and then homogenized and lyophilized. RNA was extracted using an RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) and quantified by NanoDrop® (NanoDrop Technologies, Wilmington, DE, USA). cDNA was obtained and amplified with Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Ltd., Paisley, UK) according to the manufacturers recommendations, and run on an Applied Biosystems 7300 Real-Time detection system (Applied Biosystems, Life Technologies Ltd., Paisley, UK). The following primers were used: Alt a 1 (5'-AGG AACCTACTACAACAGCC-3'; 5'-GTACCACTGTGGTCTCAA-3') and 18S rRNA (5'-GTCGTAACAAGGTTCCGTAGGT-3'; 5'-CAAAGGGAAG AAAGAGTAGGGT-3') as an endogenous control, as described by Li et al. [16]. The fold change of the corresponding mRNA of these genes was normalized with the endogenous control 18S, and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$), as described by Livak and Schmittgen [17]. The amplification was carried out in quadruplicate.

2.3. Extracts and purified proteins

A mixture of spores and mycelia of *A. alternata* (ALK-Abello, Madrid, Spain) and lyophilized kiwifruit were extracted with phosphate-buffered saline (0.1 M sodium phosphate, pH 7.0 and 0.5 mol/L NaCl; 1:5 (w/v), 1 h, 4 °C). After centrifugation (12000×g, 30 min, 4 °C), the supernatant was dialyzed (cut-off point 3.5 kDa) and freeze-dried. Protein concentration was quantified according to the Bradford method. Thaumatin-like proteins (Kiwi-PR5-P81370 (Uniprot), Pru p 2.0202-ACE80955.1 (NCBI

Protein), and Musa 4-AFK29763.1 (NCBI Protein)) were purified as previously described [18]. Recombinant Alt a 1 was purchased from Bial Aristegui (Bilbao, Spain) [19].

2.4. Immunohistochemistry assays

To detect the presence of Kiwi-PR5 and Alt a 1 proteins, samples were recovered after 7 days post-infection (dpi). Small, hand-cut pieces of infected kiwifruits were fixed with 4% formaldehyde in PBS pH 7.4 at 4 °C overnight. After PBS washing, the tissues were cut into 30–40 µm sections with a vibratome under water and dried down on 10-well multiwell slides (Fisher Scientific Inc., Pittsburgh, USA). The sections were permeabilized by dehydration in a series of methanol (30%, 50%, 70% and 100%, 5 min each) and re-hydrated (70%, 50%, 30% methanol, PBS; 5 min each). After washing with PBS, tissue sections were incubated overnight at 4 °C with specific antibodies: monoclonal anti-Alt a 1-Alexa 488-conjugated (1:50, Bial Aristegui, Bilbao, Spain) and polyclonal anti-PR5-Alexa 550-conjugated (1:50, [20]). Sections were mounted with glycerol:PBS (1:1) and observed with a Leica TCS-SP8 confocal microscope, using the laser excitation lines of 488 and 561 nm. Collection of 3D stacks was optimized to the maximum Z resolution.

2.5. Immunoprecipitation assays

Dynabeads® M-280 Tosylactivated (10^7 Dynabeads; approximately 20 µg/ml; Invitrogen, Oslo, Norway) were resuspended by vortexing, and washed with PBS (10 mM pH 7.4). Purified protein (20–50 µg/ml) was incubated overnight with the activated Dynabeads at 37 °C with slow rotation. After incubation, the excess protein was recovered in the supernatant fraction. The coated beads were then washed four times alternately with PBS pH 7.4 BSA 0.1% (2×), 0.1 M borate buffer pH 9.5 (1×) and PBS pH 7.4 (1×). After that, protein extract (1000 µg/ml) or purified protein (20 µg/ml) was incubated with the coated beads in PBS pH 7.4 BSA 0.1% overnight at 4 °C with slow rotation. After extensive washing, coated beads were resuspended in Laemmli buffer and separated in 15% SDS–PAGE, following the immunodetection assay protocol outlined below.

2.6. Immunodetection assays

Coated Dynabeads incubated with protein extract or purified proteins were separated by 15% SDS–PAGE, and replica gels were electrotransferred onto polyvinylidene difluoride (PVDF) membranes. After blocking (Sigma–Aldrich, St. Louis, MO, USA), membranes were immunodetected with rabbit polyclonal antibodies raised against peach PR5 (anti-PR5; 1:10000, Carlos Pastor, Madrid, Spain) or Alt a 1 (anti-Alt a 1, 1:100000; Bial Aristegui, Bilbao, Spain), and then incubated with the detection antibody alkaline phosphatase-conjugated anti-rabbit IgG (1:5000, Sigma, St. Louis, MO, USA) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma, St. Louis, MO, USA).

2.7. Endo-β-1,3-glucanase activity

Endo-β-1,3-glucanase activity was assayed using the method described by Menu-Bouaouiche et al. [21], with minor modifications, using carboxy-methylated Pachyman (CM-Pachyman, Megazyme, Wicklow, Ireland) as substrate. Kiwi-PR5 (20 µg/ml) was incubated for 0, 5, 10, 30 and 60 min in 100 µL of 1% (w/v) CM-Pachyman in 50 mM sodium acetate, pH 5.0, 100 mM NaCl containing 0.05% (w/v) sodium azide. One millilitre of 0.1% (w/v) tetrazolium blue, 50 mM NaOH, and 0.5 M sodium potassium tartrate was added to samples and heated in boiling water. The

quantity of liberated D-glucose equivalents was estimated by absorbance at 660 nm. Assays were performed in triplicate. Results are expressed as nkat/mg of protein (1 kat corresponds to the formation of 1 mol of D-glucose equivalents).

2.8. Statistical analysis

SPSS 17.0, Statgraphics Centurion XVI and GraphPad Prism 6.01 software programs were used for statistical analysis. Results were compared by applying the non-parametric Kruskal–Wallis (with

Dunn's correction) for multiple comparisons, contingency tables and Spearman correlation, when appropriate. *P*-values lower than 0.05 were considered significant in all the analyses.

2.9. Molecular modelling of the Kiwi-PR5–Alt a 1 complex

The crystal structures recently determined for Alt a 1 [4] and Kiwi PR5 [22] proteins were used. Poisson–Boltzmann electrostatic potentials using AMBER atomic charges and radii assigned with PDB2PQR [23] were computed with the APBS program [24] to solve

Fig. 1. (A) Immunolocalization of Alt a 1 in ungerminated spores, detected by specific monoclonal antibody, showing fluorescence signal in red (Anti-Alt a 1-Alexa 550) in an overlay with DIC (Overlay) and an orthogonal projection of a 3D stack. (B) Fold change in the Alt a 1 relative gene expression levels for kiwifruit inoculated with *Alternaria* spores ($10^6/20 \mu\text{l}$) for 1, 2, 5 and 7 days. Results are expressed in fold change compared to the expression of 18S, used as housekeeping gene. Mean values and S.D. (bars) of four independent assays are shown (*P*-value < 0.05). (C) Immunolocalization of Alt a 1 in the pulp of kiwifruits at 7 dpi with *Alternaria* spores ($10^6/20 \mu\text{l}$), by fluorescence signal (Anti-Alt a 1-Alexa 550) and the overlay with DIC in a 3D projection (3D projection).

the non-linear Poisson–Boltzmann equation in sequential focusing multigrid calculations. The potentials were obtained at 3D grids made of 161^3 points with a step size of about 0.5 Å at 298.15 K and 0.150 M ionic concentration with dielectric constants of 4 for proteins and 78.54 for water. Numerical output was processed in scalar OpenDX format and mapped onto the protein molecular surfaces and rendered with PyMOL [25].

Protein–protein docking model structures for the Kiwi-PR5–Alt a 1 complex were obtained with RosettaDock (rosettadock.graylab.jhu.edu/docking [26]) and PatchDock (bioinfo3d.cs.tau.ac.il/PatchDock/ [27]) servers. In both cases, the best-score and low-energy models were selected.

3. Results

3.1. Alt a 1 was detected in kiwifruits inoculated with Alternaria, despite the fact that no hyphae development was observed

Spores from *A. alternata* were isolated from kiwifruits (*Actinidia deliciosa*, variety Hayward, cultivated in an ecological field), and its identity was confirmed by ITS rDNA sequencing. The isolated

fungus was used to study the role of Alt a 1 in the saprobic state in the colonization of the non-host fruit kiwifruit.

Firstly, Alt a 1 was localized inside ungerminated spores by immunofluorescence with specific monoclonal antibody in confocal microscopy (Fig. 1A), observing the most intense signal in the spore wall (Fig. 1A, orthogonal projection).

Kiwifruits from a local market were inoculated with 10^8 of the isolated spores in 20 µl drops (Supplementary Data Figure). As expected, no hyphae development could be observed until 14 dpi by staining with methylene blue (data not shown). Despite the fact that hyphae were not observed, the presence of Alt a 1 was confirmed in the kiwi pulp after 7 dpi by quantitative rtPCR (Fig. 1B), and by immunofluorescence using a specific monoclonal antibody in confocal microscopy (Fig. 1C).

Thus, Alt a 1 was found in the pulp of kiwifruit despite the fact that no hyphae development was observed.

Alt a 1 was capable of interacting with a kiwi PR5-thaumatin- like protein (Kiwi-PR5)

Pull down assays were carried out to find potential plant receptors for Alt a 1. Dynabeads® M-280 Tosylactivated (Invitrogen,

Fig. 2. (A) Dynabeads® M-280 Tosylactivated were coated with Alt a 1 (Alt a 1-Dynabeads) and incubated with kiwifruit extract (Kw-ext) and purified kiwifruit PR5 (Kiwi PR5, Kw-PR5; banana PR5, Ba-PR5; peach PR5, Pe-PR5). The result was separated by SDS–PAGE. Coomassie staining (Coomassie), and replicas blotted with polyclonal antibodies against Alt a 1 (anti-Alt a 1; dilution 1:10⁵) and PR5 (anti-PR5; dilution 1:10⁴) are shown. (B) Dynabeads® M-280 Tosylactivated were coated with Kiwi PR5 (Kiwi PR5 Dynabeads) and incubated with Alt a 1 (5 µg) and *A. alternata* extract (20 µg Alt-ext). The result was separated by SDS–PAGE and stained with Coomassie (Coomassie). Grey (–) boxes indicate control proteins. White (+) boxes indicate immunoprecipitated proteins.

Oslo, Norway) were coated with Alt a 1 (Bial Aristegui, Bilbao Spain) and incubated with a kiwifruit protein extract (Fig. 2A). A principal band around 26 kDa was observed when the immunoprecipitated proteins were separated by SDS–PAGE and stained with Coomassie. This band was identified by mass fingerprinting as the PR5-thaumatin like protein (P81370). The accuracy of the retained protein identification was further supported by immunodetection using specific polyclonal antibodies against PR5 (Anti-PR5). The higher molecular weight bands of the retained which can be observed in the Coomassie staining corresponded to typical aggregates of the members of this family, as it was confirmed by the detections with PR5 antibodies.

To validate the specificity of this interaction, Kiwi-PR5 was purified from fruits and used to coat Dynabeads M-280 Tosylactivated® (Fig. 2B). Kiwi-PR5-coated beads were incubated with *Alternaria* protein extract and purified Alt a 1. When the retained proteins were separated by SDS–PAGE, a single band of 14 kDa was observed on Coomassie, confirming that Alt a 1 was the unique binding protein.

In order to assess whether the ability of Alt a 1 to bind Kiwi-PR5 was also the case with other PR5, additional pull-down assays were performed using other members of this protein family, such as peach PR5(ACE80955.1) [28] and banana PR5 (AFK29763.1) [20]. In both cases, it was confirmed that Alt a 1 was able to bind other

Fig. 3. Model structure of the Kiwi-PR5–Alt a 1 complex. (A) Ribbon diagram of the best RosettaDock docking structure. Alt a 1 is coloured pale yellow, Kiwi-PR5 is coloured green, and acidic residues in the catalytic cleft are shown as sticks with C atoms in violet and O atoms in red. (B and C) Poisson–Boltzmann electrostatic potential mapped onto the molecular surface of Alt a 1 (B) and Kiwi-PR5 (C) at the geometry of the complex in A. The scale bar at the bottom refers to electrostatic potential values in units of kT per unit charge (k , Boltzmann's constant and T , absolute temperature). (D) Inhibition of enzymatic activity of Kiwi-PR5 in the presence of Alt a 1. Endo- β -1,3-glucanase test using a carboxy-methylated Pachyman as substrate. Generated D-glucose equivalents were estimated by measuring the absorbance at 660 nm. Results are expressed as the mean of assays performed in triplicate in nkat mg^{-1} of protein.

PR5s, as shown by Coomassie and immunodetection with specific antibodies (Fig. 2A).

3.3. *Alt a 1* inhibited the enzymatic activity of kiwifruit PR5

The interaction between kiwi PR5 and *Alt a 1* was supported by molecular modelling (Fig. 3A–C). By using the experimental X-ray structures of *Alt a 1* [4] and Kiwi-PR5 [22], different protein–protein docking calculations were performed. The best complex candidates revealed the formation of *Alt a 1*–kiwi-PR5 aggregates in which the electrostatically negative cleft of kiwi-PR5, where the antifungal enzymatic activity occurs, was partially blocked with *Alt a 1* protein that faced an electrostatically positive prominent region.

This structural feature provided the fungal protein with an electrostatically driven affinity for the kiwi PR5 that resulted in the

inhibition of its enzymatic activity. This was confirmed by measuring the kiwi PR5 β -glucanase activity in the presence of *Alt a 1* (5 μ g; Fig. 3D). *Alt a 1* was able to inhibit the enzymatic activity of PR5, although full inhibition was not observed.

These results reveal why both proteins were visualized together in kiwi fruits (7 dpi). Kiwi-PR5 and *Alt a 1* co-located in the pulp by immunofluorescence and confocal microscopy (Fig. 4).

4. Discussion

In this study, hyphal development was not observed on kiwi-fruit 14 dpi with spores of *A. alternata*. Spores appeared to remain dormant until fruit senescence, when the growth of *Alternaria* was induced. By contrast, the presence of *Alt a 1* was detected in kiwi pulp several days after inoculation, despite the absence of hyphae. To date, the role of *Alt a 1* remains unknown, although it seems to

Fig. 4. Immunolocalization of *Alt a 1* (A. Anti-*Alt a 1*-Alexa 550, 1:100) and PR5 (B. Anti-PR5-Alexa 488, 1:50) and overlay (C. 3D projection) of both fluorescence signals in kiwi pulp (7 dpi).

be related to virulence and pathogenicity [29]. Alt a 1 has also been described as the most representative secreted protein at the beginning of the germination process [19]. The present results demonstrate that, even when spores had not yet germinated, they were in fact producing Alt a 1, highlighting the importance of this protein for the development of the fungus.

The presence of the fungal spores induced the plant response by dramatically increasing the expression of plant defense proteins [30]. Hence, it is possible that the presence of Alt a 1 induces the expression of the kiwi PR5, a plant defense family, being co-located in the pulp and interacting with it.

The PR5 or thaumatin-like family compromise proteins with molecular masses around 20–30 kDa and a very stable three-dimensional structure maintained by 8 disulfide bridges [31]. PR5 protein expression is induced by fungus attack, due to the β -glucanase activity that takes place at a characteristic cleft, which is rich in acidic residues, conferring a strong negative electrostatic potential on the active site [18,20,28,31,32]. By computational modelling, Alt a 1 seemed to be bound to a surface region of the kiwi PR5 protein that had a complementary electrostatic nature. This binding resulted in the partial inhibition of PR5-enzymatic activity.

Although the role of Alt a 1 in infection has not been completely resolved by this study, the protein appears to be secreted in the early stages of colonization, before the development of hyphae. In non-host species such as kiwifruit, it may be that the physiological action of Alt a 1 is stopped by its interaction with PR5, which would in turn inhibit, partially, the anti-pathogenic activity of the PR5 proteins.

From the point of view of human health, the presence of Alt a 1 in apparently healthy kiwifruits is highly relevant. Kiwi PR5-TLP (known as Act d 2) and Alt a 1 have been characterized as major allergens from the fruit and *Alternaria*, respectively [18,33]. In fact, *Alternaria* has been described as one of the principal causes of severe asthma in the USA [34]. Thus, the results presented in this study suggest that *Alternaria*-allergic patients may experience an allergic crisis after ingesting infected kiwis. However, further experimental evidence would be required to determine whether this is indeed the case.

In summary, the present study reveals Alt a 1 as a competitive inhibitor of the PR5-TLP family, which may be particularly relevant in both fungal infection and in the processes involved in human allergic responses.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2014.02.044>.

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3.2 Molecular Dynamics of Major Allergens from *Alternaria*, Birch Pollen and Peach

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Doctoral candidate's contribution to this work:

Preliminary structural study of dimers of Alt a 1 and analysis of protein-protein interfaces

Structural comparison of these dimers with those of the 4AUD structure of Alt a 1

Study of the geometry of the β -barrel over the MD simulation in monomeric Alt a 1

Analysis of the intermolecular -SS- bridge in Alt a 1 dimers over the MD simulation

Study of the structural stability of epitopes in these dimers derived from MD trajectories

Preparation of plots for the analysis of MD trajectories for Alt a 1 structures

Molecular Dynamics of Major Allergens from *Alternaria*, Birch Pollen and Peach

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Abstract: In the search for factors that make a protein allergenic (an issue that remains so far elusive) some common features of allergens such as small size, high stability and lipid binding are recognized in spite of their structural diversity. Other relevant but still poorly understood feature is their capability to form homodimers. We investigated by means of Molecular Dynamics (MD) calculations the stability in solution of several dimers of three major allergens

from *Alternaria* mold, birch pollen, and peach fruit known to play essential roles in allergic disease. By running 20 ns MD simulations we found essential properties on solution that provide information of interest on their dimerization, stability of their epitopes and dynamical features of ligand binding cavities. Our results show that three essential allergen proteins display a distinct behavior on their trends to form homodimers in solution.

Keywords: Allergens · Epitopes · Molecular dynamics · Protein models · Protein structures

1 Introduction

Allergy is a health problem with about 25% of the population in developed countries affected. By the end of 2013, the Allergome database^[1,2] listed 2400 sources described as allergenic to humans and about 1000 allergens have been classified and assigned to a reduced number of protein families.^[3] Experimental structure is available for one hundred different allergen proteins which have been classified into 20 structural families.^[4] However, despite the increase of clinical, biological and chemical studies on allergy, the factors that make a protein allergenic remain elusive. Defining characteristic features of allergens is essential to discriminate the properties that distinguish them from structurally related innocuous proteins. Although known allergen proteins show structural diversity, some common features are recognized: most allergens are relatively small, rather stable and well-structured proteins^[4,5] and lipid binding is found for more than 50% of the major allergens.^[6]

Other relevant but still poorly understood feature of allergens is their capability to form homo-oligomers, particularly homodimers. A computational analysis of 55 allergen X-ray structures found that 80% of them can exist as symmetric dimers or oligomers in crystals.^[7] This report raised the suggestion that many of these molecular complexes aggregates could be transient dimers difficult to observe at normal concentrations in cells but becoming favored if concentration increases locally as it should occur on antigen-presenting cells or antigen-antibody complexes.^[7] We have recently extended the oligomerization analysis to 84 allergen X-ray structures using crystal information together with a new computational tool that uses evolutionary information to address dimer formation.^[8] Our study lowered the proportion of allergens with oligomeric states down to

66% although it still seems clear that oligomerization is a common feature of allergens.^[9]

While most studies to derive physicochemical or structural features of allergens have focused on their 3D structure, much less attention has been paid to their dynamic properties in solution. We address here the exploration of three major allergens from *Alternaria alternata* mold (Alt a 1), birch (*Betula verrucosa*) pollen (Bet v 1), and peach (*Prunus persica*) fruit (Pru p 3) by means of Molecular Dynamics (MD) simulations. The reasons why we chose these major allergens for our study are the following: (i) they are structurally different proteins that play roles in food allergy, a subject in which we have been working for years, (ii) they are major allergens that have been used as models in allergic disease, (iii) IgE-epitope regions have been reliably characterized in the three cases. *Alternaria* is one of the principal species associated with allergy, especially chronic asthma. Its major allergen, Alt a 1, is a protein with a unique dimeric β -barrel structure that appears to define a new protein family found in fungi with as yet unknown function.^[10] *Alternaria* infects a wide diversity of plants including fruits and recent results suggested the possibility that patients with chronic asthma could suffer from an al-

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lergic reaction when they eat contaminated fruit.^[11] The major birch pollen allergen Bet v 1 is the main elicitor of airborne allergies as 50–90% of patients affected by pollen allergies are sensitized to Bet v 1. Up to 90% of these patients also exhibit allergic cross-reactions to food allergens contained in fruits and vegetables. Bet v 1 is the most extensively studied allergen as it has been used as a model system in allergy (for a review see Reference^[12]). Experimental evidence on the existence of dimeric Bet v 1 has been very recently reported^[13] and its physiological ligand identified.^[12] The major peach allergen, Pru p 3, has been characterized as the major food allergen in the adult population in the Mediterranean area and it has been also used as a model system in food allergy.^[14,15] Pru p 3 is a prototypical member of nonspecific lipid transfer proteins (nsLTPs), a panallergen family widely distributed throughout plants with a particularly stable fold that explains their resistance to proteolysis.^[16] Although there is no evidence in vivo for Pru p 3 dimerization, the crystal structure of this protein is a dimer.^[17]

MD simulations on monomeric allergen proteins can be found in the literature (see for instance References^[16–23]) but these calculations have been customarily intended to assess the stability of ligand binding to allergens.^[17,18,20,22,23] However, to the best of our knowledge, no MD calculations for allergen dimers have been reported so far. In the current study we addressed the stability in solution of different oligomer structures of Alt a 1, Bet v 1, and Pru p 3 major allergens by means of 20 ns MD simulations. Besides addressing the stability of the different protein assemblies, we investigated also the structural flexibility of ligand binding sites in solution. A further relevant point in our MD calculations was the exploration of the dynamical stability of IgE-epitope regions identified before for these three allergens. We aimed thus to gain insight into the structure and dynamics properties of different oligomeric states in solution of representative proteins which play a major role in allergic diseases.

2 Computational Methods

2.1 Initial Geometries

The coordinates for initial geometries were the following. The crystal structure of Alt a 1, PDB entry 3 VOR,^[10] was used for the monomer. Dimer and tetramer geometries were generated with rotation matrices and translation vectors included in the PDB file. The dimer thus obtained shows two N-terminal cysteines at position 30 forming an intermolecular disulfide bridge. Whereas other four cysteines (residues 74, 89, 128, and 140) forming intramolecular disulfide bridges are conserved among all sequences of Alt a 1 homologs, Cys30 is only conserved among the closest homologs.^[10] For this reason, we decided to study a dimer with the constraint to keep the intermolecular disulfide bond along the MD simulation and a second dimer

leaving unbonded both Cys30 residues. Another crystal structure of an Alt a 1 variant, PDB entry 4AUD, already a dimer,^[24] was also used. This protein has a 97.6% sequence identity with 3 VOR but lacks six amino acids at the N-terminal segment that includes Cys30.

Three crystal structures of Bet v 1 differing in the amino acid at position 5 were used as initial geometries for monomers: PDB entry 1BV1 that is the protein with the genetically encoded tyrosine at position 5^[25] and PDB entries 4BK6 and 4BK7 that have cysteine and phenylalanine, respectively, at position 5.^[13] The dimer geometry constructed with the rotation matrix and translation vector given in the 1BV1 file displays the IgE epitope regions at the protein-protein interface (see below). Since allergens induce cross-linking of IgE antibodies bound to high affinity receptors on mast cells or basophil surfaces, one would expect that an allergen dimer that triggers cross-linking should have the antigenic regions located at opposite ends on the surface. For this reason, we used a second dimer predicted by the Evolutionary Protein-Protein Interface Classifier (EPPIC) approach^[8] that shows the proper orientation of epitopes (see below). A third Bet v 1 dimer corresponding to the dimeric structure of 4BK6 (cysteine at position 5) and showing proper orientation of epitopes was also utilized.

The crystal structure of Pru p 3, PDB entry 2ALG,^[17] was selected for the initial geometry of monomer. This PDB file has already two chains that were used for a first dimer of Pru p 3. Since this dimer has the IgE epitope regions near the protein-protein interface (see below), we used a second dimer predicted by EPPIC that has the proper orientation of epitopes at opposite ends of the protein surface. In addition, initial geometries for complexes of monomer and two dimers with palmitate were obtained upon superposition of palmitate molecule with the ligand in the 2ALG entry (laurate).

Protein–protein interfaces in all the dimers were analyzed with Protein Interfaces, Surfaces and Assemblies (PISA) software^[26,27] using the EBI-PISA server (www.ebi.ac.uk/msd-srv/prot_int/pistart.html) to compute interface areas as well as to determine residues and interactions (hydrogen bonds and salt bridges) in the interfaces.

2.2 Molecular Dynamics Calculations

MD calculations using the CHARMM 3.1 force field^[28] were performed with NAMD 2.9.^[29] Initial structures were immersed in periodic rectangular solvation boxes with water molecules added according to the TIP3P model^[30] together with Na⁺ and Cl[−] ions added to counter the total charge of the protein systems while providing 0.150 M salt concentration. The particle mesh Ewald summation method^[31] was used for long-range electrostatics and a 10 Å cutoff was set for short-range non-bonded interactions. The following set of calculations were performed for every system: (1) first optimization along 5000 conjugate gradient minimization steps; (2) equilibration of water for 100 ps at 2 fs time

steps, 298 K and 1 atm with all atoms fixed in proteins; (3) keeping same time step, T and P, simulation runs during 20 ns (10 million steps) in the NPT ensemble. Langevin dynamics for the T control and Nosé-Hoover Langevin piston method for the P control were employed. Output results were stored every 5000 steps rendering thus trajectories composed of 2000 frames which were processed and analyzed with VMD 1.9.1,^[32] Carma,^[33] and MDTRA^[34] programs. Molecular graphics were prepared and rendered with PyMOL 1.6.^[35]

3 Results and Discussion

Given that the aggregation states of the three allergens studied amount to a total of 17 protein systems, we resorted to a limited length of MD simulations in explicit solvent (20 ns) in order to get the total computational burden within affordable limits. Before proceeding further, we firstly assessed that this simulation time was adequate for our goals by testing in the main dimers of Alt a 1, Bet v 1, and Pru p 3 proteins (see below) that the trajectories provided stabilized values of the properties analyzed.

Data on protein-protein interfaces of the dimers investigated were obtained at three frames of MD simulations: initial (time = 0), middle (time = 10 ns), and final (time = 20 ns) structures. This information is summarized in Table 1 where we present interface areas, number and types of residues in the interface and number of intermolecular hydrogen bonds and salt bridges at the interface. Detailed data on the properties gathered in Table 1 are given in the Supporting Information where all the interacting residues, hydrogen bonds and salt bridges are specified in Tables S1 and S2 (Supporting Information). Root mean square fluctuations (RMSF) per residue computed in the MD simulations for all the aggregation states are shown in Figure 1. In what follows, MD results for every allergen together with their cor-

responding data in Table 1 and Figure 1 are presented and discussed separately.

3.1 Alt a 1 Allergen

Figure 2 displays initial geometries for monomer, dimer, and tetramer states of Alt a 1 protein from the crystal structure in PDB entry 3 V0R^[10] as well as the dimer in PDB entry 4AUD.^[24] This latter protein is a variant lacking the N-terminal tail that contains Cys30. In dimeric Alt a 1, this residue can be covalently linked to the equivalent residue from the second chain (Figure 2A). While it has been claimed that dimer stabilization should be due to this presumed disulfide linkage,^[10,36] mass spectra obtained under denaturing solution conditions have provided evidence that Alt a 1 dimerization is not mediated by the disulfide bond.^[7] It must be also noted that whereas four cysteines that make intramolecular disulfide bonds (residues 74, 89, 128, and 140) are conserved among all the Alt a 1 homologs, Cys30 is only conserved among the closest homologs. We studied (i) the dimer with the intermolecular disulfide bond kept along the MD simulation ("dimer SSbridge" in what follows), (ii) the equivalent dimer without that link (named just "dimer") and (iii) the dimer in PDB entry 4AUD that lacks six N-terminal amino acids including residue 30 (named "dimer (4AUD)"). Results are shown in Figure 2E–2H and Table 2.

The rather small changes in radii of gyration R_g (Figure 2E) indicate that protein size is steadily maintained over the simulation time, particularly for the three dimers that show small standard deviations about 0.1 Å, half those of monomer and tetramer (Table 2). While "dimer" and "dimer SSbridge" show virtually indistinguishable R_g values along the simulation time, "dimer (4AUD)" has a slightly greater size which in turn could indicate a slightly weaker interaction between monomers. This is confirmed by interface data in Table 1. Whereas "dimer" and "dimerSSbridge"

Table 1. Interface areas (Å²), number of residues, hydrogen bonds (HB) and salt bridges (SB) in protein-protein interfaces of dimers of Alt a 1, Bet v 1, and Pru p 3 allergen proteins at three frames of MD 20-ns simulations.

Dimer	Initial structure, time = 0				Middle structure, time = 10 ns				Final structure, time = 20 ns			
	Area	Residues[a]	HB	SB	Area	Residues[a]	HB	SB	Area	Residues[a]	HB	SB
Alt a 1 dimer	1361	39 (8c 12p 19np)	9	0	1590	45 (10c 14p 21np)	14	0	1333	41 (8c 13p 20np)	10	0
Alt a 1 dimer SSbridge	1361	39 (8c 12p 19np)	8	0	1427	41 (9c 12p 20np)	12	0	1393	41 (10c 11p 20np)	9	0
Alt a 1 dimer 4AUD	770.3	24 (5c 7p 12np)	1	0	852.0	28 (6c 8p 14np)	3	6	914.8	30 (7c 8p 15np)	4	8
Bet v 1 dimer1 Y5	822.0	27 (10c 7p 10np)	3	0	417.9	17 (7c 4p 6np)	4	0	312.2	16 (6c 3p 7np)	1	2
Bet v 1 dimer2 Y5	443.2	12 (6c 2p 4np)	3	1	388.1	12 (6c 2p 4np)	5	2	394.7	11 (4c 3p 4np)	5	2
Bet v 1 dimer C5	510.2	17 (8c 3p 6np)	6	2	650.9	18 (9c 2p 7np)	7	2	683.5	22 (12c 3p 7np)	6	3
Pru p 3 dimer1	453.8	16 (3c 7p 6np)	10	0	484.7	17 (3c 7p 7np)	13	0	469.1	17 (3c 7p 7np)	10	0
Pru p 3 dimer1 PLM	453.8	17 (3c 7p 7np)	10	0	432.5	19 (3c 7p 9np)	12	0	494.8	18 (3c 7p 8np)	12	0
Pru p 3 dimer2	330.6	11 (1c 4p 6np)	3	0	389.3	14 (1c 3p 10np)	2	0	371.8	13 (1c 4p 8np)	5	0
Pru p 3 dimer2 PLM	330.6	11 (1c 4p 6np)	3	0	114.2	8 (2c 2p 4np)	0	0	141.7	8 (3c 1p 4np)	0	4

[a] Number of: c = charged, p = polar, np = nonpolar residues in parentheses

Figure 1. RMSF per residue of α carbons obtained in 20 ns MD simulations for oligomer states of (A) Alt a 1, (B) Bet v 1, and (C) Pru p 3 proteins.

have rather similar interface areas, numbers of interacting residues, and intermonomer hydrogen bonds, "dimer (4AUD)" shows much smaller values of these properties. However, the latter dimer presents a high number of salt bridges. The lack of six N-terminal residues in the 4AUD variant allows for a close proximity of two acidic (E35 and D37, 3 VOR numbering) and one basic (R103) residues in one chain and the counterpart residues in the other chain which permits formation of salt bridges (see Table S1 in the Supporting Information). As a consequence, interface area

and number of residues making contacts in the interface increase in "dimer (4AUD)" along the MD simulation although they remain below those of "dimer" or "dimerSS-bridge" (Table 1).

Unlike other proteins with a β -barrel fold or other major allergens such as Bet v 1 or Pru p 3, Alt a 1 lacks a clear binding cavity as its β -barrel core seems too narrow to harbor ligands. It has been speculated that the binding of a ligand might induce conformational changes resulting in the opening of the β -barrel or that in solution Alt a 1 had

Figure 2. Oligomer states of Alt a 1 protein. (A) “Dimer”: dimer of protein with PDB code 3 V0R. C30 and epitope residues shown as sticks. (B) Monomer with residues in the inner side of the β -barrel defining distances d1 (F45–F99), d2 (F60–L106), and d3 (I62–L108) shown as green sticks. (C) “Dimer (4AUD)”: dimer of protein with PDB code 4AUD. Epitope residues shown as sticks. (D) Tetramer of protein 3 V0R. C30 residues shown as sticks. (E–H) Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1, d2, and d3 inside the β -barrel in the monomer. (G) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Alt a 1 monomer and dimer. (H) *RMSD* of non-hydrogen atoms of C30 in monomer and dimers without (blue) and with (red) intermolecular disulfide bridge between C30 residues.

an internal cavity.^[10] We explored these possibilities by computing three distances within the β -barrel (two at the ends and one at the center, Figure 2B) along the MD simu-

lations. These interatomic distances show consistent values among aggregation states (Table 2). End distance d1 is between 4.1 and 4.9 Å with standard deviations ~ 0.5 Å, cen-

Table 2. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Alt a 1 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	<i>RMSD</i>			Interatomic distances inside the barrel [a]			
	Protein	Epitope [b]	C30 residue [c]	d1	d2	d3	R_g [d]
Monomer	1.880 (0.259)	0.987 (0.146)	6.620 (1.676)	4.136 (0.463)	4.156 (0.447)	5.125 (1.024)	15.56 (0.211)
Dimer chain A	1.317 (0.134)	0.984 (0.162)	2.748 (0.932)	4.351 (0.481)	3.978 (0.324)	4.563 (0.644)	19.39 (0.096)
Dimer chain B	1.265 (0.165)	0.880 (0.155)	2.676 (0.817)	4.065 (0.446)	3.853 (0.337)	4.769 (0.716)	
Dimer SSbr ch A	1.272 (0.145)	0.978 (0.200)	1.469 (0.508)	4.327 (0.473)	3.839 (0.303)	4.756 (1.028)	19.37 (0.095)
Dimer SSbr ch B	1.360 (0.153)	0.963 (0.153)	1.422 (0.483)	4.395 (0.444)	3.941 (0.315)	4.579 (0.769)	
Dimer (4AUD) ch A	1.470 (0.141)	1.069 (0.227)	–	4.815 (0.714)	4.101 (0.367)	3.894 (0.360)	19.86 (0.110)
Dimer (4AUD) ch B	1.314 (0.140)	1.040 (0.177)	–	4.905 (0.686)	4.193 (0.393)	4.126 (0.429)	
Tetramer chain A	1.964 (0.301)	–	6.112 (1.811)	4.187 (0.482)	3.957 (0.349)	5.067 (1.085)	25.49 (0.258)
Tetramer chain B	1.545 (0.278)	–	5.192 (1.867)	4.067 (0.445)	3.855 (0.294)	4.687 (0.799)	
Tetramer chain C	1.672 (0.381)	–	5.096 (2.134)	4.253 (0.432)	3.891 (0.297)	4.625 (0.694)	
Tetramer chain D	1.842 (0.351)	–	5.058 (2.294)	4.076 (0.469)	3.773 (0.256)	4.539 (0.539)	

[a] d1 = Cε1(F45)–Cζ(F99) distance, d2 = Cζ(F60)–Cδ1(L106) distance, d3 = Cδ(I62)–Cδ2(L108) distance: see Figure 2B. [b] Epitope *RMSD* not computed for the tetramer. [c] Alt a 1 protein with PDB code 4AUD lacks this residue. [d] Values refer to the whole molecular assembly.

tral distance d2 is between 3.8 and 4.2 Å with standard deviations ~0.3 Å, and end distance d3 is between 3.9 and 5.1 Å with greater standard deviations between 0.5 and 1.1 Å. As indicated by the latter deviations, d3 has much larger oscillations than d1 or d2 (Figure 2F), which suggests a greater opening of the β-barrel end corresponding to the I62–L108 pair at the cup-like region of the protein (Figure 2B). However, the overall magnitude of these distances (Table 2) along simulation time suggests that it is unlikely that in solution the cavity inside the β-barrel could harbor a ligand in any of the molecular aggregates analyzed.

Compared to other proteins, allergens are highly structured^[4–6] and it is also known that in contrast to IgG, IgE binds mainly to structured proteins^[4]. Therefore, one would expect IgE epitopes to locate in structured regions of allergens. Epitope location in β-strands^[10,36] (Figure 2) and our MD results show that this is indeed the case for Alt a 1. Average values of root means square deviations (*RMSDs*) and their standard deviations (Table 2) reveal a consistently smaller motion of epitope regions than the proteins as a whole along simulation time (Figure 2G). This is even more apparent in the monomer where the epitope region has an average *RMSD* about half that of the protein (Table 2, Figure 2G). The smaller *RMSD* values of epitope regions with respect to protein also holds for the dimer formed by the variant (“dimer (4AUD)” data in Table 2). The IgE epitope identified in Alt a 1 spans the sequence segments 41–50 and 54–63.^[36] The *RMSF* per residue plot (Figure 1A) shows that the regions covered by these residues display small fluctuations particularly in “dimer” and “dimerSSbridge” which would represent the preferred state for IgE cross-linking. The four regions showing large *RMSF* peaks in these dimers (sequence segments 64–70, 90–94, 112–116, and 131–136, Figure 1A) correspond to loops joining adjacent β strands that locate far from the protein-protein interface, specially the 131–136 region which is the farthest loop from the interface (horizontal extreme ends in Figure 2A). In contrast, all residues in tetrameric Alt

a 1 show overall larger fluctuations that indicate a greater mobility with respect to the remaining aggregation states. This is in agreement with the behavior revealed by the greater *RMSD* values (Table 2) and the larger oscillations of R_g in the tetramer (Figure 2E). Nevertheless, considering even the peaks associated to mobile loops in dimers or tetramers, the small *RMSF* values (Figure 1A) reveal a significant structural stability for the aggregation states of Alt a 1.

On the other side, the overall smaller *RMSD* values in dimers than in monomer or tetramer (Table 2) indicate that dimers are significantly more rigid in solution. Interestingly, the dimer with the disulfide bond between Cys30 residues (“dimer SSbr” data) is not more rigid than the dimer without this linkage (“dimer” data) a result that should confirm the earlier suggestion that Alt a 1 dimerization is not mediated by the disulfide bond.^[7] Even though Cys30 shows an obviously greater motion in the dimer without its disulfide bond (*RMSD* ~2.7 Å) than in the dimer with it (*RMSD* ~1.4 Å), this residue is much more flexible in monomer (*RMSD* = 6.6 Å) or in tetramer (*RMSD* > 5.1 Å) states, a result that indicates a far greater conformational freedom of the N-terminal tail in monomer or tetramer than in dimers (Figs. 2A–2D): the oscillations of *RMSD* for Cys30 are much greater in the monomer than in the dimers regardless disulfide bond formation (Figure 2H).

3.2 Bet v 1 Allergen

Although birch pollen Bet v 1 is probably the best characterized allergen as it has been used as a model system in research on allergy, the allergenic significance of its dimerization has been controversial (see reviews in References^[12,13]). Very recently, a combination of crystallographic, mass spectrometric, and immunological methods has provided evidence on dimeric Bet v 1 having a non-canonical incorporation of cysteine at position 5 instead of genetically encoded tyrosine.^[13] In addition, a crystal structure for a variant with phenylalanine at position 5 was also report-

Figure 3. Dimers and monomer of Bet v 1 protein. (A) "Dimer1 Y5": crystallographic dimer of protein with PDB code 1BV1. Y5 and epitope residues shown as sticks. (B) "Dimer2 Y5": same as (A) for the EPPIC-predicted dimer. (C) "Dimer C5": crystallographic dimer of protein with PDB code 4BK6. C5 and epitope residues shown as sticks. (D) "Monomer": canonical monomer with residues inside the cavity defining distances d1 (F22–F30) and d2 (F64–Q132) shown as green sticks. (E–H). Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1 and d2 inside the cavity in the monomer. (G) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Bet v 1 monomer and dimers. (H) *RMSD* of non-hydrogen atoms of Y5.

ed. Based upon a number of experimental results, the authors proposed that residue 5 should play a relevant role in

the allergenic properties of this protein.^[13] Figure 3 displays initial geometries for two dimers of canonical Bet v 1 pro-

tein (PDB code 1BV1), the crystallographic one ("dimer1 Y5" in what follows, Figure 3A) and that predicted by EPPIC^[8] on evolutionary data grounds ("dimer2 Y5", Figure 3B), the non-canonical dimer ("dimer C5", PDB code 4BK6^[13], Figure 3C) and the canonical monomer ("monomer", Figure 3D). Residues corresponding to the reported IgE epitope of Bet v 1^[25] are located near the protein-protein interface in "dimer1 Y5" whereas they are properly located for cross-linking of IgE antibodies in both "dimer2 Y5" and "dimer C5" (Figures 3A–3C). MD results for these dimers together with "monomer" and non-canonical monomers with cysteine ("monomer C5") and phenylalanine ("monomer F5") at position 5 are presented in Table 3 and Figures 3E–3H.

Changes in radii of gyration R_g for the three monomers are indistinguishable (average $R_g = 15.7$ Å with standard deviations below 0.1 Å) but clear differences are found for the three dimers, not in their average R_g values between 22.7 and 22.9 Å but in their oscillations (Figure 3E) reflected into their large standard deviations (Table 3). Only non-canonical "dimer C5" shows a steady behavior while both "dimer1 Y5" and "dimer2 Y5" display large oscillations of R_g that suggest floppy structures, particularly "dimer1 Y5" which has a standard deviation ~ 1 Å. These results point to a stronger inter-monomer attraction in the structure of "dimer C5" than in both "dimer1 Y5" and "dimer2 Y5" structures. Interface data in Table 1 confirm this point. Although the initial structure of the crystallographic "dimer1 Y5" has a greater interface area than "dimer2 Y5" and "dimer C5", this latter dimer increases its interface area and number of interacting residues, hydrogens bonds and salt bridges as the simulation progresses (Table 1). Motion in solution brings together side chains of C5 residues in the two protein chains and "dimer C5" becomes linked by an intermolecular disulfide bridge that forms at about 5 ns (Figure 3E). In contrast, many intermolecular contacts are broken along the simulation in the crystallographic "dimer1 Y5" and the interface area at the final structure is only 38% of the area at the initial structure (Table 1). The EPPIC-predicted "dimer2 Y5" shows a proper location for epitope residues

but it has a geometry with a smaller protein-protein interface (Figure 3B) which in turn involves less intermolecular contacts than in crystallographic dimers. However, the formation of five hydrogen bonds and two salt bridges (Table 1 and Table S1 in the Supporting Information) along the simulation gives an increased stability to "dimer2 Y5" with respect to "dimer1 Y5".

Bet v 1 has a huge hydrophobic cavity that runs parallel to the large α -helix (Figure 3D). This protein is known to bind a great diversity of ligands in vitro such as flavonoids and fatty acids^[37,38], and quercetin-3-O-sophoroside has been recently identified as its natural ligand.^[12] With the aim to assess the flexibility of this pocket in solution, we computed two interatomic distances at both ends of the cavity (Figure 3D) along the MD simulation (Figure 3F). The four residues chosen to monitor both distances are involved in binding different ligands within the hydrophobic pocket.^[38] The innermost distance d1 corresponding to the F22-F30 pair has values between 4.8 and 5.4 Å with standard deviations between 0.4 and 0.8 Å. The outermost distance d2 corresponding to the F64-Q132 pair shows much greater values between 7.0 and 8.1 Å and larger standard deviations between 1.3 and 2.2 Å (Table 3). While d1 remains steady over simulation time, d2 shows large oscillations (Figure 3F) suggesting a greater flexibility in the outer end of the cavity. In agreement with available evidence, both distances show values compatible with binding ligands of moderate-large size.^[37,38] However, our MD results suggest that an end of the ligands should remain anchored at the narrower inner end of the cavity (d1 distance) while being permitted by the protein structure to move at the opposite end (d2 distance). It must be noted that F30 has been identified as a key residue in the coordination of distinct ligands.^[38] Our results also show that cavity properties in the dimers are comparable to those of monomeric forms (Table 3).

In agreement with that anticipated by R_g values and confirmed by *RMSF* plots (Figure 1B), the low average *RMSDs* ~ 1 Å and their small standard deviations ~ 0.1 Å reveal a very low mobility in the three monomers. Residues in

Table 3. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Bet v 1 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	RMSD			Interatomic distances inside cavity [a]		
	Protein	Epitope	Residue 5	d1	d2	Rg[b]
Monomer	1.103 (0.120)	0.991 (0.185)	0.964 (0.349)	4.967 (0.594)	7.005 (1.735)	15.73 (0.060)
Monomer C5	1.036 (0.120)	1.190 (0.179)	0.908 (0.346)	4.767 (0.422)	7.055 (1.846)	15.66 (0.055)
Monomer F5	1.103 (0.106)	1.138 (0.207)	1.336 (0.840)	5.033 (0.526)	7.817 (1.760)	15.68 (0.078)
Dimer1 Y5 chain A	3.237 (1.032)	3.809 (1.490)	2.439 (1.007)	5.268 (0.775)	8.114 (1.841)	22.94 (0.887)
Dimer1 Y5 chain B	2.790 (0.932)	2.973 (0.960)	2.240 (0.838)	4.881 (0.622)	7.416 (1.819)	
Dimer2 Y5 chain A	1.736 (0.395)	1.792 (0.477)	1.199 (0.432)	5.386 (0.703)	7.202 (2.120)	22.80 (0.437)
Dimer2 Y5 chain B	1.646 (0.405)	1.760 (0.436)	1.176 (0.453)	4.907 (0.473)	8.030 (1.683)	
Dimer C5 chain A	1.702 (0.446)	1.733 (0.504)	0.963 (0.462)	4.780 (0.531)	7.645 (1.264)	22.66 (0.279)
Dimer C5 chain B	1.872 (0.476)	1.993 (0.525)	1.048 (0.409)	5.067 (0.551)	8.054 (2.217)	

[a] d1 = C ζ (F22)–C ζ (F30) distance, d2 = C ζ (F64)–O ϵ 1(Q132) distance: see Figure 3D. [b] Values refer to the whole molecular assembly.

them exhibit *RMSF* values below 1 Å with the exception of sequence segments 59–64, 90–93, and 107–109 that correspond to loops joining adjacent β strands. In the dimers, these loops happen to locate at positions far from the dimerization interface (loops at vertical extreme ends in Figures 3A–3C) and hence they show also *RMSF* peaks in the dimers though with far greater values than in monomers (Figure 1B). The main IgE epitope identified in Bet v 1 is composed of residues 42–52 plus 70, 72, 76, 86, and 97.^[25] In contrast to Alt a 1, *RMSD* values for epitope residues are now similar to those of whole proteins although with slightly greater standard deviations ~ 0.2 Å (Table 3). A common feature is that epitope residues are located in regions with low structural fluctuations (Figure 1B). Both *RMSD* changes (Figure 3G) and *RMSF* values (Figure 1B) reveal a greater structural mobility for the crystallographic “dimer1 Y5” and much lower for the EPPIC-predicted “dimer2 Y5” in agreement with the discussion on protein-protein interfaces above. Interestingly, although some epitope residues locate on loop regions (Figure 3B and 3C), the dimers with proper orientation of epitopes (“dimer2 Y5” and “dimer C5”) have average *RMSDs* similar for proteins and epitopes (Table 3): “dimer2” and “epitope dimer2” plots in Figure 3G are indistinguishable. On the contrary, the dimer with epitopes located near the protein-protein interface (“dimer1 Y5”) has greater *RMSDs* and larger oscillations of epitope residues (Figure 3G). Given the location of these residues in this dimer and its evolution along the simulation discussed above with regard to protein-protein interfaces, this is another result associated to the greater instability of “dimer1 Y5”.

Finally, residue at position 5 in monomers has different features when it is tyrosine or cysteine (*RMSD* < 1.0 Å, 0.35 Å standard deviation, Table 3) and when it is phenylalanine (*RMSD* = 1.34 Å, 0.84 Å standard deviation). In dimers with properly oriented epitopes (“dimer2 Y5” and “dimer C5”), the residue at position 5 locates at the protein-protein interface (Figure 3B and 3C) and it therefore moves with less flexibility (smaller *RMSD* values, Table 3) than in “dimer1 Y5” where residue 5 locates at outer sides of the structure (Figure 3A). As a consequence, residue 5 has far greater oscillations (Figure 3H) and greater *RMSF* values (Figure 1B) in “dimer1” than in “dimer2”. On the basis of these MD results, it could be conjectured that the presumed allergenic role of residue 5^[13] might involve stabilization of dimeric structures that allow for IgE cross-linking. This should be supported by the smaller mobility suggested by *RMSD* data of this residue as compared to that of proteins as a whole (Table 3).

3.3 Pru p 3 Allergen

Major peach allergen, Pru p 3, has been used as a model in food allergy^[15,39] and is a prototypical member of nsLTPs, proteins widely distributed in plants with a particularly stable fold and a large hydrophobic tunnel with open ends

at opposite sides of the surface able to harbor a great variety of lipidic ligands.^[18,40] The allergenic properties of Pru p 3 have been thoroughly studied^[14–17] and its IgE epitopes have been characterized by using different techniques.^[14] Figure 4 displays initial geometries of dimeric crystal structure of Pru p 3^[17] (“dimer1” in what follows) that has epitope residues at a location not suitable for IgE cross-linking (Figure 4A), a dimer predicted by EPPIC^[8] (“dimer2” with the proper orientation of epitope residues (Figure 4C), the monomer (Figure 4B), and the protein-palmitate complex (Figure 4D). MD results for monomer, “dimer1”, and “dimer2” together with their corresponding complexes with palmitate (PLM) chosen as a representative ligand of nsLTPs are presented in Table 4 and Figures 4E–4J.

Changes in radii of gyration R_g (Figure 4E and Table 4) and *RMSF* values (Figure 1C) are indistinguishable for the monomer irrespective of the presence of PLM. For “dimer1”, R_g results with and without palmitate are also similar but the standard deviations are slightly smaller in the presence of the ligand and in fact, *RMSF* values show that the complex has smaller fluctuations in this dimer. On the contrary, while uncomplexed “dimer2” present R_g changes and *RMSF* per residue values not very different from “dimer1”, the presence of palmitate yields a larger size and far greater oscillations: compare “dimer2” and “dimer2 PLM” plots in Figure 1C and in Figure 4E or the corresponding average values in Table 4. These results point to a destabilizing effect of the ligand on the geometry of “dimer2”. Protein-protein interface data (Table 1) show that whereas interface areas, number of residues making contacts and hydrogen bonds in the interface of “dimer1” are very similar with and without the ligand, the presence of palmitate in “dimer2” gives place to far smaller interface areas and less interacting residues as the simulation progresses. Moreover, while a few more contacts and slightly greater interface areas occur in uncomplexed “dimer2” along the simulation, just the opposite happens in the presence of the ligand. The geometry of “dimer2” predicted by EPPIC on evolutionary grounds has a weak intermolecular attraction noticed in the small interface area and the low number of interacting residues in the initial structure (Table 1) that turns out even weaker with palmitate. Since no particular feature is seen in the structure of this system that could explain this effect, it seems just a consequence of the increased motion produced by the presence of a flexible ligand (see next paragraph) in an otherwise weak protein-protein aggregate. The large hydrophobic cavity (tunnel) is one of the main features of nsLTPs.^[18,40] This tunnel runs parallel to the larger α -helix and is flanked by the unstructured C-terminal tail (Figure 4). In order to assess its flexibility, we computed along the MD simulation two distances between residues that are involved in the interaction with palmitate and have besides their side chains at opposite locations at both ends of the cavity (Figure 4B and 4D). Depending on the structure considered, the outermost distance d1 between

Table 4. Average values of *RMSD* of all non-hydrogen atoms, selected interatomic distances, and radius of gyration (R_g) for different assemblies of Pru p 3 allergen protein obtained in MD 20 ns simulations. Standard deviations in parentheses. All values in Å.

	<i>RMSD</i>			Interatomic distances inside the tunnel[a]		
	Protein	Epitope	PLM	d1	d2	R_g [b]
Monomer	0.974 (0.105)	1.096 (0.216)	–	6.576 (1.498)	4.933 (1.455)	12.03 (0.077)
Dimer1 chain A	1.075 (0.175)	1.392 (0.432)	–	5.213 (0.952)	3.968 (0.611)	16.89 (0.089)
Dimer1 chain B	0.994 (0.145)	1.081 (0.342)	–	5.535 (0.763)	4.472 (1.033)	
Dimer2 chain A	3.128 (1.759)	3.071 (1.719)	–	5.158 (1.025)	3.945 (0.831)	17.15 (0.346)
Dimer2 chain B	2.785 (1.548)	2.688 (1.516)	–	6.057 (1.084)	4.389 (1.217)	
Monomer PLM	1.077 (0.137)	1.245 (0.260)	2.603 (1.278)	8.727 (1.217)	4.764 (1.201)	12.08 (0.074)
Dimer1 PLM ch A	1.147 (0.152)	1.255 (0.213)	2.878 (1.240)	7.311 (1.665)	5.320 (1.717)	17.19 (0.130)
Dimer1 PLM ch B	1.124 (0.236)	1.378 (0.239)	2.661 (1.047)	7.901 (1.174)	6.362 (1.437)	
Dimer2 PLM ch A	5.732 (1.895)	5.763 (1.975)	4.608 (1.124)	6.876 (2.374)	6.519 (2.078)	19.63 (0.819)
Dimer2 PLM ch B	5.635 (1.986)	5.506 (1.955)	4.385 (1.264)	6.443 (2.826)	5.964 (1.101)	

[a] d1 = N δ 2(N35)–C δ 1(I77) distance, d2 = O γ (S55)–C γ 2(I81) distance: see Figure 4B. [b] Values refer to the whole molecular assembly.

N35 and I77 shows values between 5.2 and 6.6 Å in the absence of ligand and between 7.3 and 8.7 Å in the presence of PLM. The innermost distance d2 corresponding to the S55–I81 pair has values between 3.9 and 4.5 Å in the absence of ligand and between 4.8 and 6.5 Å in the presence of PLM (Table 4). Both ends display thus an opening in the presence of ligand though it is noticeably greater at the outer end corresponding to d1. A feature of both distances is their great standard deviations that in turn suggest a great mobility. In fact, plots in Figs. 4F and 4G reveal large oscillations, particularly for the outermost distance d1. In the absence of ligand (Figure 4F), the innermost distance d2 starts with large oscillations but it stabilizes with a sudden decrease at a conformational change of I81 occurring between 9.26 and 9.27 ns (Figure 5) and remains steady afterwards oscillating with small amplitude around a distance below 4 Å. In the presence of ligand (Figure 4G), both distances separate at about 6 ns and remain oscillating steadily around values d1 ~9 Å and d2 ~4.5 Å. Compared with the uncomplexed monomer, these larger values indicate a considerable opening of the outermost end of the tunnel.

RMSD values (Figure 4H and 4I and Table 4) reveal again a completely different behavior of “dimer2” as compared to monomer and “dimer1”. In the absence of PLM (Figure 4H), “dimer2” stabilizes at about 4 ns and remains with large oscillations around *RMSD* ~2 Å. In the presence of ligand (Figure 4I), *RMSD* of “dimer2” displays large oscillations without signs of stabilization. The IgE-epitope is composed of residues 35, 37, 39, 40, 42–44, 70, and 74–79.^[14] *RMSF* plots (Figure 1C) show no particular features for these residues.

Figure 4. Dimers and monomer of Pru p 3 protein. (A) “Dimer1”: crystallographic dimer of protein with PDB code 2ALG. Epitope residues shown as sticks. (B) Monomer protein with residues inside the tunnel defining distances d1(N35–I77) and d2 (S55–I81) shown as green sticks. (C) “Dimer2”: same as (A) for the EPPIC-predicted dimer. (D) Same as (C) with a palmitate molecule shown as cyan sticks inside the tunnel. (E–J). Results of 20 ns MD simulations. (E) Radius of gyration. (F) Interatomic distances d1 and d2 inside the tunnel in the uncomplexed monomer. (G) Same as (F) in the protein-palmitate complex. (H) *RMSD* of non-hydrogen atoms of proteins and epitope regions of Pru p 3 monomer and dimers for uncomplexed systems. (I) Same as (H) for protein-palmitate complexes. (J) *RMSD* of non-hydrogen atoms of palmitate. **Figure 5.** Change of interatomic distance d2 inside the tunnel in uncomplexed Pru p 3 monomer. Decrease in d2=O γ (S55)–C γ 2(I81) distance at the geometry change of side chains occurring between 9.26 ns (green) and 9.27 ns (cyan).

In both “dimer1” and “dimer2”, *RMSD* changes of epitope residues are rather similar to those of the dimers as a whole. In agreement with the discussion above, high average *RMSDs* ~3 Å for uncomplexed “dimer2” and ~5.6 Å for its complex with PLM as well as great standard deviations near 2 Å in both cases reveal that “dimer2” is a clearly unstable structure. In contrast, the monomeric form and

"dimer1" exhibit low *RMSDs* with small standard deviations (Table 4) and a steady behavior over the simulation time which is nearly the same for epitope residues. These features agree with the well-known structural stability characteristic of nsLTPs.^[16] As for the mobility of palmitate (Figure 4J and Table 4), *RMSD* values show no clear stabilization although in both monomer and "dimer1" average values ~ 2.7 Å indicate less flexibility than in "dimer2" where again greater average values ~ 4.5 Å (Table 4) point to an unstable structure.

4 Conclusions

MD simulations aimed at examining the stability in solution of oligomeric states of three major allergen proteins draw the following conclusions.

Alt a 1 forms a rather stable dimer in solution without formation of inter-monomer disulfide linkage between Cys30 residues. The identified epitope of Alt a 1 locates in a structured region with greater rigidity than the dimer as a whole. The narrow cavity inside the β -barrel remains steadily small over the simulation time which makes unlikely that it might harbor any ligand.

Bet v 1 forms dimers in solution with proper orientation of epitopes for IgE cross-linking and similar stability to that of monomer. Even though some epitope residues locate in loop regions, their mobility is comparable to that of proteins as a whole. The large hydrophobic pocket has an inner end with less flexibility than its outer end which shows a large opening, features present in monomeric and dimeric forms. As for residue 5 for which a role in the allergenic properties of Bet v 1 has been proposed, our results point to a stabilizing effect on inter-monomer attraction. MD data suggest a greater stability of the dimer with cysteine instead of genetically encoded tyrosine at position 5.

Pru p 3 in solution forms no stable dimers with proper orientation of epitopes for IgE cross-linking. The hydrophobic tunnel of Pru p 3 shows an inner end less flexible than its outer end which displays a large opening in solution, a feature that holds in the presence of palmitate although in this case, a larger opening is found in the outer end. MD results indicate that the dimer with proper orientation of epitopes is not stable in solution, a feature which also holds in the complex with palmitate. Since the stable crystallographic dimer has epitope residues at a location not suitable for IgE cross-linking our MD study hints at a monomeric form as the more likely with regard to the allergenic properties of Pru p 3.

Summarizing, MD results presented in this work show that Alt a 1, Bet v 1, and Pru p 3, three proteins that play an essential role in allergy disease, have in common (i) very stable monomeric structures, (ii) epitope residues with flexibility lower than or similar to that of proteins as a whole in spite of spanning part of loop regions, and (iii) formation of homodimers. The three allergens differ in (i) the stability of

the dimers presenting epitopes with a proper orientation for IgE cross-linking: great in Alt a 1, moderate in Bet v 1, and extremely low in Pru p 3 and (ii) the flexibility of internal cavities: rigid and small in Alt a 1, rigid at an end and flexible at the opposite end in Bet v 1, and overall flexible in Pru p 3. Three essential allergens are thus shown to display a distinct behavior on their trends to form homodimers in solution.

Competing Interests

The authors declare that no competing interests exist.

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3.3 Computational study of pH-dependent oligomerization and ligand binding in Alt a 1, a highly allergenic protein with a unique fold

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Doctoral candidate's contribution to this work :

Structural analyses of Alt a 1 oligomers and rendering of molecular graphics with PyMOL

Docking calculations of quercetin and alternariol to monomeric and dimeric Alt a 1

Docking calculations of quercetin and alternariol to tetrameric Alt a 1 at different pH's

Experimental assays of quercetin-binding

Experimental assays to settle the pH-dependence of tetramer formation and ligand-binding

Writing of experimental Results and Methods sections of the manuscript

Abstract

Alt a 1 is a highly allergenic protein from *Alternaria* fungi responsible for several respiratory diseases. Its crystal structure revealed a unique β -barrel fold that defines a new family exclusive to fungi and forms a symmetrical dimer in a butterfly-like shape as well as tetramers. Its biological function is as yet unknown but its localization in cell wall of *Alternaria* spores and its interactions in the onset of allergy reactions point to a function to transport ligands. However, at odds with binding features in β -barrel proteins, monomeric Alt a 1 seems unable to harbor ligands because the barrel is too narrow. Tetrameric Alt a 1 is able to bind the flavonoid quercetin, yet the stability of the aggregate and the own ligand binding are pH-dependent. At pH 6.5, which Alt a 1 would meet when secreted by spores in bronchial epithelium, tetramer-quercetin complex is stable. At pH 5.5, which Alt a 1 would meet in apoplast when infecting plants, the complex breaks down. By means of a combined computational study that includes docking calculations, empirical pKa estimates, Poisson-Boltzmann electrostatic potentials, and Molecular Dynamics simulations, we identified a putative binding site at the dimeric interface between subunits in tetramer. We propose an explanation on the pH-dependence of both oligomerization states and protein-ligand affinity of Alt a 1 in terms of electrostatic variations associated to distinct protonation states at different pHs. The uniqueness of this singular protein can thus be tracked in the combination of all these features.

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Keywords

Allergenic proteins · Oligomerization · Protein-ligand binding · Electrostatic potentials · Molecular Dynamics · Ligand-protein docking

Introduction

Alternaria is a genus of common molds that invade crops at pre- and post-harvest stages causing considerable agricultural spoilage due to rotting of fruits and vegetables. *Alternaria* sp. are also associated with respiratory allergies and severe asthma. Their spores can reach levels of thousands per cubic meter of air during spring and summer months becoming a risk factor for respiratory diseases [1-7]. *A. alternata* is the most important member and one of the principal fungal agents associated with allergic disease. In the U.S., its presence is one of the most common causes of respiratory disorders, particularly in children and young adults [4-6]. In Southern Europe, over 20% of patients with a history of respiratory allergy are sensitized to *A. alternata* [7].

Alt a 1 is the major allergen of *A. alternata* [8-11]. It is a highly allergenic protein that leads to IgE-mediated hypersensitivity in more than 90% of patients with *Alternaria* sp. allergy [12]. Alt a 1 was cloned and the expressed recombinant allergen used to measure IgE and IgG antibody responses in *Alternaria*-sensitized patients [8-10]. Four linear epitopes were identified by synthetic overlapping peptides spanning the whole sequence of Alt a 1 and IgE binding was evaluated with sera from patients with *Alternaria*-induced allergy [13]. Two of these epitopes displayed consistent reactivity whereas two other regions showed weak IgE binding [13].

The high-resolution crystal structure of Alt a 1 reveals a unique 11-stranded β -barrel architecture that forms a "butterfly-like" homodimer [14]. Four cysteines form intramolecular disulfide bonds that contribute to the high temperature stability of the protein while an N-terminal cysteine (C30) is able to form an intermolecular disulfide bond that links the two subunits in the dimer [14]. While not a prerequisite for allergenicity, dimerization might enhance the allergenic potential of a protein, as only one type of IgE should be required for cross-linking [15]. As shown below, the spatial location of the main epitopes in the dimeric structure is consistent with a putative cross-link interaction with IgE. The secondary structure elements identified through the recently reported NMR backbone ^1H , ^{15}N , and ^{13}C chemical shift assignment of Alt a 1 in solution are in close agreement with the crystal structure [16]. Because searches with several structural alignment methods predicted rather low similarities, it has been claimed that the Alt a 1 structure is unique and defines a new protein family exclusively found in fungi [14]. As the structural study presented in the current work suggests, that uniqueness could also be tracked in the combination of oligomerization, intermolecular ligand-binding, and pH dependence features of this singular protein.

The biological function of Alt a 1 is still unknown. A search for 3D fragments did not show any functional relationship to enzymatic sites, ligand-binding sites, or DNA-binding templates [14]. Alt a 1 is localized in the cell wall of *Alternaria* sp. spores [17] which access the respiratory tract to mediate allergic reactions. This localization is consistent with a previous suggestion that Alt a 1 would be involved in spore germination [18]. Considering the binding properties known for other β -barrel proteins, a function as a transporter of small ligands could be hypothesized. However, the β -barrel in Alt a 1 seems too narrow to harbor ligands. Although it was speculated that in solution the protein

could feature an internal cavity [14], recent Molecular Dynamics (MD) calculations showed that the dimensions of the β -barrel remain small and fluctuate very little along simulation time [19]. The possibility that higher-order oligomeric assemblies such as tetramers could increase the flexibility of Alt a 1, which in turn should be related with its ligand-binding features was also tentatively suggested without further elaboration [14]. Indeed, we propose here that this seems to be the case.

On the basis of our experimental results showing that Alt a 1 protein binds the flavonoid quercetin and that its aggregation state is pH-dependent, we present here a computational study of Alt a 1 intended to elucidate its possible binding mode. We achieved this by means of docking calculations, computation of Poisson-Boltzmann electrostatic potentials, empirical pKa predictions, and MD simulations for distinct protein assemblies. By taking in consideration protein-quercetin complexes at different pH values, we suggest a possible binding mode of Alt a 1 and provide detailed insight into the pH dependence of both oligomerization states and ligand-binding abilities. Based upon all these results, we finally propose a picture on the possible protein stages which could be relevant to investigate physiological interactions of Alt a 1 involved in the onset of allergic reactions.

Results and Discussion

Quaternary structure of Alt a 1 suggests a putative binding site in the tetrameric assembly

The crystal structure of Alt a 1 include residues 28 to 157 traced into electron density [14]. Searches for related topologies using PTGL provided no match for Alt a 1 architecture. This unique topology features an 11-stranded β -barrel fold with one 3-residue 3_{10} helix (Fig. 1). Four cysteines forming intramolecular C74-C89 and C128-C140 disulfide bridges are conserved among all sequences of Alt a 1 homologs whereas a fifth N-terminal cysteine C30 is only conserved among the closest homologs [14]. Three residues (Y46, D114, and V139) have 0.50 occupancy in electron density map thus indicating two alternative conformations. While both conformations are similar in V139, they differ noticeably by torsion at C α position in D114 and particularly in Y46 (Fig. 1). As shown below, one of the two side chain orientations of this tyrosine is involved in ligand-binding.

PISA predicts two quaternary structure aggregates: a tetramer with total buried area 11290 Å² and solvation free energy change upon assembly of -186 kcal mol⁻¹ (Fig. 2a) and a dimer in a butterfly-like shape with total buried area 4870 Å² and solvation free energy change of -106 kcal mol⁻¹ (Fig. 2b). Some small compounds originated from the crystallization solution are present in the crystal structure. Although one would consider it unlikely that their spatial locations arising from symmetry operations that set oligomerization states could bear physiological significance, the location of two molecules of one of these compounds (4-hydroxy-2,5,6-triaminopyrimidine, Fig. 2c) is illuminating. They are located in the interface between two pairs of subunits in the middle of a barrel-like substructure made of β -strands from two different chains in tetramer (Fig. 2d). Moreover, this location displays a β -stacking geometry with two neighbor side chain rings of Y46 residues from different

chains just in only one of their two possible conformations (B conformation in the crystal structure file). Taken together, these features led us to tentatively assume that this inter-monomer barrel could be a putative binding site of Alt a 1 in its tetrameric state.

An intermolecular disulfide bridge is needed to stabilize dimeric Alt a 1

While its geometry clearly suggests that the butterfly-like dimer is linked by an intermolecular disulfide bond between C30 residues in the two chains (Fig. 2b) [14], evidence that Alt a 1 dimerization is not mediated by that link has been also reported [15]. As said above, it should be further considered that C30 is only conserved among the closest homologs of Alt a 1 [14]. To investigate the role played by C30 on the dimer stability in solution, we performed 100-ns MD simulations on the dimeric structure with and without that intermolecular disulfide bridge. Exploratory MD calculations during 20-ns simulation times had previously shown no other difference than larger RMSD values of backbone atoms that increased with time in the dimer without disulfide link [19]. However, by extending simulation time to 100 ns, we found here a significant increase in backbone RMSD values at ~50 ns in the dimer without disulfide link that finally breaks down at $t = 71$ ns (Fig. 3a). Interestingly, the structural regions corresponding to the two main IgE epitopes, segments 41-50 and 54-63 [13] that span strands $\beta 1$ and $\beta 2$ together with loop $\beta 1\beta 2$ (Figs. 1b and 2b), show much smaller motion (Fig. 3b: compare scale with Fig. 3a). Furthermore, the intermolecular disulfide bond has no apparent effect on the mobility of epitope regions even at the time immediately before breaking (Fig. 3b). When time fluctuation (RMSF) of residues are compared, the absence of the intermolecular link reflects into overall greater fluctuations but RMSF values of epitope residues are consistently low in the presence and in the absence of the disulfide bridge (Fig. 3c). One could speculate that local rigidity of epitope structural regions should be beneficial for cross-linking IgE interaction.

Docking calculations predict a high-affinity tetrameric Alt a 1-quercetin complex

In the course of our preliminary work to identify the natural ligand of Alt a 1, our experimental results (to be published) show that at pH = 6.5 Alt a 1 tetramerizes in the presence of quercetin while at pH 5.0-5.5 the assembly apparently breaks down. pH = 6.5 is the value found in culture supernatant of bronchial epithelium [45] and hence it represents the expected acidity which Alt a 1 would meet with when it is secreted by *Alternaria* spores in bronchial epithelium. pH = 5.0-5.5 is the value in the apoplast of ripe fruit [46] in which Alt a 1 would enter when infecting plant cells. It is worth noting that similar pH-dependence of both aggregation states and binding abilities are known for other proteins. It is the case of transthyretin, an amyloidogenic protein for which a crystal structure corresponding to the tetrameric form of a complex just with quercetin has been recently reported [47]. This tetramer structure shows two binding sites at inter-subunit interfaces and disrupts at pH 4.6 [48].

Based on the mentioned quercetin-binding evidence, 3D structures of Alt a 1-quercetin complexes were explored *in silico* by means of docking calculations. We firstly selected as search

space a large 80 Å edge cube centered at the center of the tetramer enclosing the whole assembly structure and performed AutoDock Vina calculations to dock quercetin. The two best docking complex geometries (poses with lowest binding affinity free energies of -9.2 and -8.8 kcal/mol) proved to correspond to the two inter-chain sites in which two molecules of the crystallographic ligand are present in the PISA-predicted tetrameric structure (Fig. 2a). With this result, we then selected as search space a 20 Å edge cube centered at the middle point between aromatic rings of Y46 residues in neighbor chains (Fig. 2d) enclosing this site and performed new calculations to dock both quercetin and alternariol (Fig. 4a). *Alternaria* species produce over 70 secondary metabolites toxic to plants and some of them have been reported to be also toxic to humans and animals. Alternariol and alternariol methyl ether are the two most important mycotoxins produced by these fungi [49,50].

Vina docking method allows for ligand flexibility [33]. While quercetin has freedom of rotation about the bond between 1-benzopyran-4-one and catechol rings, alternariol is a conjugate molecule (Fig. 5a) rigid in docking calculations although it is known to distort slightly from planarity upon intermolecular hydrogen bonding of OH groups [51]. Quercetin docked to tetrameric Alt a 1 has its catechol ring rotated a torsion angle of 67° with respect to benzopyran ring while docked alternariol remains planar (Fig. 4b). Most stable docked complexes predicted by Vina for this tetramer confirmed the binding site at the center of the inter-monomer barrel-like substructure identified above (Fig. 2). Y46 residues in conformation B from the two chains flank the site displaying a π -stacking arrangement with both ligands (Fig. 4c). Nine residues from each chain are within 4.5 Å neighborhood of quercetin which is also hold by three hydrogen bonds: two of them link both catechol hydroxyls to Nδ2 and Oδ1 atoms of Asn 61 in chain A and the third one links carbonyl oxygen of quercetin to Oε1 atom of Gln 141 in chain A (Fig. S1 in Supplementary Material). Superposition of quercetin and alternariol complexes showed nearly coincident locations for both ligands (Fig. 4b, 4c, and 4d). Best docking modes of quercetin and alternariol showed affinity free energies of -9.3 and -9.5 kcal/mol, respectively. Taking these values as estimates of protein-ligand binding free energies would predict complex dissociation constants 0.13 and 0.093 μM, respectively. Separate dockings for the two possible sites in the tetramer gave exactly the same results. These two sites are symmetry-related by a rotation about an arbitrary axis joining their centers by 90° (Fig. 4d).

Docking of quercetin to monomeric Alt a 1 was also explored. To this end, we firstly selected as search space a large 50 Å edge cube centered at the center of the β -barrel enclosing the whole structure. The two best docking geometries (poses with lowest affinity free energies of -6.5 and -5.8 kcal/mol: Fig. S2 in Supplementary Material) corresponded approximately to the spatial locations of two molecules of the crystallographic ligand present in the monomer crystal structure (PDB id. 3V0R [14]). Whereas neither of these two poses lie inside the β -barrel (Fig. S2 in Supplementary Material), that of lower energy displays quercetin close to Y46 at a spatial location which corresponds to the barrel-like inter-chain site when monomers aggregate to form the tetramer (Fig. 2d and 4c).

Additional docking calculations with the butterfly-like dimer and search spaces defined by a 50x50x80 Å box enclosing the whole structure found similar sites, neither of which are near the protein-protein interface in the dimer. Comparing docking affinity energies, structural features of binding sites, and protein-ligand interactions in the different oligomeric Alt a 1-quercetin complexes it seems apparent that the inter-chain barrel-like site in the tetrameric assembly (Fig. 4) is most favorable for binding.

Predicted pKa shifts show a different pH-dependent pattern for different aggregation states of Alt a 1

pKa values of all residues with ionizable side chain groups were obtained with the empirical predictor Propka 3.1 [34,35] for the three aggregation states of Alt a 1 (Table 1). The implementation in Propka 3.1 of new algorithms for modeling noncovalently coupled titrational events allow to predict the effect on pKa values of the spatial proximity of titratable groups that can influence the titration of each other. This improved version of the pKa predictor takes thus account on coupling effects between amino acid side chains and also incorporates effects between ligands and interacting residues [35].

Alt a 1 has a total of 38 titratable amino acids (arranged in ascending order of standard pKa's in water): 11 aspartates, 5 glutamates, 1 histidine, 1 cysteine not involved in intramolecular disulfide bonds, 8 tyrosines, 9 lysines, and 3 arginines (Table 1). At neutral pH (unprotonated histidine) this yields a total charge -4 for Alt a 1 protein. Among these 38 residues, 12 in tetramer, 8 in dimer, and 4 in monomer display pKa shifts from model values about 1.0 pKa unit or greater (they are highlighted in Table 1). Most of these residues are located at interfaces between subunits in the butterfly-like dimer and more prominently in the tetramer. One might therefore conjecture that changes of protonation states produced by pH variations could significantly affect oligomer stability. A remarkable exception to this location is the case of R48 and Y55 in the dimer that have their side chains highly exposed to the environment (Fig. S3 in Supplementary Material). If one considers that these two residues are within the 41-50 and 54-63 epitope regions proposed for Alt a 1 [13], it is tempting to speculate that R48 and Y55 could play a prominent role in the interaction with IgE. Other points regarding these pKa shifts ≥ 1.0 units are worth to note (Table 1): 2 out of the 3 residues with shifts ≥ 2.0 in tetramer (E44 and Y46) are at or near the putative binding site in the inter-subunit interface (Fig. S3b); Y46, which is here proposed to play a major role in protein-ligand interaction, has shifted pKa values only in tetramer; C30, which forms the intermolecular disulfide bond that stabilizes dimeric Alt a 1 as mentioned above, has shifted pKa values only in the dimer; R48, which is speculated to be involved in IgE interaction, has identical shifted pKa values in the three aggregation states.

As said, two ionizable groups at nearby spatial positions can influence titration of each other so that they titrate coupled. Propka 3.1 models such noncovalently coupled events by predicting pKa values in both of the two possible titrations thus yielding alternative protonation states [35]. In these cases, the method identifies which residues are interacting through backbone or/and side chain hydrogen bonding and Coulombic interaction. Sets of residues found to have alternative protonation

states in aggregation states of Alt a 1 were: E44 and D137 only in tetramer, C30 only in dimer, and both Y38 and Y118 in the three states (Table 2). Since pKa's were computed without imposing intermolecular disulfide bridge in the dimer, the alternative pKa values of C30 in the two chains just reflect the close proximity of SH groups which form the disulfide link. Coupled alternative pKa's arising from vicinity of Y38 and Y118 in monomer (Fig. S4 in Supplementary Material) also reveal their mutual interaction. Alternative pKa's for E44 and D137 in tetramer arise from their mutual interaction and from interactions (Coulombic and hydrogen bonding as well: data not shown) with R48, N133, and D137. Interestingly, all these residues are located at both ends of the barrel-like inter-chain substructure in the tetramer which is here proposed as the putative binding site of Alt a 1 (Fig. 5). As for these results, one might expect that changes in the protonation states of acidic E44 and D137 (occurring at pH 5.5 - 6.5) associated to their interaction with basic R48 (Table 2, Fig. 5) would affect protein-protein interfaces and hence the own tetramer stability.

A major feature of all these pKa predictions is that tetrameric Alt a 1 displays varying protonation states just between pH 5.0 and 6.5, interval of interest to further study the molecular processes that eventually lead to *Alternaria*-induced allergy or respiratory disorders. As a consequence of those different protonation states, the total charge of tetrameric Alt a 1 changes considerably between pH 5.0 and 6.5 whereas that of the dimer changes only 2 units and the total charge of monomeric Alt a 1 remains constant in that pH interval (Table 3).

Poisson-Boltzmann electrostatic potentials of tetrameric Alt a 1 show pH-dependent major variations

Considering the great variations of total charge with pH found in tetrameric Alt a 1 and aimed to explore charge distribution over the protein surface, we computed Poisson-Boltzmann (PB) electrostatic potentials of this aggregate with ionizable side chains at protonation states corresponding to pH 5.5, 6.0, 6.5, and 7.0. The electrostatic nature of the surface shows marked differences (Fig. 6a and 6b). The outer surface exhibits large patches of negative potential that span particularly over the middle hole in the structure at pH 7.0. In contrast, these areas show a much more positive potential at pH 5.5 (Fig. 6a). The inner surface region in the barrel-like inter-chain substructure also exhibits noticeable differences at the putative ligand-binding site. This site is dominated by a strongly negative potential that spans over the whole interface at pH 7.0 whereas it is lined with neutral/positive potential regions at pH 5.5 (Fig. 6b). The 0.0 PB electrostatic potential isosurface divides space around a molecule in negative and positive spatial domains (Fig. 6c). In the case of tetrameric Alt a 1 and despite its intricate shape, this isosurface shows a qualitatively distinct feature at pH 7.0 and 5.5. Whereas at neutral pH the middle hole is largely accessible because the 0.0 isosurface changes locally very close to the structure matching the protein topography, at pH 5.5 the isosurface extends outwards from the structure (Fig. 6c).

Given the large pKa differences noticed above, we performed new docking calculations of quercetin and alternariol to tetrameric Alt a 1 now with the protonation states corresponding to pH 5.5, 6.0, 6.5, and 7.0. Except for the conformation of some hydroxyl hydrogens, geometries of docked quercetin at all those pH values are rather similar (Fig. S5 in Supplementary Material; the same was found for docked alternariol: data not shown). The PB electrostatic potential computed for quercetin molecule mapped onto the molecular surface shows slightly positive dominant character when (Fig. 6d), although it has a smaller range of values than protein (see scale bar in Fig. 6). Thus, in complexes in which the binding site is more negative, i.e. at higher pH's (Fig. 7), one might expect a stronger protein-ligand affinity than at lower pH's. This is in agreement with the aforementioned result that Alt a 1 tetramerizes in the presence of quercetin at pH 6.5 but not at pH 5.5. Finally, it must be noted that the interfaces between subunits in the tetramer leave enough room for access of ligands (Fig. 7).

MD simulations show a pH-dependent pattern for stability of tetrameric Alt a 1-quercetin complex

The stability of tetrameric Alt a 1-quercetin complexes in solution at different pH values was addressed by means of 100-ns MD simulations. A first set of MD calculations were performed for a model dimer composed of two neighbor chains forming the barrel-like binding site (Fig. 4c) taken from tetramer complexes prepared with docking calculations at different pH's as explained above. A second set of MD calculations were performed for the full tetrameric assembly of these complexes with quercetin. Given the considerable computational effort involved in tetramer forms (over 74,000 atoms and 50 million simulation steps), they were restricted to the complexes at the two most representative pH values mentioned above: 5.5 and 6.5. In order to compare the pH-dependence pattern provided by MD simulations for dimer and tetramer forms, the results analyzed here refer to the dimeric interface with quercetin bound in the putative binding site. In what follows, it must be taken into account that all the RMSD values analyzed (Fig. 8), including those of the ligand, were computed along the trajectories upon aligning whole protein backbones, not the ligand.

In the dimer form, RMSD's of protein backbone atoms at both pH's display an overall similar pattern just until the complex at pH 5.5 breaks down sharply at 77.2 ns (Fig. 8a). The ligand exhibits a markedly different behavior. At pH 6.5, RMSD's of non-hydrogen quercetin atoms show values ~ 5 Å during the first 60 ns that decrease down to ~ 3 Å during the next 30 ns ending up again at ~ 5 Å (Fig. 8b). At pH 5.5, quercetin RMSD values are initially very low (< 2 Å) and increase then at 35 ns showing greater variations until this dimer disrupts at 77.2 ns (Fig. 8b).

In the tetramer form, RMSD's of protein backbone atoms at pH 6.5 increase during the first half of the simulation and then stabilize at values ~ 4 Å (Fig. 8c). Note that despite these RMSD's refer now to the whole tetrameric system, their magnitude is similar to those of the dimeric system. At pH 5.5, these RMSD's remain low during nearly the first half of the simulation but they increase rapidly around 50 ns and tetramer complex breaks down at 50.6 ns (Fig. 8c and Fig. S6 in Supplementary Material). The behavior of quercetin predicted by MD simulations for this tetramer form is again

rather different. At pH 6.5, RMSD's of non-hydrogen quercetin atoms increase considerably during the first half of the simulation and then decrease stabilizing at about 5 Å during the second half of the simulation (Fig. 8d). At pH 5.5, quercetin RMSD values are again initially low but they show intense oscillations at 45 ns just before breaking at 50.6 ns (Fig. 8d). Marked oscillations in RMSD plots for quercetin (particularly in tetramer complex) might be viewed as a consequence of the motion of large protein systems in which, besides, the long N-terminal tails (Fig. 4c and 4d) have a great mobility in solution.

It must be stressed that MD simulations for both dimer and tetramer forms agree in predicting system breakdown at pH 5.5 and system stability at pH 6.5. Given the differences in mobility, structure complexity, and aqueous environments involved in dimer and tetramer systems on one side, and the fact that initial structures for the dimeric interface analyzed are the same in both cases (pKa predictions do not change protein structure), this MD result should be associated with different protonation states underlying both pH's. The shorter simulation time at which breakdown occurs in the tetramer with respect to the dimer at pH 5.5 hints at a slightly greater lability of the tetrameric complex. If one recalls that 5.5 is the expected pH that Alt a 1 would meet in the apoplast when infecting plant cells and that 6.5 is a pH typical of bronchial epithelium where the protein would be secreted by *Alternaria* spores, these MD results suggest that tetrameric Alt a 1-quercetin complex should be dynamically unstable in the location of Alt a 1 in infected plants and stable in infected humans. In any event, it is obvious that the complex breakdown process predicted by these MD calculations must be investigated deeper, which is beyond the scope of this computational report on Alt a 1 protein.

Finally, it is noted that protein-ligand nonbonded energies computed from these MD trajectories also reveal the pH-dependence discussed. Plots of electrostatic and van der Waals protein-ligand interaction energies in both dimer and tetramer forms show significant changes just during 5-10 ns before complex breakdown at pH 5.5 whereas they behave steadily at pH 6.5 during that time span (Fig. S7 in Supplementary Material). In the dimer form, both energies are similar during most of the time at both pH's except just in the last 5 ns at pH 5.5 in which the electrostatic term increases sharply even becoming repulsive (Fig. S7a) whereas the vdW term shows no noticeable changes (Fig. S7b). In the tetramer form, the interaction energies differ during most of the time although they show most marked changes during the last 10 ns before breakdown at pH 5.5, time span in which both electrostatic (Fig. S7c) and vdW (Fig. S7d) energies become much less attractive at this pH than at 6.5. As far as these non-bonded protein-ligand energies computed over MD simulations are concerned, these results suggest again that Alt a 1-quercetin complex should be favored at pH 6.5 but not at 5.5. The variation of protein-ligand electrostatic energies along MD simulations is thus in agreement with the discussion on PB electrostatic potential features presented above.

Conclusions

Alt a 1, a highly allergenic protein from *Alternaria* sp. fungi is particularly interesting because of its structural peculiarities [14]. The monomer shows a unique β -barrel fold that is at odds with other proteins featuring this substructure as the barrel seems too narrow to harbor any ligand. Alt a 1 forms a highly symmetric butterfly-like homodimer with a geometry which favors an intermolecular disulfide bond but evidence against this link has been also reported [15]. Studies of IgE-binding with sera from *Alternaria*-sensitized patients allowed to identify two major epitope regions [13] that display a structural organization appropriate for cross-linking in IgE-interaction. Alt a 1 is predicted to form also stable tetrameric assemblies but no other information regarding this oligomer is available. The biological function of Alt a 1 is still unknown yet its localization in cell wall of *Alternaria* spores [17] that mediate allergic reactions and its possible role in spore germination [18] should be consistent with a function of transporter of small ligands but, as said, the β -barrel is apparently unable to bear ligands. Further, our experimental results reveal that the tetrameric assembly of Alt a 1 binds quercetin but both the own aggregation state and ligand binding abilities are pH-dependent, a behavior which, incidentally, is also known for other quercetin bearing proteins [47,48]. It seems thus that the different oligomers could play different roles, a feature that we think should be particularly relevant to study physiological interactions of Alt a 1 involved in the onset of allergic reactions.

Our computational study on the structural features of Alt a 1 conducted within a research programme aimed at elucidating physiological interactions involved in *Alternaria* allergy led us to propose the following tentative picture. Alt a 1 tetrameric assembly is favored in the presence of an as yet unknown ligand which is well represented by the flavonoid quercetin. The corresponding tetramer-ligand complex displays two symmetric barrel-like substructures composed of β -strands from different chains that we suggest is the binding site of Alt a 1. The ligand locates at the interface between subunits in the middle of the barrel flanked by nearby aromatic rings of two tyrosines (Y46) from different chains at a proper geometry for π -stacking interaction. This geometry corresponds precisely to one of the two alternative conformations of Y46 derived from electron density in the reported high-resolution crystal structure of Alt a 1 [14]. Different protonation states of ionizable side chain groups arising from different pH's modify protein-ligand affinity so that the quercetin complex is electrostatically favored at $\text{pH} \geq 6$. Since the pH which Alt a 1 would meet in bronchial epithelium is 6.5, the tetrameric form should be the favored state of the protein there. In contrast, MD simulations show that this complex disrupts at pH 5.5 so that one might expect that the monomer should then be the favored state. However, if one takes into consideration the suggestion that high local concentrations of protein favor dimerization [15], that the dominant state of Alt a 1 would ultimately be either monomeric or dimeric should depend on local concentration. In this regard, it must be recalled that the symmetric dimer in the butterfly-like shape (which indeed presents proper orientation of epitope regions [13] for IgE cross-linking) could be the favored state of Alt a 1 protein upon local high concentration.

Additional *in silico* work is obviously still needed to elucidate details concerning oligomerization and ligand-binding features of Alt a 1. However, we believe that the results presented here are of interest for further *in vitro* and *in vivo* work aimed at elucidating not only the biological function of this protein but also its physiological interactions related with allergy and other respiratory diseases in humans associated to *Alternaria* genus. Research along these lines is underway in our laboratory and will be the subject of forthcoming reports.

Methods

Structural analyses

The initial 3D structure of Alt a 1 was the crystal structure at 1.9 Å resolution PDB id. 3V0R [14]. Secondary structure was identified with DSSP [20,21]. The topology diagram of Alt a 1 (Fig. 1) was prepared from that initially provided for the 3V0R entry by PDBSum [22,23] web (www.ebi.ac.uk/pdbsum/). Searches for related topologies were conducted using the Protein Topology Graph Library (PTGL) [24] web (ptgl.uni-frankfurt.de/). Aggregation states of Alt a 1 were obtained with the Proteins, Interfaces, Structures and Assemblies (PISA) method [25,26] implemented in PDBePISA server (www.ebi.ac.uk/msd-srv/prot_int/pistart.html). All molecular graphics were prepared and rendered with PyMOL 1.7.6 [27].

Docking calculations

Initial geometry of quercetin was prepared with structural modeling tools in the UCSF Chimera 1.10.2 package [28] while that of alternariol was taken from its crystallographic X-ray structure [29]. Both geometries were optimized in Molecular Mechanics calculations using AMBER ff14SB force field [30] after assigning AM1-BCC atomic charges with AnteChamber [31] as implemented in Chimera 1.10.2. Structures of quercetin and alternariol as well as the different protein aggregates were prepared for docking with AutoDockTools [32]. In order to explore putative binding sites, search spaces of sizes intended to enclose the whole structures were used in a first set of AutoDock Vina [33] docking calculations in monomeric and tetrameric Alt a 1. The monomer was explored by using a search space defined by a 50 Å edge cube centered at the center of the α -barrel whereas the tetramer was explored with an 80 Å edge cube centered at the center of the assembly. In both cases, the best docking poses (i.e., those having lowest protein-ligand binding affinities) for quercetin agreed well with the spatial positions of the crystallographic aromatic ligand (Fig. 2c) in both the X-ray structure of monomeric Alt a 1 [14] and in the PISA symmetry-predicted structure of tetrameric Alt a 1. With this result, a second set of AutoDock Vina [33] calculations were performed with smaller search spaces defined now by 20 Å edge cubes centered at the position of C6 atom of the crystallographic aromatic ligand in both monomeric and tetrameric Alt a 1. This procedure was used to obtain the geometries of quercetin and alternariol docked to tetrameric Alt a 1 with different

protonation states corresponding to pH = 5.5, 6.0, 6.5, and 7.0 (see below). In all cases, the docking pose with lowest protein-ligand binding affinity was selected for further analyses.

Calculations of pKa values and protonation states

pKa values of ionizable side chain groups in Alt a 1 were computed with Propka 3.1 [34,35] program. This version of the empirical pKa predictor Propka implements a new algorithm for modeling noncovalently coupled residues that due to their spatial proximity can influence the titration of each other [35]. A first run of Propka 3.1 for the three aggregation states of Alt a 1 predicted pKa values for all ionizable side chains and identified coupled residues while a second run of Propka 3.1 gave alternative pKa values for them. Propka 3.1 output also provides a list of interactions with other residues that result in each pKa particular value.

Protonation states at pH values given in input were assigned with Pdb2pqr 2.0.0 [36,37] using Propka 3.1 as implemented in the Pdb2pqr server (nbc-222.ucsd.edu/pdb2pqr_2.0.0/). Pdb2pqr adds hydrogens as needed by the particular protonation state and optimizes local conformations to fix clashes. Pdb2pqr/Propka 3.1 output takes the form of a PQR file that is just a modified PDB file in which occupancy and B-factor items in ATOM entries are replaced with atomic charges and radii. This PQR file is then used as input to compute Poisson-Boltzmann electrostatic potentials. For compatibility with the force field used in Molecular Dynamics simulations (see below), CHARMM 3.1 atomic charges [38] were selected in Pdb2pqr calculations. Atom coordinates and charges for the different aggregates of Alt a 1 with protonation states corresponding to pH values from 4.0 to 7.0 at intervals of 0.5 pH units were obtained in this way. These tetramer structures corresponding to pH = 5.5, 6.0, 6.5, and 7.0 were those used in docking calculations to obtain complexes with quercetin and alternariol as explained above.

Poisson-Boltzmann electrostatic potentials

PB electrostatic potentials were computed for tetrameric Alt a 1-quercetin complexes at pH = 5.5, 6.0, 6.5, and 7.0 by solving the Poisson-Boltzmann equation with APBS (Adaptive Poisson Boltzmann Solver) 1.4.1 program [39]. CHARMM 3.1 charges and radii [38] were assigned to protein atoms with Pdb2pqr/Propka 3.1 as explained above. CHARMM 3.1 force field parameters including atomic charges were assigned to quercetin and alternariol with the SwissParam method [40] implemented as a separate web service in Swiss Institute of Bioinformatics (swissparam.ch/). Radii for atoms in quercetin and alternariol were taken from values for equivalent atom types in PQR files for proteins. The nonlinear PB equation was solved in sequential focusing multigrid calculations in 3D meshes of $192^3 = 7,077,888$ points (spatial grids with step size about 0.5 Å in tetramer systems) at 298.15 K and 0.150 M ionic concentration. Dielectric constants 4 for proteins and 78.54 for water were used. The numerical output of all PB electrostatic potentials were stored in OpenDX scalar data format and then mapped onto molecular surfaces of proteins and ligands and graphically rendered with

PyMOL 1.7.6 [27]. PB potentials are given in units of kT per unit charge (k , Boltzmann's constant and T , absolute temperature).

Molecular Dynamics calculations

Alt a 1 systems explored with MD calculations were the following: (a) the butterfly-like dimers with and without intermolecular C30-C30 disulfide bridge, (b) the dimeric arrangement taken from the tetramer in complex with quercetin, and (c) the full tetrameric structure complexed with quercetin. Systems (b) and (c) were subjected to MD simulations at pH 5.5 and 6.5 using the CHARMM 3.1 force field [38] were performed with the multiprocessor Linux-POWER-MPI version of NAMD 2.10 [41] in CeSViMa supercomputer of Technical University of Madrid. All systems were immersed in periodic rectangular solvation boxes with a spacing distance of 15 Å around proteins and water molecules added according to the TIP3P model [42]. Na⁺ and Cl⁻ ions were added to counter the total charge of protein systems at the different pH values while providing 0.150 M salt concentration. The total number of atoms involved in these simulations were about 49,800, 50,500, and 74,000 for systems (a), (b), and (c), respectively. The particle mesh Ewald summation method [43] was used for long-range electrostatic and a 10 Å cutoff was set for short-range non-bonded interactions. For every system, the following set of calculations were done: (1) optimization along 5,000 conjugate gradient minimization steps, (2) equilibration of water for 100 ps at 2 fs time steps at 298 K and 1 atm with all atoms except those of water fixed, and (3) simulation runs during 100 ns keeping same time step (which involves 50 million steps for every simulation), and same T and P in the NPT ensemble. Langevin dynamics for T control and Nosé-Hoover Langevin piston method for P control were used. Output results were stored every 25,000 steps rendering thus trajectories composed of 2,000 frames which were processed and analyzed with VMD 1.9.2 [44].

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Figure legends

Fig. 1. a Cartoon diagram of the crystal structure of Alt a 1 protein (PDB id 3V0R, [14]). Secondary structure assigned with DSSP. Labeling of β strands follow the notation used in Ref. [14]. Alternative conformations of side chains for Y46, D114, and V139 residues having 0.50 occupancy in the electron density are shown as sticks in yellow (conformation A) and cyan (conformation B). N-terminal C30 involved in dimer stabilization is also shown as sticks. **b** Topology diagram labelled as in **a** obtained from that given by PDBsum (www.ebi.ac.uk/pdbsum/).

Fig. 2. a Alt a 1 tetramer. Compounds originated from the crystallization solution depicted as sticks (sulfate anions in yellow and red) at spatial locations arisen from symmetry operations used to set oligomerization states. Cyan boxes mark the inter-subunit geometry of two molecules of one of these compounds. **b** Alt a 1 butterfly-like dimer. β -strands colored in different hues correspond to the two main epitope regions K41-P50 and Y54-K63 identified in the allergen [13]. The inter-monomer disulfide bridge formed between C30 residues in the two chains is represented as green sticks. **c** Structural formulas of compounds present in the crystal structure of Alt a 1: 4-hydroxy-2,5,6-triaminopyrimidine (*left*) and 8-aminocaprylic acid (*right*). **d** Upper (*left*) and side (*right*) views of the site marked in the top cyan box in **a** rotated to highlight the β -barrel substructure. Side chains of Y46 in their conformation B are represented as yellow (carbon bonds) and red (oxygens) sticks in the two subunit chains.

Fig. 3. Results from 100-ns MD simulations of the butterfly-like dimeric form of Alt a 1 with and without the intermolecular disulfide bridge between C30 residues in the two chains. RMSD of backbone atoms in **a** the whole dimer and **b** residues in the two main IgE epitope regions [13]. **c** RMSF of all residues (28-157) present in the crystal structure of Alt a 1 [14]. Shaded segments 41-50 and 54-63 correspond to the two epitope regions.

Fig. 4. a Structural formulas of quercetin and alternariol used in docking calculations. **b** Geometries of quercetin (deep blue sticks for carbon bonds) and alternariol (cyan sticks for carbon bonds) from structural superposition of the corresponding complexes with tetrameric Alt a 1. **c** Upper (*left*) and side (*right*) views of the inter-chain binding site in the superimposed structures of tetrameric Alt a 1 complexes with quercetin and alternariol (same colors as in **b**). Side chains of Y46 residues in their conformation B are depicted as yellow sticks for carbon bonds in the two chains. **d** The two binding sites in tetrameric Alt a 1 are symmetry-related by a rotation by 90° about a vertical axis through the center of the tetramer. Three views rotated by 45° are shown.

Fig. 5. Interacting residues E44, R48, N133, D137, and D145 located at both ends of the barrel-like substructure in the interface between subunits in tetrameric Alt a 1. E44 and D137 are coupled residues with alternative protonation states. Y46 side chains are shown as a reference to locate ligand position in the putative binding site. Letters after residue labels denote A and B chains.

Fig. 6. **a** PB electrostatic potential mapped onto the outer surface of tetrameric Alt a 1 with ionizable side chains at protonation states corresponding to pH 7.0 (*top*) and 5.5 (*bottom*). The structure is seen at the same orientation as the last view in Fig. 4d. **b** PB potential mapped onto the inner surface of one of the two inter-subunit interfaces forming the putative binding site. Same orientation and pH values as in **a**. Side chains of Y46 (sticks in same colors as chain ribbons) are shown as a reference to locate ligand position. **c** 0.0 isosurfaces of PB electrostatic potential at same orientation and pH values as in **a**. **d** Geometry of quercetin and PB potential mapped onto its molecular surface at four views rotated by 90° about a horizontal axis. Same scale range of PB potential used in **a** and **b**.

Fig. 7. **a** Ribbon diagram of tetrameric Alt a 1-quercetin (QUE: yellow sticks for carbons) complex with ionizable side chain groups at protonation states corresponding to pH 7.0. Side chains of Y46 (sticks in same colors as chain ribbons) in the binding site are also shown. **b** PB electrostatic potential mapped onto the surface of tetrameric Alt a 1 at same pH and orientation as in **a** in a perspective to view the location of quercetin in the binding site.

Fig. 8. RMSD results for the dimeric interface forming the barrel-like binding site in the tetrameric Alt a 1-quercetin complex at pH 5.5 and 6.5. RMSD of **a** protein backbone atoms and **b** non-hydrogen atoms in quercetin from 100-ns MD simulation in the dimer form (Fig. 4c). Dimer complex at pH 5.5 breaks down at 77.2 ns. RMSD of **c** protein backbone atoms and **d** non-hydrogen atoms in quercetin from 100-ns MD simulation in the complete tetramer form (Fig. 4d). Tetramer complex at pH 5.5 breaks down at 50.6 ns.

Table 1. pKa values of residues with ionizable side chains in oligomerization states of Alt a 1

Residue	Tetramer ^a	Dimer ^a	Monomer ^a
D	Model pKa = 3.80		
D37	3.26	3.20	3.16
D71	<u>4.85</u>	3.83	3.83
D79	3.93	3.90	3.90
D83	3.16	3.85	3.16
D96	<u>4.78</u>	4.54	4.29
D100	4.06	<u>4.86</u>	4.06
D102	<u>2.43</u>	3.05	2.43
D114	3.77	3.58	3.64
D115	4.38	3.96	3.94
D137	3.67	2.57	2.57
D145 ^b	<u>5.99</u> (AB) <u>5.65</u> (CD)	4.30	4.04
E	Model pKa = 4.50		
E35	5.06	4.96	4.94
E44 ^b	<u>7.81</u> (AB) <u>8.37</u> (CD)	4.83	4.81
E51	4.52	4.50	4.50
E82	4.39	4.41	4.39
E91	<u>5.75</u>	4.73	4.73
H	Model pKa = 6.50		
H84	6.64	6.09	6.64
C	Model pKa = 9.00		
C30 ^c	9.05	<u>8.08</u>	9.05
Y	Model pKa = 10.00		
Y38	10.05	<u>11.50</u>	9.80
Y46 ^b	<u>11.81</u> (AB) <u>12.31</u> (CD)	10.38	10.38
Y54	10.40	10.40	10.40
Y55	<u>12.12</u>	<u>12.12</u>	<u>11.93</u>
Y87	10.00	10.27	9.95
Y118	<u>11.74</u>	9.66	<u>11.30</u>
Y127	9.85	10.13	10.13
Y147	<u>11.05</u>	10.84	10.46
K	Model pKa = 10.50		
K41	9.95	10.93	10.93

K49	9.69	9.75	9.77
K63	10.33	10.37	10.37
K80	10.72	10.73	10.73
K85	10.74	10.76	10.76
K109	9.92	<u>9.43</u>	10.07
K111	10.37	10.44	10.39
K136	10.66	10.66	10.66
K155	9.90	<u>8.95</u>	9.84
R	Model pKa = 12.50		
R48	<u>13.45</u>	<u>13.48</u>	<u>13.48</u>
R103	<u>14.28</u>	<u>14.36</u>	<u>14.28</u>
R129	12.37	12.40	12.40

^a Underlined italics values represent pKa shifts about 1 pKa unit or greater

^b Different values in tetramer for chains indicated in parentheses

^c Cysteines 74, 89, 128, and 140 participate in intramolecular disulfide bridges

Table 2. Residues with alternative pKa values in oligomers of Alt a 1

Residue	Tetramer ^a	Dimer ^a	Monomer
D137	3.67 (ABCD) 5.78 (ABCD)		
E44	7.81 (AB) 8.37 (CD) 5.69 (AB) 6.26 (CD)		
C30		8.08 (A) 10.16 (B) 10.16 (A) 8.08 (B)	
Y38	9.97 (CD) 11.46 (CD)	11.50 (AB) 10.01 (AB)	9.80 11.21
Y118	11.74 (CD) 10.25 (CD)	9.66 (AB) 11.15 (AB)	11.30 9.88

Alternative pKa values are given in separate lines for each residue

^a Chains indicated in parentheses

Table 3. Total charge of oligomerization states of Alt a 1 at different pH's

pH	Tetramer		Dimer	Monomer
	Normal pKa state	Alternative pKa state		
7.0	-12	-16	-8	-4
6.5	-8	-12	-8	-3
6.0	-6	-8	-6	-3
5.5	0	+4	-6	-3
5.0	+4	+8	-6	-3
4.5	+16	+20	+6	+1

Figure 1

Figura 2

Figure 3

Figura 4

Figura 5

Figura 6

Figura 7

Fiura 8

Fig.8

Computational study of pH-dependent oligomerization and ligand binding in Alt a 1, a highly allergenic protein with a unique fold

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Supplementary Material

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Fig. S1. Binding site defined by 4.5 Å neighborhood around quercetin (sticks with carbons colored violet) docked to tetrameric Alt a 1. Carbon atoms in residues corresponding to chains A and B are colored in green and light red, respectively. Cyan dashed lines represent hydrogen bonds with their distances given in Å. Boxed labels indicate side chain residues hydrogen-bonded to quercetin. Distances colored in violet refer to hydrogen bonds with quercetin.

Fig. S2. 50Å-edge cube box defining the search space for docking calculations in Alt a 1 monomer structure. The two best geometries of docked quercetin (carbon cyan sticks) with lowest affinity free energies -6.5 kcal/mol (bottom) and -5.8 kcal/mol (top) are compared with the spatial positions of two molecules of the crystallographic ligand (4-hydroxy-2,5,6-triaminopyrimidine: carbon deep purple sticks) in the X-ray crystal structure of the monomer (PDB id. 3V0R).

Fig. S3. Residues showing pKa shifts about 1.0 pKa unit or greater in **a** dimeric and **b** tetrameric states of Alt a 1 protein. Letters A-D after residue labels denote subunit chains.

Fig. S4. Spatial location of tyrosines Y38 and Y118 in **a** monomeric, **b** dimeric and **c** tetrameric states of Alt a 1 protein. Letters A-D after residue labels denote subunit chains.

Fig. S5. Superposition of geometries of quercetin docked to tetrameric Alt a 1 at pH 5.5 (red), 6.5 (light green), and 7.0 (blue). A geometry at pH 6.0 is very similar to that at pH 6.5 and it is not distinguishable.

Fig. S6. RMSD of protein backbone atoms for the dimeric interface forming the barrel-like binding site in the tetrameric Alt a 1-quercetin complex. Results from 100-ns MD simulation of the whole tetramer complex at pH 5.5 and 6.5. The complex at pH 5.5 breaks down at 50.6 ns.

Fig. S7. Variation of protein-quercetin interaction energies in the dimeric interface forming the barrel-like binding site in the tetrameric Alt a 1-quercetin complex. Values computed from 100-ns MD trajectories over the last 50 ns before system breaking occurring at pH 5.5. **a** Electrostatic and **b** van der Waals interaction terms from MD simulation in the dimer form (see Fig. 4c). **c** Electrostatic and **d** van der Waals terms from MD simulation in the complete tetramer form.

3.4 The secret of Alt a 1, a versatile protein: pathogenic effector, allergen and flavonoid transporter

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Doctoral candidate's contribution to this work:

Quantification of Alt a 1 from *Alternaria alternata* spores

Isolation of the ligand of Alt a 1

Alt a 1-flavonoid binding assays

Characterization of the ligand activity

Preparation of figures and manuscript

Abstract

Spores of pathogenic fungi are virtually everywhere causing severe losses in crops and human diseases. Being an endophytic fungi, *Alternaria* spp. are able to produce host-selective phytotoxins. Among them, Alt a 1 is a strongly allergenic protein found in *A. alternata* causing severe asthma. Despite its well-established pathogenicity, the molecular mechanisms underlying the action of Alt a 1 and its own physiological function remain largely unknown. To gain insight into the role played by this protein in the pathogenicity of the fungus, we studied Alt a 1 production in *A. alternata* spores and investigated its activity. Alt a 1 is accumulated inside the spores and its released together with a ligand in a pH-dependent manner with an optimum production between 5.5-6.5 interval. Alt a 1 ligand was identified as a methylated flavonoid that is able to inhibit plant root growth and detoxifies reactive oxygen species. We also found that Alt a 1 changes its oligomerization state depending on the pH of the surrounding medium and that these changes release the ligand. Based upon these results, we propose that the presence of the ligand should be a pathogenicity tool to block plant defenses favoring fungal entry into plant.

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Keywords: Alt a 1, respiratory allergy, *Alternaria alternata*, endophytic fungus, flavonoids, pH, ROS

Introduction

Alternaria genus of endophytic fungi that include more than 50 species, many of them are known plant pathogens. Moreover, *Alternaria* species also cause different types of infections and disease in humans such as hypersensitivity, pneumonitis, bronchial asthma, allergic sinusitis and rhinitis(1–4). *Alternaria* spp. are distributed worldwide, particularly in warm regions, where their spores are present in the atmosphere throughout the year, peaking up in spring, summer and autumn (5).

Particularly, *A. alternata* is able to infect more than 100 plant species. Its pathogenicity could be attributed to the production of diverse host-selective phytotoxins or bioactive host-dependent metabolites, as well as to increased toxic reactive oxygen species (ROS) production, in which bring about death cell (6). Being endophytic, *Alternaria* fungi can stay on the plant tissue surface without inducing apparent symptoms in their hosts waiting the right moment to germinate. However, the molecular mechanisms underlying the interactions between endophytic fungi and their host plants remain largely unexplored (7).

Fungal flavonoid-type compounds have been previously reported (8, 9) that could play a role in plant defense against fungi. Flavonoids are well-known secondary plant metabolites involved in a variety of processes such as cell signaling, plant growth, and reproduction (10–14). Among this heterogeneous group of molecules, flavone derivatives with two *ortho*-hydroxyl groups on the B ring (catechol) are particularly interesting due to their capacity to detoxify ROS (12, 15–17) and siderophore activity (18, 19), both features relevant in plant defense strategies.

Alt a 1 (AAM90320.1, NCBI Protein Database) protein is detected in the spores of *A. alternata* (20) before the germination process (21). It has been described as the main allergen associated with chronic asthma (22) and its interaction with pathogenesis-related plant defense protein PR5 has been recently demonstrated (23) although the molecular mechanism of Alt a 1 in fungal pathogenesis still remains unknown (24). Alt a 1 is codified by a unique gene only present in *Alternaria* and related species (25) and its expression pattern has been correlated with the infection process in *Arabidopsis thaliana* (26). Alt a 1 protein form a 30 kDa homodimer that dissociates into two subunits of 14.5-16 kDa, under reducing conditions (27). The monomeric crystal structure shows a β -barrel composed of eleven β -strands which has been claimed to be a unique fold without equivalent in the Protein Data Bank (7).

Here we report results that shed light on the versatility of this singular protein. We show that Alt a 1 is not secreted in its apo-form but it transports a flavonoid compound and that its oligomerization state changes depending on both the presence of its ligand and the pH of the surrounding medium. This ligand shows positive staining with diphenylboric acid 2-aminoethyl ester (DPBA), capacity to inhibit plant cell growth, and antioxidant activity. Therefore, although the identity of this ligand has not been fully

elucidated, its flavonoid nature is demonstrated. Taken together, our results suggest that Alt a 1 ligand is involved in ROS detoxification pointing out its role in defense response in *Alternaria*.

Results

Alt a 1 is located inside Alternaria spores and is secreted carrying a flavonoid in a pH-dependent manner

Alt a 1 was located inside spores of *Alternaria* by immunofluorescence using a specific monoclonal antibody by an analysis on confocal microscopy. The most intense signal was detected in the spore cytoplasm at time 0 decreasing with elapsed time suggesting. This indicates that Alt a 1 is preformed and secreted when spores detect humidity (Figure 1A, B). Alt a 1 release is strongly pH-dependent and is accumulated mainly in the first few minutes at pH values between 5.0 and 6.5 (Figure 1C).

We have found that Alt a 1 was secreted together with a phenolic compound as confirmed by thin layer chromatography of the retained fraction using ethanol:formic acid:water (50:30:20) as eluent. DPBA staining (11) revealed a spot-dot suggesting the flavonoid nature of the ligand.

To characterize its chemical composition, the ligand was purified after ethanol extraction (70%) of spore-mycelium mixture at room temperature, followed by high-performance liquid chromatography (HPLC) (Nucleosil 120 C18, 250 x 4mm). Peaks were stained with DPBA (Figure 2A) and positive fractions were analysed by mass spectrometry (Figure 2B). Two peaks were observed ($m/z = 242.079$, 372.119) and the MS/MS experiments revealed the presence of different fragment ions with m/z values 122.3, 144.3, 154.3, 165.1, 203.3, 231.3 and 242.5 (Figure 2C).

Alt a 1 is able to bind flavonoids with high affinity

Alt a 1 is able to bind quercetin (3,3',4',5,7-pentahydroxyflavone), a well-known flavonol bearing a catechol moiety, as it was demonstrated by pull-down assays using Dynabeads® M-280 Tosylactivated. The heated fraction could be stained with DPBA (Figure 3A). In contrast, no DPBA positive peak was observed before heating (data not shown), suggesting that fluorescence could be quenched when Alt a 1 was bound. A dissociation constant $K_d = 0.7 \mu\text{M}$ was estimated for the Alt a 1-ligand complex (Figure 3B). Release of the ligand from Alt a 1 was also strongly pH-dependent (Figure 3C) with maximum amount released at pH between 5 and 6.5, just the same interval as that corresponding to Alt a 1 secretion.

The flavonoid carried by Alt a 1 shows quercetin-like activities

Quercetin and related polyhydroxylated flavones are known plant root inhibitors showing ROS scavenger activity. The presence of the ligand inhibited the growth of and also show ROS detoxification, we studied both activities in the ligand of Alt a 1 protein. Growth inhibition assays in *Arabidopsis* root

(Figure 4A) revealing thus a quercetin-like root growth inhibitory activity. Seeds were grown during six days and, the ligand of Alt a 1 was then added to the medium and root length was measured after 24 hours of treatment following the procedure previously described for quercetin (28). Therefore, the ligand of Alt a 1 protein also presents flavonoid activities.

Even more, the study ROS detoxification characteristic of catechol-bearing flavonoids, we tested the ability of Alt a 1 ligand to inhibit the activity of horse-rabbit peroxidase (HRP) using OPD as substrate and hydrogen peroxide as a cofactor necessary for proper enzyme functioning. The ligand produced a significant decrease in the amount of ROS comparable to quercetin (Figure 4B). When the HRP activity inhibition assay was performed with the ligand in the presence of Alt a 1, a slight increase of ROS was observed (Figure 4B). This result suggests that the chemical groups involved in the antioxidant activity (presumably, catechol) would be blocked by interactions with amino acid residues in the binding site of the protein.

Discussion

Alternaria species have different lifestyles, ranging from saprophytes to endophytes to pathogens (6). They are a highly successful group as fungal pathogens that cause severe losses in a wide variety of economically important crops as well as many ornamental and weed species. The responsible mechanism for this pathogenic success could be attributed to the secretion of effector protein Alt a 1 before germination outbreak.

In this work, we have found that Alt a 1 secretion occurs in the first minutes when spores detect humidity, showing to be very sensitive to the pH of the surrounding medium. The maximum release of Alt a 1 is observed at pH values between 5.0 and 6.5. This result is consistent with previous reports describing optimum pH about 6.0-6.5 for spore germination in *Alternaria* (29, 30). In contrast, an optimum pH about 4.0-4.5 was found for releasing mycotoxins (30). These pH values are typical of apoplast in ripe fruit (31).

Alt a 1 is not released in an apo-form but it carries a flavonoid ligand with properties similar to those of quercetin, a plant flavonol model (32). The protein-ligand affinity is high ($K_d = 0.7 \mu\text{M}$) and the presence of the ligand was found to be necessary for higher-order oligomerization though still in a pH-dependent manner. In fact, the tetrameric form of Alt a 1 carrying the ligand is stable at pH 6.5, whereas at pH 5.0 the tetramer breaks down to monomers releasing the flavonoid ligand to the medium.

Ligand transport has been described for a great number of allergens such as peach allergen Pru p 3, strawberry allergen Fra a 1, and birch pollen allergen Bet v 1 (33–35). Although our results strongly support the flavonoid nature of the molecular weight 372 is found for it, unfortunately it could not be

identified. Besides, relatively little is still known about the presence of flavonoids in fungi. Some strains of *Aspergillus candidus* produce chlorflavonin, a methylated flavone (36), and the presence of other flavone with several methyl and methoxy groups was detected in *Colletotrichum dematium* (9). Thus far it is known that most metabolites in fungi have antifungal activity towards phylogenetically unrelated species, and the toxicity of flavones in this process is found to decrease with the presence of methoxy groups (37, 38). Based upon similar fragmentation profiles of flavonoids, the chemical structure of the mentioned methylated flavones from fungi and the experimental evidence reported here on its activity, we hypothesize that the ligand of Alt a 1 is a molecule with $C_{20}H_{20}O_7$ composition and flavone-based structural formula (Figure 5). Although there are several positional isomers compatible with this proposal, this framework structure fulfils relevant conditions: (i) the catechol moiety (B-ring) coincides with that of quercetin (hydroxyls in positions 3' and 4') and is free to perform plant root growth inhibitory and ROS scavenger activities, (ii) incorporates methoxy and methyl substituents found in fungal flavones, and (iii) its molecular weight is 372. Further work will be devoted to elucidate which of the six positional isomers in Figure 5 is the actual structure of the ligand of Alt a 1.

In summary, the following picture on the versatility of Alt a 1 can be drawn. When *Alternaria alternata* germinates, the infected plant expresses pathogenesis-related proteins and produces ROS as a defense response. Alt a 1 is mainly released as a tetramer carrying its flavonoid ligand but when the complex reaches the plant apoplast, the mildly acidic pH 5.5 there brings out aggregate breakdown and the protein changes to the ligand. As a monomer, Alt a 1 interacts with plant defense proteins such as PR5 (23) and the free flavonoid is able to detoxify ROS.

Methods

Fungal growth

Alternaria spores were isolated from kiwifruits by scraping the surface and then cultured on PDA medium (potato dextrose agar; Difco™ Becton Dickinson and Company, Sparks, MD, USA) with cefotaxime (200µg/ml) (Calbiochem®, Merck KGaA, Darmstadt, Germany), as previously described (23). After 8 days, *Alternaria* spores were recovered with sterile water and stored at -80°C in 20% glycerol.

Immunohistochemistry assays

Spores of *A. alternata* were fixed with 4% formaldehyde in PBS, pH 7.4 at 4°C, 15 min. After PBS washing the spores were permeabilized by 30 cycles of freezing and thawing. After washing with PBS, the spores were incubated overnight at 4°C with specific monoclonal anti-Alt a 1 antibodies (1:50, Bial Aristegui, Bilbao, Spain) and revealed by anti-mouse Alexa 488-conjugated antibodies (Molecular

Probes). The specimens were mounted with glycerol:PBS (1:1) and observed with a Leica TCS-SP8 confocal microscope, using the laser excitation lines of 488 and 561 nm.

Production of Alt a 1 and its ligand

Spores of *A. alternata* (10^6) were incubated during 1 hour at 25°C in medium with different pH's (5.0, 6.5, and 7.4) at several times (5, 10, 20, 30, and 60 min). Supernatants were coated in ELISA (Enzyme-linked immunosorbent assay) plates for 2 hours at 37°C. After blocking, plates were washed and incubated with a polyclonal anti-Alt a 1 antibody (1:1000, Bial Aristegui), and HRP-IgG antibodies (1:30000, Sigma-Aldrich, Madrid, Spain).

The presence of the ligand of Alt a 1 was quantified in the supernatants by specific flavonoid staining with diphenylboric acid 2-aminoethyl ester (DPBA; 0.25% w/v) (11) and reading fluorescence at 485/535 nm in a microplate reader (Spectrafluor Fluorometer TECAN GeniosPro; TECAN group, Männedorf, Switzerland). The amount of ligand released was quantified using a standard curve of known amounts of quercetin (Sigma-Aldrich).

Isolation of Alt a 1

Alt a 1 was purified from spores and mycelium mixture of *A. alternata* (ALK-Abelló, Denmark). Briefly, Alt a 1 was purified from defatted mixture and extracted with PBS buffer (0.1 mM sodium phosphate pH 7.4, 1.5M NaCl; 1:5 (w/v); 1 h at 4°C). The extract was dialysed against H₂O, freeze-dried and fractionated by anion-exchange chromatography on a Waters AccellTM Plus QMA Sep-Pak^R cartridge (Waters Corp, Milford, MA, USA). Elution was carried out with 20 mM ethanolamine, pH 9.0, and the retained material was then eluted with 0.75 mM NaCl in the same buffer (1 ml/min). The Alt a 1 enriched fraction was repurified by phase reverse-HPLC on a Nucleosil 300-C4 column (7x250mm; particle size 5 µm: Tecknokroma, Barcelona, Spain). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% for 5 min and 10-100% over 150 min; 1 ml/min).

Purified Alt a 1 was quantified by means of the commercial bicinchoninic acid test (Pierce, Cheshire, UK) and purity was measured by SDS-PAGE, mass spectrophotometric analysis with a Biflex III Spectrometer (Bruker-Franzen Analytik, Bremen, Germany), and fingerprinting after tryptic digestion, using standard methods.

Isolation of the ligand carried by Alt a 1

The ligand was extracted with ethanol (70%) from the spore-mycelium mixture at room temperature. After centrifugation, the supernatant was separated by HPLC (Nucleosil 120 C18, 250x4mm, Tecknokroma) with a gradient of acetonitrile (10-85% in 30 min) at 0.5 ml/min. Spectra were recorded at 254 and 280 nm. Peaks were analysed by DPBA staining (0.25% w/v) (11) and quercetin (0.01 µg/µl) was used as a positive control. Data were recorded using a fluorescence microplate reader (Spectrafluor Fluorometer TECAN GeniosPro) at 485/535 nm.

Alt a 1- flavonoids binding assays

To test the Alt a 1 capacity to bind flavonoids, the isolated ligand and quercetin (Sigma-Aldrich) as a model were used. Immunoprecipitation assays were performed using Dynabeads® M-280 Tosylactivated (Invitrogen, Barcelona, Spain) conjugated with Alt a 1 (0.2 µg/µl) and incubated with quercetin and its ligand (0.01 µg/µl). Dynabeads were conjugated following manufacturer's instructions. After intensives washes, Alt a 1-Dynabeads were heating for 10 min at 95°C and the binding was confirmed by staining of the supernatant with DPBA.

To study the kinetics of the Alt a 1-flavonoid binding, increasing amounts of the protein were incubated with constant amounts of quercetin and ligand (0.25 µg) and stained with the fluorescent dye DPBA (0.25% w/v). The result was dotted onto a nitrocellulose membrane washed with water and visualized using an Alexa Fluor 488 laser in a Bio-Rad Pharos FXTM Plus Molecular Image. Dot intensity was calculated using the software of the instrument. All tests were performed in three independent assays. In the calculation of the dissociation constant (K_d) of Alt a 1-flavonoid (F) complex, $K_d = \frac{[\text{Alt a 1}][\text{F}]}{[\text{Complex}]}$, protein concentration [Alt a 1] was known, free flavonoid [F] was calculated by extrapolating the fluorescence obtained to a standard curve constructed with increasing amounts of flavonoid, and the concentration of the bound system i.e. [Complex], corresponded to the difference between initial [F] and free [F], measured as explained.

Mass spectrometry analysis of the ligand of Alt a 1

Equal volumes of the sample (in ethanol) and the matrix solution (10 mg/ml 2, 5-dihydroxybenzoic acid in ethanol) were mixed for a few seconds and then 1 µl of the mixture was spotted on the 800 µm AnchorChip target (Bruker-Daltonics) and allowed to dry at room temperature. For the analysis of flavonoid without a matrix, flavonoid solutions (1 µl) were applied directly to MALDI target.

MALDI experiments were performed on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics) with a smartbeam laser. Samples were analysed in the positive ion detection and delayed extraction reflector mode in the mass range between m/z 100 and 1000. The experimental parameters

used were as follows: pulsed ion extraction 250 ns, laser frequency 1000Hz, ion source 1 of 19.0 kV, ion source 2 of 16.60 kV, lens 8.79 kV, reflector 1 of 21.0 kV and reflector 2 of 9.77 kV. Typically, 1000 laser shots were summed into a single mass spectrum. The laser intensity was modified according to the threshold energy necessary for the ionization of sample, and sample also was evaluated by different laser intensity (25 to 90%).

UHPLC-ESqTOF of the ligand of Alt a 1

The analysis was performed in a microTOF-Q II mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization source in positive ion mode. Typical instrument settings were as follows: capillary voltage, 4500 V; capillary exit, 130 V; and dry gas temperature, 180°C; dry gas flow, 4 l/min. Spectra were obtained in positive ion mode. The mass spectrometer was used to carry out two scans: a full-mass scan between 50 and 1000 m/z at a repetition rate of 2 Hz, and an MS-MS scan of the most abundant ions in the full-mass scan. Argon was used as the collision gas. Collision energy was ramped at between 5 and 25 eV. Mass calibration was performed using sodium formate clusters (10-mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

Plant root growth inhibition assays

Assays with *Arabidopsis* were performed using Columbia ecotype. All seedlings were sown under sterile conditions on vertically oriented 12 cm square plates containing half-strength Murashige and Skoog (MS1/2) with 0.05% MES, 1% sucrose and 1% plant-agar (Duchefa Biochemie B.V.) plates under a 16-h light/8-h dark photoperiod at 21-23°C. To determine the antimitotic activity of the ligand, seeds were cultivated in the D-Root system and primary root length was determined as previously described (28). Data were statistically analysed using the t-Student function.

ROS inhibition assays

The inhibition of ROS activity was performed following the method previously published (39) with minor modifications using Alt a 1, the flavonoid ligand of Alt a 1, quercetin and the Alt a 1-ligand complex. Horseradish peroxidase 5 ng/ml (Sigma-Aldrich) was incubated with 0.03 µg/µl of the flavonoids, hydrogen peroxide (50 mM) and the substrate OPD (Thermo Scientific, Rockford, IL, USA). The activity was measured at 492 nm after 30 min of the reaction. The inhibition (%) was calculated considering as 100% of activity the value obtained for the sample without flavonoid. All tests were performed in triplicated.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 and Graph Pad 6. The *t* test, Kruskal-Wallis test, one-way ANOVA, and two-way ANOVA with corrections for multiple comparisons were used when applicable. p-values < 0.05 were considered significant for all assays.

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Legends to figures

Figure 1: Alt a 1 protein is located inside *A. alternata* spores. (A) Immunohistochemistry of Alt a 1 in spores at different times. The protein was detected using specific antibodies (Alexa 488) and labeled in green. (B) Spores containing Alt a 1 were quantified at different times and represented as percentage of Alt a 1-positive spores. (C) Alt a 1 release is pH-dependent. Spores (10^6) were incubated at different pHs and the quantity of Alt a 1 protein was quantified by ELISA using a specific polyclonal antibody.

Figure 2: Alt a 1 is released transporting a flavonoid ligand. (A) The ligand ($0.06 \mu\text{g}/\mu\text{l}$) purified from Alt a 1 was incubated with DPBA (specific flavonoid staining, 0.25% w/v). Fluorescence intensity was measured in the TECAN GeniosPro system ($\lambda_{\text{exc}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 535 \text{ nm}$). (B) MS-MALDI TOF analysis of the ligand. (C) MS/MS product ion mass spectrum.

Figure 3: Alt a 1 binds its ligand in a concentration-dependent manner. (A) Dynabeads M-280 Tosylactivated were coated with Alt a 1 and incubated with its ligand. The percentage of Alt a 1-Dynabeads bound to ligand is represented. (B) Increasing amount of Alt a 1 (0.0-0.50 μg) was incubated with its ligand (1 μg) and stained with DPBA and dot-blot onto nitrocellulose membrane. (C) Spores (10^6) of *A. alternata* were incubated at different pH's during 1 hour at 25°C . The presence of the ligand was detected with DPBA staining (0.25% w/v) in the TECAN GeniosPro system ($\lambda_{\text{exc}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 535 \text{ nm}$). Quercetin was used as flavonoid model.

Figure 4: Effect of the ligand of Alt a 1 in *Arabidopsis thaliana* roots. (A) Root growth inhibition by treatment with the ligand of Alt a 1 measured as percentage of inhibition with respect to root growth without ligand ($n = 12$). *P –values < 0.05 by T-Test. (B) HRP activity inhibition measured as percentage of inhibition with respect to total HRP activity ($n = 5$). *P –values < 0.05 by T-Test.

Figure 5: Proposed structure for the ligand of Alt a 1. Structural formula of the flavone compound with $\text{C}_{20}\text{H}_{20}\text{O}_7$ composition and molecular weight 372 proposed for the flavonoid ligand of Alt a 1. The catechol moiety (B-ring) of quercetin involved in plant root growth inhibition and ROS detoxification is preserved. The six positional isomers indicated in the table regarding two methyl and two methoxy substituents in the A-ring are compatible with the proposed structure.

Figure 1

B

C

Figure 2

Figure 3

Figure 4

Figure 5

3.5 The immunoactivity of Alt a 1, a fungal β -barrel allergen

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Doctoral candidate's contribution to this work:

Quantification of Alt a 1 from *Alternaria alternata* spores at different pH values

Quantification of the IgE-binding ability

Cell culture assays

Measurement of the immunogenic capacity of Alt a 1

Immunoprecipitation and size exclusion chromatography

Writing of a first draft of the manuscript and elaboration of figures

Abstract

Alt a 1 protein is the major allergen from the fungus *Alternaria alternata* and responsible for chronic asthma, yet little is known about its physiological role and immunological activity. Our main purpose was to investigate the mechanism through which Alt a 1 induces an allergic response in bronchial epithelium. Its recently reported crystal structure was claimed to be found exclusively in fungi and had no equivalent in the Protein Data Bank. Although Alt a 1 has in fact a unique topology, data obtained *in silico* by using different structural alignment methods show that this allergen presents some structural relationships with a number of other β -barrel proteins such as human lipocalin 2 (LCN2). Besides, our experimental data demonstrate that Alt a 1 is also able to interact with LCN2 as a transporter through the epithelium. Results reported here are significantly relevant for elucidating the molecular mechanisms that lead to allergic sensitization to Alt a 1. Once Alt a 1 was in the basolateral side, it is able to induce an inflammatory response inducing activation of immune cells and imbalance towards Th2 response. Our findings help elucidate the molecular events that lead to the immune response to Alt a 1 and can be also of interest for the development of new immunotherapeutic strategies.

Short summary: Alt a 1 is able to interact with LCN2 in the presence of a flavonoid, passing across the bronchial epithelium and skewing immune response towards Th2.

Keywords: Alt a 1, fungal allergen, LCN2, bronchial epithelium, β -barrel fold, *Alternaria alternata*

Additional information

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Introduction

Alt a 1, the major allergen of *Alternaria alternata*, has been described as the most important fungal agent associated with respiratory allergies and severe asthma (1, 2). In Southern Europe, over 20% of the patients with a history of respiratory allergy were sensitized to this fungus (3), and in some cases, they were monosensitized to Alt a 1 (1, 2, 4, 5).

Alt a 1 is a heat-stable, 30 kDa homodimer protein which dissociates into 14.5- and 16-kDa subunits under reducing conditions (6). Its crystal structure shows an all- β architecture composed of 11 β -strands arranged in the form of a β -barrel which has been reported not to have equivalent in the Protein Data Bank (PDB) (4). Besides hydrophobic and polar interactions between subunits, Alt a 1 dimer is presumably linked by a disulfide bond between Cys30 residues in the two chains (4).

Little is known regarding the immunological properties of Alt a 1. The mechanisms of sensitization to Alt a 1 are poorly understood and most data have focused on allergic reactions of the respiratory tract rather than on the initial steps of allergic sensitization. It could be hypothesized that sensitization occurs when allergens interfere with our immune system, and hence the nature of the allergens should be highly relevant in this process. It is worth mentioning here that only a limited number of protein families are actually able to induce allergic sensitization.

The characteristics that make a protein an allergen have been investigated for years (7). It was long thought that the answer would be somehow encoded in the aminoacid sequence itself. However, the characterization of epitopes in a large number of allergens has demonstrated that very often they correspond to conserved residues between members of the same protein family, regardless of their allergenicity (8). Other biochemical features of proteins that could be related to their allergenic behavior have been demonstrated only in a few cases such as enzyme activity in Der p 1 (9) or papain (10) but these relationships are far from widespread acceptance.

Studies on structural motifs that might be involved in the allergic response are still relatively scarce even though there is increasing evidence pointing to the existence of conserved folds with similar biological function in allergens. In this regard, the case of Der p 2, a major allergen from dust mite, has been well-studied. This protein belongs to the cystein protease family but it shares structural homology with the ML-domain protein MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signalling complex that plays a key role in activation of innate immunity (11). Der p 2 has an internal cavity occupied by a hydrophobic ligand in an immunoglobulin-like structure which has been shown to be essential to direct interaction with TLR4 and subsequent induction of immune response (12). The chase for ligands in allergology is attracting increasing interest in recent

years as illustrated for instance in the identification of quercetin as a natural ligand of Bet v 1, the major birch pollen allergen (13).

In that endeavor, we add here the case of Alt a 1. Aimed to study the relationship between its allegedly unique structure and its behaviour as a respiratory allergen, we show in this report that Alt a 1: (i) bears a certain structural resemblance to other β -barrel proteins, particularly some lipocalins, (ii) also carries a ligand, and (iii) is able to pass across the bronchial epithelium and interact with human lipocalin 2 (LCN2) in the presence of that ligand. Once Alt a 1 is located in the basolateral side, it could interact with other immune cells immunomodulating towards Th2-response. These results not only are helpful to elucidate the molecular events involved in immune response to the presence of allergens but they could be also of interest in the search for new immunotherapeutic strategies.

Results

Alt a 1 bears structural resemblance to other β -barrel proteins such as LCN2

The crystal structure of Alt a 1 protein (4) was used to firstly explore structural similarities by means of the systematic structural comparison tool available in the PDB. This search identified only four proteins (entries 2Q03, 2LFU, 4RLC, and 3Q6L). Being the β -barrel the major structural feature of Alt a 1, those four proteins displayed in fact that substructure. However, since barrel structures are frequently found in porins and other membrane proteins as well as in proteins that bind mainly hydrophobic ligands, we widened the structural similarity analysis while restricting the search to ligand-binding targets such as fatty-acid binding proteins (FABPs) and siderophore-binding proteins such as lipocalins.

To this end we searched for entries in the PDB having 3D similarity with the four proteins mentioned in the preceding paragraph and compared the located structures with Alt a 1. All candidates found were indeed β -barrel proteins. This analysis was accomplished with three methods that follow rather different approaches to the structural alignment problem: FATCAT (13), CE (14) and TM-Align (15). The most significant results are collected in Tables 1 and 2. The particular superpositions of Alt a 1 and two representative members of the two types of ligand-binding β -barrel proteins here considered are displayed in Fig. 1

When needed, FATCAT allows for a reduced number of rigid-body movements around pivot points (twists) to identify superimposable structural regions in the proteins being compared. Resorting to one or more twists is penalized in computing the superposition score. On the contrary, both CE and TM-Align methods achieve structural alignments by keeping the structures rigid. The significance levels of their score parameters may be summarized as follows (13–15). In FATCAT, a P-value = 0.05 sets the threshold to consider that two structures have some relationship and lower P-values are associated to closer structural resemblance. In CE, a Z-score > 4.5 means that proteins belong to a single structural family, a value between 4.5 and

4.0 indicates superfamily or fold-level structural similarity, and a Z-score between 4.0 and 3.5 suggests just some structural relationship. In TM-Align, a TM-score > 0.5 reveals that the structures share the same fold, a value between 0.5 and 0.45 indicates highly significant similarity and a TM-score between 0.45 and 0.35 hints at some structural similarity. Therefore, lower P-values and higher Z-score and TM-score values mean better structural similarities.

Table 1 gathers a selection of β -barrel proteins displaying structural similarity with Alt a 1 considered significant according to the preceding score intervals. Additional information is also given in Table 2 for structural superpositions of Alt a 1 and two FABPs (rat odorant-binding protein and dog allergen Can f 4, PDB codes 3FIQ and 4ODD, respectively) together with human LCN2 (PDB code 1L6M) included as a lipocalin of particular interest in humans, as discussed below. Note in Table 1 that (a) there are several proteins with structural resemblance to Alt a 1 besides the four entries initially identified by the 3D similarity tool of the PDB, some of them showing greater resemblance than 4RLC and 3Q6L, (b) the three methods predict similar significant relationships, (c) there are a number of allergens, and (d) FATCAT needs no twists for detecting structural relationships for more than half of the proteins.

Note in Table 2 the large coverage of Alt a 1 residues and the reasonable RMSD in most of the structural comparisons although in the case of Alt a 1/LCN2 comparison, the three methods predict an overall weaker resemblance. As for these results (Tables 1 and 2, Fig. 1) and according to the significance levels found, one might conclude that the claimed uniqueness of the β -barrel fold in Alt a 1 (4) does not preclude the existence of structural relationships with other proteins, particularly high in some cases. However, it must be stressed that this resemblance refers to 3D architecture but not to protein topology as a search for topological relationships using PTGL (17) (results not shown) gave no results. This suggests that even though the β -barrel scaffold in Alt a 1 bears merely spatial similarities to FABPs and some lipocalins, the major allergen from *Alternaria* seems in fact to have a unique topology.

The pH of the medium has effect on IgE-binding to Alt a 1

At present, two oligomeric states of the Alt a 1 structure have been characterized. The most stable in thermodynamic terms is the tetramer that is also stabilized by the presence of ligands similar to the well-known flavonol quercetin. This aggregate defines a 3D structure in which the IgE epitope regions identified for Alt a 1 are buried. The second assembly is a symmetric dimer in a "butterfly-like" shape stabilized by an intermolecular disulfide bridge. This dimer, that binds no ligand, displays the IgE epitopes on the protein surface spatially arranged in such a manner that they favor cross-linking interaction with the immunoglobulin.

It has been demonstrated (16) that the oligomerization state of Alt a 1 happens to depend on the pH in the surrounding medium. To address the influence of pH on the antigen-antibody interaction, the IgE binding capacity of Alt a 1 was measured at different pH's. As

shown in Fig. 2A, higher recognition was obtained when Alt a 1 was coated at pH 6.5, a result confirmed with on coating plates with supernatant from spores germinated at different pH (Fig. 2B). Alt a 1 was secreted initially at pH 4 and its release decreased at pH 7. Upon measuring IgE-binding ability using sera from patients allergic to *Alternaria alternata*, Alt a 1 was able to bind IgE only between pH 5.5 and 7.5.

Alt 1 is secreted by Alternaria alternata spores when they contact bronchial epithelium.

To study the behaviour of Alt a 1 in bronchial epithelium, normal human bronchial epithelial cell line (NHBE, Lonza) was grown in appropriate media, following the manufacturer's recommendations. Cells were seeded in collagen-coated transwell. After 3 days, the airlift was established (ALI), the apical medium were removed and cells were grown in the differentiation medium for 20 days. Differentiated monolayer was characterized using specific antibodies against B-tubulin IV (to evaluate ciliogenesis) and against MUC5A (to test the presence of goblet cells) (17). After that, *Alternaria alternata* spores (10^6) were deposited on the apical side and the induction of gene expression of characteristic epithelial cytokines (TSLP, IL33 and IL25) was quantified by real time PCR at different times (Fig 3A). Thymic stromal lymphopoietin (TSLP) production was especially increased in the first hours while production of IL25 and IL33 was more moderate.

Release of Alt a 1 over time was quantified by ELISA assays (Fig 3B). Alt a 1 secretion was produced in the first hours, although no hyphal development was observed by trypan blue staining (data not shown). Alt a 1 secreted from spores in the apical side was transported through the monolayer to basolateral side. Thus, basolateral supernatants were recovered over time and Alt a 1 was quantified by the same ELISA assays. This way, the maximum amount of Alt a 1 was observed after 10 hours (Fig 3C). By contrast, no disturbance could be observed in monolayer permeability by phalloidin staining (data not shown).

Alt a 1 retains its immunological activity after crossing epithelia.

After crossing epithelium, Alt a 1 was able to interact with immune cells, leading to activation and inducing the allergic process. Thus, the immunogenic capacity of Alt a 1 was assessed by measuring the proliferation of human peripheral blood mononuclear cells (PBMCs).

As shown in Figure 4A, PBMCs were activated at concentrations lower than 2.5 $\mu\text{g/ml}$ of Alt a 1. When higher amounts were used, induction of cell death was produced as tested by trypan blue staining. Therefore, Alt a 1 was able to induce a PBMCs activation. PBMC activation was produced in a concentration dependent manner and amounts larger than 2 $\mu\text{g/ml}$ resulted in induction of death cell.

With the aim to attempt the characterization of the PBMC activation pathway, human

monocyte-derived cell line (THP1-Xblue) was used. This cell line was transfected with a reporter expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by transcription factors NF- κ B and AP-1. THP1-XBlue™ cells respond to ligands for TLR2, TLR1/2, TLR2/6, TLR4, TLR5 and TLR8. Transcription of NF- κ B and AP-1 was detected upon stimulation with Alt a 1. This effect was completely inhibited by anti-TLR2 specific antibodies (Invivogen) but not by anti-TLR4 antibodies (Invivogen) (Fig 4B). In the case of PMBCs, both antibodies inhibited PMBCs proliferation (Fig 4C), so we found different responses to both types of THP-1 and PBMCs cells.

Alt a 1 binds to LCN2 only in the presence of quercetin

LCN2 expression was increased in the presence of *Alternaria alternata* spores as measured in the supernatant of NHBE culture by ELISA (Fig 5A). Based on the structural relationship between LCN2 and Alt a 1 as well as on the induction of LCN2 expression in the presence of spores, we studied the possibility that both proteins could mutually interact as an entry mechanism for the allergen.

Pull down assays were performed with Dynabeads M-280 Tosyl-activated coated to Alt a 1 and incubated with LCN2 (Sigma- Aldrich, Madrid, Spain). When the retained was separated by SDS-PAGE and stained with Coomassie (Fig 5B), a band around 20 kDa was observed. This result confirmed that Alt a 1 is able to bind to LCN2.

Human lipocalin binds siderophores with chelating groups (catechol) similar to those of quercetin and the presence of this flavonol also influences the capacity of Alt a 1 to oligomerize (18). For this reason, we studied the possible Alt a 1-LCN2 interaction in the presence and in the absence of quercetin (Fig 5C). Equal amounts of Alt a 1 and LCN2 were separated in exclusion size chromatography with and without quercetin, using buffers at different pHs. By comparing elution profiles, we found that both proteins were in fact able to interact yielding an apparent heterodimeric form only when quercetin was present (Fig 5D).

Discussion

When *Alternaria alternata* spores arrive at bronchial epithelium, no hyphae development is observed but TSLP mRNA expression is induced. At that stage, Alt a 1 is released in high amounts and secreted in tetrameric form carrying a flavonoid ligand as previously described (16). However, when the allergen crosses the monolayer through a transporter-mediated route assisted by LCN2, local variations of pH in the medium and the presence of this lipocalin lead to tetramer breakdown releasing the monomeric subunits. Since it is known that local monomer concentration may promote formation of transient dimers (provided that the protein has symmetry-stabilized dimers as it is the case for Alt a 1), this oligomeric state would enable the allergen to interact with the antibody. This interaction is

besides favored by the location of epitope regions on the protein surface in proper orientation for crosslinking. This way, Alt a 1 acquires IgE-binding capacity and is thus able to trigger the immune response.

Although Alt a 1 has been characterized as a unique β -barrel protein, our structural comparison study revealed a structural resemblance to a greater number of β -barrel proteins than initially expected (4). Three methods that follow different algorithmic approaches to the structural alignment problem (FATCAT, CE, and TM-Align) were employed so that the combination of their score parameters allowed us to quantify that resemblance. Among the newly identified β -barrel proteins structurally related with Alt a 1, there are lipocalin-type allergens. As for the particular case of human LCN2 protein, while the three methods yielded score parameters that did not indicate the closest structural resemblance with Alt a 1, their values were still within the range that suggests structural relationship. However, we also found that from a topological standpoint, Alt a 1 structure has apparently no related known topological motifs, a result which hints at a topological rather than architectural uniqueness of the *Alternaria* allergen.

LCN2 is a protein involved in host defense. Its expression has been reported in healthy tissues being upregulated in acute and chronic inflammatory diseases (18, 19) such as allergy. The role of LCN2 in inflammation process is still unclear, but it is known that NF- κ B acts as a positive regulator of LCN2 expression (20). We have observed in this work that the presence of *Alternaria* spores induces increase in LCN2 in the apical side of the epithelium polarized monolayer as a short term response. In the presence of a siderophore-like ligand such as quercetin, LCN2 could interact with Alt a 1 forming a heterodimer in the apical side of bronchial mucose. In this heterodimeric form, Alt a 1 and LCN2 can be recognized by the LCN2 receptor expressed on the apical surface of polarized epithelial cells (21, 22), thus allowing endocytosis of the allergen through the bronchial epithelium. In the late endosome, the LCN2-Alt a 1-quercetin complex may dissociate due to the acidic pH in this compartment (Figure 6).

Once inside cell, Alt a 1 could reach the basolateral side of the monolayer showing its full immunogenetic activity such as IgE binding, PMBC activation and induction of NF- κ B expression in THP1-XBlue™ cells. This way, one may conclude that it is Alt a 1, not its ligand, which was able to activate immune cells. This ability is mediated by TLR2 and TLR4 route as confirmed by antibodies inhibition assays.

Thus, the results reported in the current work are significantly relevant for elucidating the molecular mechanisms that lead to allergic sensitization to Alt a 1. As described, Alt a 1 not only revealed a certain structural relationship with LCN2 identified by different structural alignment methods, but the allergen can also interact with LCN2 as a transporter through

epithelium. Once located in the basolateral side, Alt a 1 is able to induce a inflammatory response inducing activation of immune cells and the imbalance towards a Th2 response.

Methods

Structural analyses

The recently reported crystal structure of Alt a 1 protein (PDB entry 3V0R, (4)) was used for structural comparisons performed with the “Sequence & Structure Alignment” tool implemented in the PDB. The following structural alignment methods were employed: (a) FATCAT, a method that optimizes a flexible structural alignment of fragment pairs minimizing the number of rigid-body movements (twists) around pivot points (14); (b) CE, a method based on a combinatorial extension (CE) algorithm of a structural alignment path defined by aligned fragment pairs (15); and (c) TM-Align, an algorithm to identify the best structural alignment between protein pairs that combines the template modeling (TM) score rotation matrix and dynamic programming (16). The significance of structural relationships was quantified by means of their corresponding score parameters: P-value in FATCAT (14), Z score in CE (15), and TM score in TM-Align (16). In parallel, a search for topological relationships with Alt a 1 was performed using the Protein Topology Graph Library (PTGL) tool (17).

The initial 3D similarity search for Alt a 1 identified only four proteins that were subsequently used as reference for wider searches. Among the proteins sampled this way, we finally selected a representative set of members to illustrate the structural relationship of Alt a 1 with a number of other β -barrel proteins. LCN2 protein (human lipocalin 2, PDB entry 1L6M, (29)) was separately chosen because it has a β -barrel architecture and is highly expressed in the lung, where Alt a 1 sensitization occurs.

Human samples

Sera from patients allergic to *A. alternata* were kindly provided by the Allergy Service of the Clinical Hospital of Barcelona. Buffy coats (cell-enriched plasma) samples were obtained from four healthy donors at the Transfusion Centre (Madrid, Spain) for isolation of peripheral blood mononuclear cells (PBMCs). The study was approved by the Ethics Committees of the Transfusion Centre (Madrid, Spain) and the Technical University of Madrid (Madrid, Spain).

Isolation of Alt a 1 protein

Alt a 1 was isolated from *Alternaria alternata* spores collected from a ecologic crop as previously described (23).

pH influence on IgE recognition

To study the effect of pH on IgE recognition, 10^6 spores of *A. alternata* or purified Alt a 1 ($5\text{ }\mu\text{g/}\mu\text{l}$) were coated in ELISA plates during 2 hours at 37°C in media with different pH's. After blocking, plates were washed and incubated with a polyclonal anti-Alt a 1 antibody (1:1000, Bial Aristegui) or a pool of sera of patients sensitized to *A. alternata*. The correct secondary antibody was used linked to HRP. Data were gathered by measuring absorbance at 405 nm in a microplate reader. All tests were performed in triplicate.

Bronchial epithelial cells culture

Primary normal human bronchial epithelial cells (NHBE) were purchase from Lonza (Walkersville, Md). Cells were growth in BEGM medium (Lonza) containing SingleQuot (Lonza) with L-glutamine (Lonza), 10% heat-inactivated fetal bovine serum (Thermo Scientific Hyclone, UK Ltd), and $100\text{ }\mu\text{g/ml}$ antibiotics (Sigma-Aldrich S.A.). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and were passaged weekly upon reaching confluence.

Cells were seeded in 24-well Transwell® inserts (Inc., Tewksbury MA, USA) coated with collagen type IV (Sigma-Aldrich S.A) for 1 hour at 37°C and a density of 5×10^5 cells/well. Initially, the cells were kept submerged in a 50:50 mix (vol: vol) of BEGM:DMEM and the medium was replaced every two days until cells reached confluence. Between days 4 and 7, the apical medium was removed while the basolateral medium was changed daily. Assays were performed between days 18 and 21 after the air-liquid interface was established.

Immunohistochemistry

Cells were fixed in paraformaldehyde 4% for 15 min at 4°C and washed three times with PBS. Cells were blocked for one hour with casein blocking buffer (Sigma-Aldrich S.A.) and then incubated in primary antibody at 4°C overnight. Cells were washed three times with PBS and incubated for one hour in secondary antibody at room temperature. Images were taken on a Zeiss LSM 710 confocal microscope. Ciliogenesis was evaluated by occurrence of the β -tubulin marker using an anti- β IV tubulin antibody (Pierce) and mucus secretion was evaluated using anti- MUC5B antibody produced in rabbit (Sigma-Aldrich S.A.).

Transport studies across epithelial cells

Cell monolayers of epithelial bronchial line were used in transport studies when values of TEER reached a plateau exceeding $300\text{ }\Omega\text{cm}^2$. 10^6 spores of *A. alternata* were added to the apical side of the Transwell® inserts and it was incubated at 37°C , 5% CO_2 . After 2, 4, 6 and 24 h, basolateral and apical supernatants were collected and Alt a 1 content was determined by ELISA with specific polyclonal anti-Alt a 1 antibody (1:1000, Bial Aristegui, Bilbao, Spain).

The amount of Alt a 1 was quantified using a standard curve of known quantities of quercetin. All tests were performed in triplicate. The integrity of the cell monolayer was tested by final staining with specific polyclonal antibody anti-tubulin.

Quantification of cytokine expression by real time-PCR

Expression of epithelial cytokines TSLP, IL33 and IL25 in the presence of 10^6 spores of *A. Alternata* spores was determined by real time-PCR. Bronchial epithelial cells were recovered from the Transwell® inserts (Corning Inc.) and mRNA was isolated as previously described (24).

The reverse transcription was carried out using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions and cDNA was amplified using the Power SYBR Green PCR Master Mix (Life technologies, UK Ltd) according to manufacturer's recommendations and ran on an Applied Biosystems 7300 real-time detection system (Life technologies). cDNA was amplified using the following primers: TSLP (F: 5'-GGGTGTCCACGTATGTTCCCT-3'; R: 5'-GCCGATTGCTACAAGAGACGG-3'), IL25 (F: 5'- GAGTCCTGTAGGGCCAGTGAAG-3'; R: 5'-CGCCGGTAGAAGACAGTCTGG-3'), IL33 (F: 5'-GGTACTCGCTGCCTGTCAAC-3'; R:5'-ACCATCAACACCGTCACCTG-3'), and elongation factor EF1 as an endogenous control (F: 5'-CTGAACCATCCAGGCCAAAT-3'; R: 5'-GCCGTGTGGCAATCCAAT-3').

The amount of target mRNA expression was normalized with endogenous control EF-1 and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta Ct}$), as described by Livak and Schmittgen (25). Changes in gene expression were calculated with respect to untreated NHBE cells. All amplifications were carried out in triplicate from three different experiments.

PBMCs proliferation

PBMCs were isolated from 4 buffy coats to density gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cultures were established in triplicate in 96-well plates (Costar, NY, USA) at 2×10^5 cells per well for proliferation analysis in a total volume of 200 μ l of RPMI media (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum, PenStrep cocktail (50 U/ml-50 μ g/ml) in the presence of different Alt a 1 quantities (2.5, 5, 25 μ g/ml). LPS (Sigma-Aldrich S.A.) and PBS were used as a positive and negative control respectively. PBMCs were incubated with the stimuli for 5 days at 37°C, 5% CO₂.

Proliferation percentage was calculated in a Neubauer chamber and the results were expressed as stimulation index (SI) calculated as the ratio between stimulated and non-stimulated PBMCs. A result was considered positive when this value was higher than 2.

NF- κ B/ AP1 activation

The transfected cell line THP1-XBlue™ (Invivogen, Toulouse, France) derived from the human monocytes THP-1 cell line was used following manufacturer's instructions. In brief, THP1-XBlue cells were seeded at a density of 1×10^6 cells/ml in cRPMI medium (RPMI 1640, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, PenStrep cocktail (50 U/ml-50 µg/ml) and 200 µg/ml of Zeocin). Cells were treated with Alt a 1 (5 µg/ml), ligand (0.5 µg/ml) and Alt a 1-ligand complex (5 µg/ml + 0.5 µg/ml) for 24h. LPS (Sigma-Aldrich S.A.) and PBS were used as positive and negative controls, respectively. 20 µl of cell suspension (~200,000 cells) per well were added to a flat-bottom 96-well plate containing 180 µl of QUANTI-Blue™ per well and incubated for six hours. Soluble Embryonic Alkaline Phosphatase (SEAP) levels were determined using a spectrophotometer at 620 nm. The results were expressed as stimulation index (SI) calculated as the ratio between stimulated and non-stimulated cells.

To determine the receptor implicated in molecular recognition, cells were previously incubated with 1 µg of polyclonal antibodies anti-TLR2 and anti-TLR4 (Invivogen).

Immunoprecipitation assays

Dynabeads® M-280 Tosylactivated (Invitrogen, UK Ltd) were conjugated with Alt a 1 (0.2 µg/µl) and incubated with LCN2 (0.01 µg/µl) following manufacturer's instructions. After extensive washing, coated beads were resuspended in Laemmli buffer and separated in 15% SDS-PAGE.

Size exclusion molecular chromatography

Size exclusion chromatography was performed at pH 6.5 on an Enrich™ SEC 70 column (Bio-Rad). The column was equilibrated in ammonium acetate and the chromatography was carried out at a flow rate of 0.65 ml/min, measuring absorbance at 280 nm. The column was calibrated using standard proteins with known molecular weight. Elution profiles of 50 µg of Alt a 1 and 50 µg of Alt a 1 plus 50 µg of LCN2 were measured in the presence and in the absence of quercetin.

Statistical analyses

GraphPad Prism 6.01 software was used for statistical analyses. Results were compared by applying Wilcoxon test for paired samples. p-values lower than 0.05 were considered significant in all the analyses.

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Figure legends

Figure 1. Alt a 1 shows structural resemblance to lipocalins and FABPs. Ribbon diagrams of structural superpositions: **A)** Alt a 1 (magenta) and rat odorant-binding lipocalin (PDB code: 3FIQ, pale green) obtained with CE structural alignment method. **B)** Alt a 1 (magenta) and an insect FABP (PDB code: 1MDC, pale orange) obtained with FATCAT (no twists) method.

Figure 2: IgE binding capacity of Alt a 1 depending on pH. **A)** Alt a 1 (5 $\mu\text{g}/\mu\text{l}$) was coated in ELISA plates at different pH's. The recognition was evaluated with a pool of sera from patients sensitized to *A. alternata*. **B)** Fungal spores were incubated at different pH's and the amount of Alt a 1 in the medium was evaluated by ELISA with a specific antibody (PAb) and a pool of sera from patients sensitized to *A. alternata*.

Figure 3: Alt a 1 secretion in contact with bronchial epithelial cells. **A)** TSLP, IL25 and IL33 relative gene expression levels. Results expressed in fold change comparing non-stimulated with stimulated conditions. In all cases, data represent the average of three independent experiments. **B)** Alt a 1 (μg) quantified in the apical side of NHBE monolayer by ELISA assays. **C)** Accumulation of Alt a 1 (μg) measured in the basolateral side by ELISA assays. Results correspond to four independent experiments. Mean and SD (bars) values are shown.

Figure 4. Immunological activity of Alt a 1. **A)** Dose-response curve of PBMCs activation incubated with different amounts of Alt a 1 for 5 days at 37°C, 5% CO_2 . **B)** PBMCs were previously incubated with specific antibodies against TLR2 and TLR4. **C)** THP1. All results expressed as stimulation index calculated as ratio between stimulated and non-stimulated cells. Statistical significance ($p < 0.05$) obtained with Wilcoxon test for paired samples ($n=5$) is shown.

Figure 5: LCN2 and bronchial epithelial cells. **A)** LCN2 release is induced by the presence of *A. alternata* spores. Amount of LCN2 measured by ELISA assays in the apical side of the transwell. **B)** Dynabeads M-280 Tosylactivated were coated with Alt a 1 and incubated with LCN2. The result was separated by SDS-PAGE and stained with Coomassie Blue R250. **C)** Elution profile comparison of Alt a 1 and Alt a 1 plus LCN2 with and without quercetin (5 μg) in ammonium acetate pH 6.5. **D)** Ribbon diagram of an initial structure of the Alt a 1-LCN2 complex modeled upon superposition of Alt a 1 and one subunit in LCN2 dimer, removal of this subunit, and geometry refinement to fix steric clashes. Alt a 1 is shown in blue and LCN2 in orange.

Figure 6: Proposed model for the molecular mechanism followed by Alt 1 to cross through the bronchial epithelium. See text in Discussion section.

Table 1. β -barrel proteins with experimental structure and structural similarity to Alt a 1

Scores of structural alignments obtained with FATCAT, CE, and TM-Align structural comparison methods. Results ranked by FATCAT *P-value*; N_{tw} is the number of twists in this method

Protein	PDB code	FATCAT <i>P-value</i>	N_{tw}	CE <i>Z-score</i>	TM Align <i>TM-score</i>
Uncharacterized protein (<i>Shewanella denitrificans</i>)	2Q03	3.20×10^{-4}	0	4.25	0.490
Insect FABP (<i>Manduca sexta</i>)	1MDC	4.75×10^{-4}	0	3.70	0.469
Heparin-binding antigen (<i>Neisseria meningitidis</i>)	2LFU	5.03×10^{-4}	0	4.42	0.496
Rat odorant binding protein 1 (<i>Rattus norvegicus</i>)	3FIQ	9.19×10^{-4}	0	4.58	0.460
Dog Can f 4 allergen (<i>Canis lupus</i>)	4ODD	1.04×10^{-3}	0	4.58	0.445
Milk whey trichosurin (<i>Trichosurus vulpecula</i>)	2R74	2.46×10^{-3}	0	4.07	0.447
Fluorescent FABP-like (<i>Coturnix japonica</i>)	4I3B	2.47×10^{-3}	1	3.70	0.448
Rat odorant binding protein 3 (<i>Rattus norvegicus</i>)	3ZQ3	3.64×10^{-3}	0	4.07	0.439
Chicken liver FABP (<i>Gallus gallus</i>)	1TVQ	3.67×10^{-3}	0	3.50	0.394
Small molecule transport prot. (<i>Pseudomonas feruginosa</i>)	4RLC	3.71×10^{-3}	1	3.89	0.476
Rat intestinal FABP (<i>Rattus norvegicus</i>)	1IFC	5.08×10^{-3}	1	3.70	0.452
Bovine BLG in complex with retinoic acid (<i>Bos taurus</i>)	1GX9	5.66×10^{-3}	0	4.25	0.439
Caprine BLG, orthorhombic form (<i>Capra hircus</i>)	4OMW	7.53×10^{-3}	0	4.07	0.438
Apo bradavidin2 (<i>Bradyrhizobium diazoefficiens</i>)	4GGR	8.69×10^{-3}	0	3.89	0.491
Sheep BLG, P3121 group (<i>Ovis aries</i>)	4NLI	8.93×10^{-3}	0	4.07	0.435
Human adipocyte FABP 4 (<i>Homo sapiens</i>)	3Q6L	1.06×10^{-2}	1	3.83	0.452
Violaxanthine de-epoxidase (<i>Arabidopsis thaliana</i>)	3CQN	1.31×10^{-2}	1	3.70	0.411
Female hamster pheromone (<i>Mesocricetus auratus</i>)	1E5P	1.56×10^{-2}	1	4.42	0.450
Dog Can f 2 allergen (<i>Canis lupus</i>)	3L4R	2.21×10^{-2}	1	4.07	0.436
Major horse allergen Equ c 1 (<i>Equus caballus</i>)	1EW3	3.01×10^{-2}	1	4.07	0.434
Human lipocalin 2 LCN2 (<i>Homo sapiens</i>)	1L6M	3.02×10^{-2}	1	3.70	0.399
Ferriheme nitrophorin 4 (<i>Rhodnius prolixus</i>)	4HPA	3.52×10^{-2}	1	3.70	0.457

Table 2. Parameters of structural superposition of Alt a 1 and some β -barrel proteins

Scores of structural alignments computed with FATCAT (*P-value* and number of twists N_{tw}), CE (*Z-score*), and TM Align (*TM-score*) structural comparison methods together with number of residues of Alt a 1 (percentage of the total in parentheses) and RMSD in the superposition

Protein	FATCAT		CE		TM-Align	
<i>Protein type</i>	<i>P-value</i>	N_{tw}	<i>Z-score</i>		<i>TM-score</i>	
<i>(PDB code)</i>	<i>N.res</i>	<i>RMSD</i> (Å)	<i>N.res</i>	<i>RMSD</i> (Å)	<i>N.res</i>	<i>RMSD</i> (Å)
Insect FABP (1MDP)	4.75×10^{-4}	0	3.70		0.469	
	80 (62%)	3.08	92 (71%)	4.37	89 (68%)	3.76
Chicken FABP (1TVQ)	3.67×10^{-3}	0	3.50		0.394	
	84 (65%)	2.78	74 (57%)	3.48	80 (62%)	4.05
Rat odorant binding protein (3FIQ)	9.19×10^{-4}	0	4.58		0.460	
	79 (61%)	3.12	84 (65%)	3.61	91 (70%)	3.94
Dog allergen Can f 4 (4ODD)	3.67×10^{-3}	0	3.50		0.394	
	84 (65%)	2.78	74 (57%)	3.48	80 (62%)	4.05
Human LCN2 (1L6M)	3.02×10^{-2}	1	3.70		0.399	
	89 (68%)	3.41	77 (59%)	4.16	92 (71%)	4.67

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

4. DISCUSSION

DISCUSSION

The characterization of most plant and fungal allergens from both biochemical (biological activity) and physiological (role in the source organism) points of view is still rather incomplete. However, the molecular mechanisms underlying protein activities and physiological roles are often converged in nature. It is therefore quite possible that identifying the role of allergens in their natural sources may help better understand the mechanism of allergic sensitization in humans. For this purpose, the fungus *Alternaria alternata*, a pathogen that causes major losses in agricultural crops, was selected as a model.

Alt a 1, the major allergen of *Alternaria alternata* present inside the spores, is not secreted in an apo-form but it carries a methylated flavonoid similar to quercetin (a plant flavonol model). However, if one analyzes the X-ray crystallographic structure of Alt a 1 deposited in the PDB [1], it is difficult to predict a binding site in the monomer. On the other side, the study of possible oligomerization states of the protein demonstrates that Alt a 1 shows two major aggregates: a “butterfly state” that corresponds to the dimeric form and a “basket state” that corresponds to the tetrameric form. The conversion between them is dependent on the pH of the surrounding medium and on the presence of a flavonoid-like ligand.

At pH 6.5, which Alt 1 would meet when secreted by spores in bronchial epithelium, the protein is in its “basket state” and carries two units of the flavonoid ligand bound at two dimeric interfaces in the tetramer. However, at pH 5.5 which Alt a 1 would meet in plant cell apoplast when infecting plants, the tetramer breaks down releasing monomeric units and the ligand. The monomer of Alt a 1 acts as an effector, interacts with plant defense proteins (PR5) produced by the plant as a response to the presence of fungal spores, and inhibits PR5 activity [2]. At the same time, the free flavonoid can react with reactive oxygen species produced by the plant in response to the presence of the pathogen, and detoxifies the system.

On the contrary, spores of *Alternaria alternata* in air suspension, maybe lifted by rain, can be inhaled reaching the human bronchial epithelium where pH is about 6.5. One may assume then that Alt a 1 is released mostly in the “basket state” carrying the flavonoid ligand. However, it seems reasonable to think that the assembly product (tetramer) and its component units (monomers) are at equilibrium even though it can be largely displaced by pH and other environmental local effects. In contact with bronchial epithelium, spores also induce the activation of innate immune system, increasing the release of human lipocalin 2 (LCN2). In the presence of the ligand, heterodimeric LCN2-Alt a 1-flavonoid ligand complex is stabilized, which suggests that the presence of LCN2 should also provoke that the mentioned equilibrium is displaced towards the monomeric form so that the stable LCN2-Alt a 1 complex is ultimately formed. This complex could be recognized by

megalin, the endocytic receptor of LCN2 expressed on the apical surface of polarized epithelial cells [3], allowing endocytosis of the allergen through the bronchial epithelium. In late endosome, the complex dissociates because of the acidic pH there, which in turn implies that Alt a 1 cannot be in tetrameric form in this subcellular compartment. From here the lipocalin is returned to the surface by recycling vesicles whereas Alt a 1 might follow the same path or it might be processed for being presented by MHC II on the basolateral side. Since transient allergen dimers are known to form depending just on local increased concentration of monomers provoked by spatial constraints [4], Alt a 1 could be able to pass through the epithelium in dimeric form as far as the allergen present in the basolateral side is recognized by specific IgE of allergic patients. This way, Alt a 1 might cross the bronchial epithelium in endocytic vesicles and reach the basolateral side in the “butterfly state”. This fact, together with increase in Th2 cytokine production such as TSLP cause imbalance towards a Th2 response.

Figure 1: Model for the action mechanism of Alt a 1

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5. CONCLUSIONS

CONCLUSIONS

1. Alt a 1 protein from the fungus *Alternaria alternata* is an effector able to interact with plant defense proteins from kiwi (PR5) as a competitive inhibitor.
2. Stable monomeric structures, flexibility of epitope regions lower than the remaining of the structure, and capacity to form homodimers are common characteristics shared by three major allergens: Alt a 1, Bet v 1 and Pru p 3. However, they differ in dimer stability and in the flexibility of internal cavities.
3. Alt a 1 tetrameric assembly is favored in the presence of a ligand at $\text{pH} \geq 6$, but this complex breaks down at $\text{pH} 5.5$ into monomer units.
4. Alt a 1 is released carrying a ligand with $\text{C}_{20}\text{H}_{20}\text{O}_7$ composition and properties that indicate a flavonoid nature. Based upon its physical and chemical characteristics, a structure is proposed for the ligand.
5. *Alternaria alternata* spores induce the expression of TSLP and Alt a 1 protein is able to activate the immune system towards a TLR route.
6. *Alternaria alternata* spores induce the expression of human lipocalin 2 (LCN2).
7. Alt a 1 and LCN2 show a structural relationship and both are able to interact forming heterodimers.
8. The study of the physiological mechanism of Alt a 1 in plants has enabled to clarify the molecular mechanisms that induce an allergic response in bronchial epithelium.