UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

FACOLTÀ DI FARMACIA

DOTTORATO DI RICERCA
IN
SCIENZA DEL FARMACO
XXIV CICLO 2008-2011

STUDY OF PROTEINS AND PEPTIDES FROM PLANT FOODS AND THEIR POSSIBLE EFFECTS ON HUMAN HEALTH

Tutors:
Ch.ma Prof.ssa
STEFANIA ALBRIZIO

Ch.ma Dott.ssa
MARIA ANTONIETTA CIARDIELLO

CANDIDATA:
DOTT.SSA IVANA GIANGRIECO

COORDINATORE DEL DOTTORATO:
Ch.ma Prof.ssa
MARIA VALERIA D'AURIA

INDEX

ABBREVIATIONS

A: Asthma

(n)Act c 10: (natural) LTP1 from *Actinidia chinensis*

(n)Act d 10: (natural) LTP1 from *Actinidia deliciosa*

(n)Act d 5: (natural) kiwellin from *Actinidia deliciosa*

ANG: angioedema

(n)Api g 2: (natural) LTP1 from *Apium graveolens*

(r)Ara h 9*: (*recombinant) LTP from *Arachis hypogaea*

ARG-C: proteinase that preferentially cleaves the C-terminal side of arginine residues

(n)Art v 3: (natural) LTP1 from *Artemisia vulgaris*

ASP-N: proteinase that preferentially cleaves the N-terminal side of aspartic acid or glutammic acid

BCIP: 5-bromo-4-chloro-3-indolyl-phosphate

BSA: bovin serum albumin

CD: Circular Dichroism

(n)Cit s 3: (natural) LTP1 from *Citrus sinensis*

COX-2: cyclooxygenase-2

(r)Cor a 8: (recombinant) LTP1 from *Corylus avellana*

CrD: Crohn's Deasese

CRD: component resolved diagnosis

CW: cell wall

D: Diarrhea

DBPCFC: Double-Blind Placebo Controlled Food Challenge test

DMSO: Dimethyl Sulfoxide

2D-NMR: Bidimensional Nuclear Magnetic Resonance

E: Edema

EC-LPS: *Escherichia Coli* Lipopolysaccharide

ELISA: Enzyme-Linked ImmunoSorbent Assay

EST: expressed sequence tag

FPLC: Fast Protein Liquid Chromatography

(n)Fra a 3: (natural) LTP1 from *Fragaria ananassa*

GI: gastrointestinal symptoms

HFIP: hexafluoroisopropanol

(n)Horvu: (natural) LTP2 from *Hordeum vulgare*

ICAM-1: Inter-Cellular Adhesion Molecule 1

ISAC: Immuno Solid-phase Allergen Chip

ISAC79: ISAC with 79 spotted allergens

ISAC103: ISAC with 103 immobilized allergens

ISAC-Exp96: experimental ISAC with 96 immobilized allergens

(n)Lac s 1: (natural) LTP1 from *Lactuca sativa*

(n)Len c 3: (natural) LTP1 from *Lens culinaris*

(n)Lyc e 3: (natural) LTP1 from *Solanum lycopersycum*

(n)Lyc 7 kDa: (natural) LTP2 from *Solanum Lycopersycum*

LTP: Lipid Transfer Protein

(n)MAIZE: (natural) LTP2 from *Zea ma*is

(n)Mal d 3: (natural) LTP1 from *Malus domestica*

MALDI -TOF: Matrix Assisted Laser Desorption Ionization Time Of Flight

(n)Mor n 3: (natural) LTP1 from *Morus nigra*

NCBI: National Center for Biotechnology Information

NBT: Nitro Blue Tetrazolium

NR: No Result

O: Oral symptoms

OAS: Oral Allergy Syndrome

(n)Ory s 14: (natural) LTP1 from *Oryza sativa*

(n)ORYSJ: (natural) LTP2 from *Oryza sativa*

P: skin itching

(r)Par j 2: recombinant LTP1 from *Parietaria judaica*

PBS: phosphate buffer saline

(n)Pla or 3: (natural) LTP1 from *Platanus orientalis*

(n)Pru ar 3: (natural) LTP1 from Prunus *armeniaca*

(n)Pru av 3: (natural) LTP1 from *Prunus avium*

(n)Pru d 3: (natural) LTP1 from *Prunus domestica*

(n)Pru p 3: (natural) LTP1 from *Prunus persica*

(n)Pru du 3: (natural) LTP1 from *Prunus dulcis*

(n)Pyr c 3: (natural) LTP1 form *Pyrus communis*

PVDF: polyvinylidene fluoride

RP-HPLC: Reversed-Phase High-performance liquid chromatography

(n)Rub i 3: (natural) LTP1 from *Rubus idaeus*

SDS-PAGE: polyacrylamide gel electrophoresis in sodium dodecyl sulfate

SGF: Simulated Gastric Fluid

SF: soluble fraction

SIF: Simulated Intestinal Fluid

(n)Sin a 3: (natural) LTP1 from *Brassica alba*

(n)SOLTU: (natural) LTP2 from *Solanum tuberosum*

(n)SOYBN: (natural) LTP2 from *Glicine max*

SPHIAa: Single Point Highest Inhibition Achievable assay

SPT: Skin Prick Test

TBS-T: tris buffer saline tween

TFA: trifluoro acetic acid

TFE: 2,2,2- trifluoroethanol

TNF-alfa: Tumor Necrosis Factor-alpha

(n)Tri a 14: (natural) LTP from *Triticum aestivum*

U: Urticaria

URT: urticaria

V: Vomiting

(n)Vit v 1: (natural) LTP1 from *Vitis vinifera*

(n)WHEAT: (natural) LTP2 from *Triticum aestivum*

(n)Zea m 14: (natural) LTP1 from *Zea mays*

ABSTRACT

Foods can affect human health beyond basic nutritional effects. In fact, foods are a source of different molecules, including nutraceuticals, i.e. health-promoting molecules, and proteins that can cause allergic reactions. The objectives of this thesis are in the framework of a research program focused on the study of allergens and nutraceuticals from plant foods and their possible effects on human health. Kiwifruit has been chosen as a model of food endowed with beneficial effects on human health and also an important source of food allergy, although for comparative purposes other foods have sometimes been included in the study.

Four new allergens have been identified in plant food, namely green and gold kiwifruit, black mulberry and tomato. They belong to the family of Lipid Transfer Proteins (LTP) and three of them have already been included in the official list of allergens of the World Health Organization/International Union of Immunological

Societies (WHO/IUIS) with the allergen names Act d 10, Act c 10 and Mor n 3. The structural properties of these allergens have been characterized using classical biochemical methodologies. The immunological and clinical features have been investigated using both classical methodologies, such as skin prick test, prick by prick test and DBPCFC, and emerging methodologies, such as the high throughput ISAC system allowing analysis by multiplex biochip-based immunoassay. The results obtained by comparative analysis show a heterogeneous behavior of different LTPs indicating that the biochemical grouping of allergens can be misleading in the allergy diagnosis and that an improved allergy diagnosis can be obtained by testing every single patient with the most comprehensive panel of available LTPs/allergens .

The influence of different experimental conditions on the structural properties and IgE reactivity of the kiwifruit allergen kiwellin (Act d 5) was investigated by circular dichroism, skin testing, immunoblotting and ISAC microarray system. Results obtained demonstrated that the physico-chemical features of the environment can affect the

conformation and the IgE reactivity of Act d 5. This behavior suggests that the environmental conditions may affect the response of allergy diagnostic systems by modulating the pattern of exposed antigenic epitopes.

The functional characterization of kissper, a naturally occurring kiwifruit peptide, performed by immunohystochemical and ELISA experiments on human colonic mucosa deriving from biopsy carried out on subjects suffering from Crohn's desease, clearly indicated an antinflammatory effect of this molecule. This result suggests a possible use of kissper as a nutraceutical molecule in the treatment of inflammation.

INTRODUCTION

Health and disease rely on different factors. In this context, environment and food quality play an important role, being sometimes causes of positive or negative effects on human health. For instance, some foods are a source of nutraceuticals, i.e. molecules providing health benefits, beyond the nutritional aspects. Nevertheless, some subjects have to avoid some foods because they are also sources of allergic reactions.

1. Allergic deasese

Allergies are undesiderable reactions produced by the normal immune system. Allergic reactions occur when the immune system reacts against normally harmless substances present in the environment. The name "allergen" is given to a molecule that can cause an allergic reaction.

When subjects with allergic deaseses are exposed to common environmental substances, such as house dust mite or grass pollen or foods, a type of white blood cells (B lymphocytes) produce specific antibodies known as Immunoglobuline E (IgE) against protein molecules contained in that substance (sensitization). This IgE then attaches itself to another type of white blood cell (mast cells), and when the mast cells come into the contact with that substance again, they initiate a complex immune response that leads to the allergy (**Figure1).**

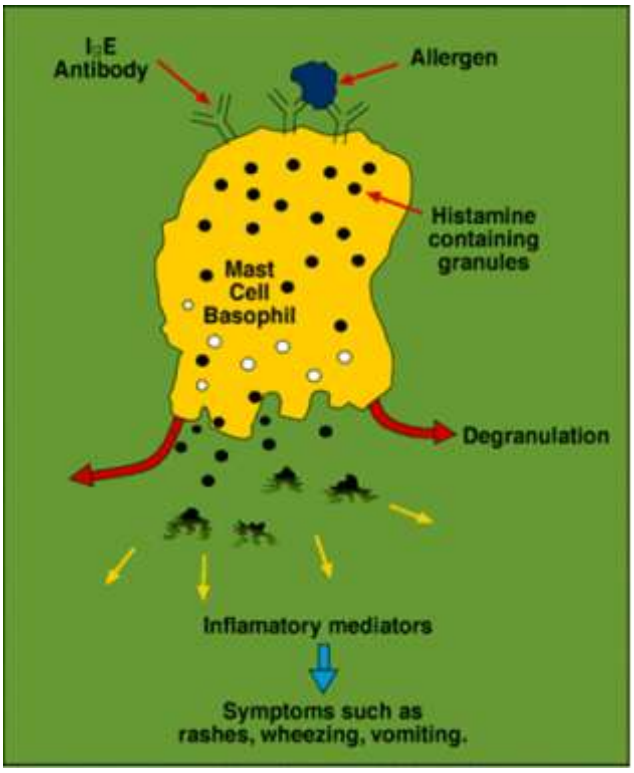

Figure 1. Release of inflammatory mediators, like histamine, from a mast cell after interaction of allergens with specific IgE present on the cell surface.

It was estimated that more than 80 million people in Europe have an allergic disease, with a dramatically increasing prevalence over the last 20-30 years. Literature data report that allergic deaseses are on the rise and are no longer confined to specific seasons, or to people living in specific areas *(1)*. The recent escalation of allergic deasese may be at least partially attributable to environmental factors: people are now exposed to several different substances, both natural and man-made. Psychological stress is also thought to be linked to allergic disorders as modern living and hectic lifestyles have an increasingly negative impact *(2)*.

The effect of allergy on sensitive individuals can be substantial, and the common symptoms include breathing difficulty, coughing, repeated sneezing, itchy, swollen and rose nose and eyes, eczema and dermatitis. In very serious cases, an anaphylactic reaction can be fatal. The list of the substances to which people are commonly allergic include house dust mite, pollens from grasses and trees, animal dander (including cat, dog and horse) moulds, foods (including tree nuts, peanuts, shellfish, fish, milk, eggs,

wheat, fruits) and latex. Different allergic subjects can show different symptoms when they are exposed to the substances they are allergic to.

A phenomenon called cross-reactivity may occur when an antibody reacts not only with the allergen that caused the sensitization, but also with homologous proteins. In food allergy cross-reactivity occurs when a food allergen shares structural similarities with a different food allergen or aeroallergen.

1.1. Food allergy

Food allergy is an abnormal response to specific protein components of food, triggered by the immune system. The reactions can range from mild symptoms, such as dermatitis, vomiting and diarrhea, to severe ones, such as the anaphylactic reaction.

Some allergens, most often from fruits and vegetables, cause allergic reactions especially if eaten when raw. However, most food allergens can still cause reactions after they have been cooked or have undergone digestion, because some of

them are resistant to high temperature and to gastric and intestinal digestion.

1.1.1. Lipid Transfer Proteins (LTP)

Plant Lipid transfer proteins (LTP) are small proteins that are divided in two sub-families according to their molecular masses, namely LTP1 those having a molecular mass of 9 kDa and LTP2 those with a molecular mass of 7 kDa. Although LTP1 and LTP2 share a common compact fold consisting of four α-helices stabilized by four disulfide bridges, the pairing partners of cysteines are not completely conserved between the two subfamilies, that also display a low overall sequence similarity (about 30% identity) *(3)*. LTPs are reported to be involved in defence mechanisms *(4)*, pollination and germination *(5, 6)*. However, the *in vivo* biological function remains elusive. LTPs are thermostable and resistant to the proteolytic digestion. Probably due to the extreme resistance to the gastrointestinal digestion, LTP is a severe food allergen that can cause anaphylaxis *(7)*.

Up to now 63 LTPS have been reported as allergens, 46 of them expressed in edible parts of plants and all of them belonging to the LTP1 protein subfamily. Among allergenic LTPs, the best characterized at the structural, immunological and clinical level is the peach LTP1, Pru p 3 (**Figure 2**). It is an allergen that can cause severe symtoms and one of the most important allergic sensitizers in the Mediterranean area. Several reviews on the topic of LTP as allergens reported preliminary evidence of a heterogeneous behavior of this group of molecules *(8, 9)*.

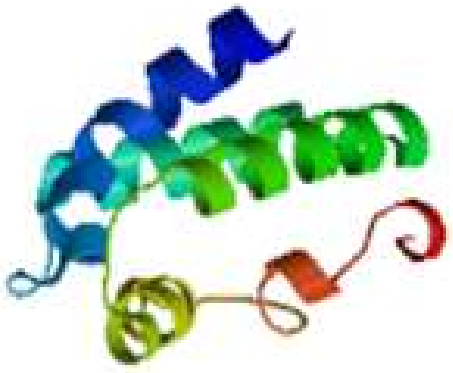

Figure 2. Molecular structure of the allergenic peach LTP1, Pru p 3.

1.2. Diagnosis of allergic deasese

To date the best treatment of allergic subjects is based on the avoidance of the allergen source. The implication is that a proper management of allergic subjects requires a highly reliable diagnosis in order to identify the molecules that each patient has to avoid. However, the allergenic molecules identified so far are much less than those actually contained in the allergenic sources. In addition, sometimes diagnostic systems produce wrong results showing false positive or negative responses.

Several *in vitro* and *in vivo* test systems are available and currently used to investigate the causes of individual allergic reactions. Usually, data from more than one test system are comparatively evaluated in the attempt to reach a reliable diagnosis. *In vivo* Skin Prick Testing (SPT) and *in vitro* blood tests are usually used for this purpose. Sometimes it is necessary to perform a provocation or "challenge test" with the suspected food, that involves the introduction of a specific food to the patient in gradually and increasing amounts and in very controlled conditions. The challenge test

must be performed in a hospital where any serious reaction can be safely managed.

The traditional test systems are based on the use of commercially available protein extracts derived from the allergy sources. However, they frequently fail to detect specific IgE because their composition can be very variable. Ripening stage, post-harvest treatments, proteolytic degradation and protocols used for the extraction significantly affect the relative amounts of many proteins and the profile of allergenic components *(10)(11)(12)*.

Since it seems impossible to obtain standardized extracts with a costant allergenic composition and containing all the allergenic proteins present in the natural source, component resolved diagnosis (CRD) has gained more attention in the recent past. In fact, the research and the commercial companies are evolving through the development of new tecnologies useful for the detection of specific IgE against purified recombinant or natural allergens. Among the novel metodologies we find the increasing use of diagnostic systems allowing the simultaneous measurement of IgE

antibodies specific for different individual allergens (i.e. multiplex analysis) with the same serum sample *(13)*.

1.2.1. ISAC microarray system

ISAC (Immuno Solid-phase Allergen Chip) is an *in vitro* diagnostic system useful for semiquantitative analysis of immunoglobulin E (IgE) in the serum samples *(14)(15)*. In contrast with the traditional systems, the ISAC system do not use any protein extract. It uses only purified allergens (component resolved diagnosis, CRD) allowing a better standardization of the tests. It is a chip containing four identical reaction chambers (**Figure 3A**) and each chamber is a microarray where purified natural and recombinant allergens are immobilized separately (**Figure 3B**). The IgE contained in the serum of an allergic subject recognize one or more immobilized allergens on the microarray (**Figure 4A**) and the interaction is revealed using an antibody, conjugated with a fluorescent probe, specific for human IgE (**Figure 4B**).

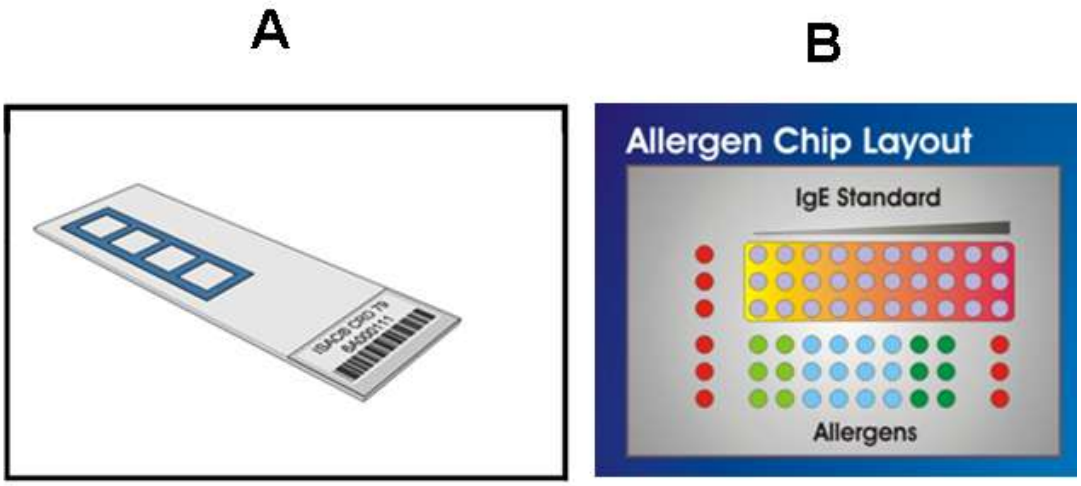

Figure 3. ISAC system. Chip with 4 reaction chambers (A) and purified allergens spotted on the microarray (B).

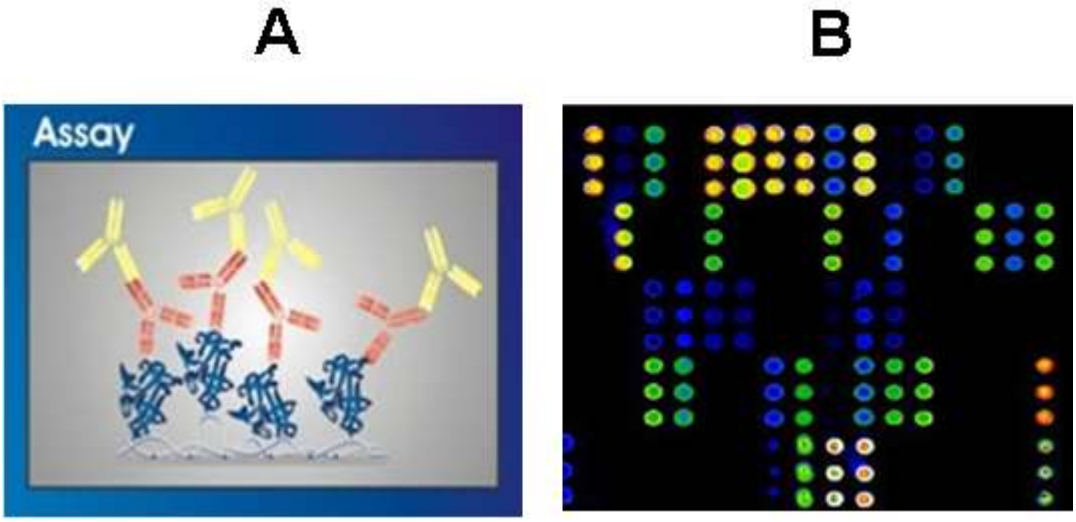

Figure 4. IgE-allergen interaction is revealed by a fluorescent dye conjugated with an anti-IgE antibody. (A) The allergenic proteins immobilized on the chip are in blue, the human IgE are in red and the anti-IgE are in yellow. (B) The fluorescence intensity is mesured with a "microarray scanner"

In some allergological centers, this system is routinely used for allergy diagnosis providing information on the subjects' sensitivity to all the allergens available on the microarray with a single test *(16, 17)*.

Few years ago the first version of the ISAC microarray become commercially available. It had 79 different allergenic proteins spotted on the microarray (ISAC79). The number of allergens immobilized on this microarray is growing and at present a microarray with 103 allergens (ISAC103) is available.

2. Nutraceuticals in plant food

Regular consumption of fruit and vegetables is generally associated with a reduction of the risk of several human pathologies, such as cardiovascular diseases, heart failure, microbial infections, cancer, Alzheimer's disease and other age-related functional degenerations *(18)(19)*. These advantages are usually ascribed to rich vitamin and antioxidants, although other kind of still unknown molecules could have beneficial effects on human health. *(20)*.

Kiwifruit is one of the foods at which different types of health-promoting effects have been ascribed and reported in the literature.

2.1. Kiwifruit

Kiwifruit is indigenous to southern Asia. The vines can be found growing wild on the edges of forests near the Yangtze River Valley. The kiwifruit plant belongs to the family *Actinidia*. Although there are around 400 varieties, the ones most widely grown for commercial fruiting purposes are *Actinidia deliciosa*, which has a green flesh, and more recently *Actinidia chinensis,* also known as the gold kiwifruit or "Chinabelle", which has a yellow flesh and is sweeter and less acidic than the green variety (**Figure 5**).

Kiwifruit is reported as a food with significant effects on human health. For instance, this fruit was used by traditional Chinese medicine for the prevention and therapy of many different kinds of cancers *(21)*. More recently, a number of experimental data suggested the presence of several

biological activities associated to kiwifruit, beyond basic nutrition.

Figure 5. Green (*Actinidia deliciosa*) and gold (*Actinidia chinensis*) kiwifruit.

For instance, *in vitro* cytotoxicity towards tumor cell lines and antimicrobial activity *(21)*, protection against oxidative DNA damage, *(22)* and cardiovascular protective properties *(23)* were reported. Data from human trials indicated that daily consumption of kiwifruit promoted laxation in ederly people *(24)*. *In vitro* experiments showed that the protein thaumatin, isolated from kiwifruit, displays antifungal and antiviral (anti-HIV) activities against human pathogens *(25)*. Unfortunately, some subjects have to avoid the consumption

of kiwifruit because it is also an important source of food allergens. Actinidin, kiwellin and thaumatin are the three most abundant proteins found in green kiwifruit *(12)*. In the gold species, kiwellin is the major protein component. Literature reports show that all these three kiwifruit proteins have allergenic properties *(25)(26)(27)*.

2.1.1. Kissper, a kiwifruit peptide with channel-like activity

It has been demonstrated that kiwellin may undergo *in vivo* and *in vitro* proteolytic processing by the protease actinidin originating KiTH and kissper *(28)*. KiTH is a 16-kDa polipeptide corresponding to the C-terminal region of kiwellin. Kissper is a 4-kDa peptide corresponding to the N-terminal region of kiwellin. Kissper can be isolated in significant amounts from the edible part of ripe green kiwifruit. The elucidation of the primary structure of kissper revealed that it is a cysteine-rich peptide of 39 residues.

2D NMR experiments indicated that kissper has a compact structure, stalilized by three disulfide bridges and only short

regions of regular secondary structure, without any evident similarity with other bioactive peptides (**Figure 6)**.

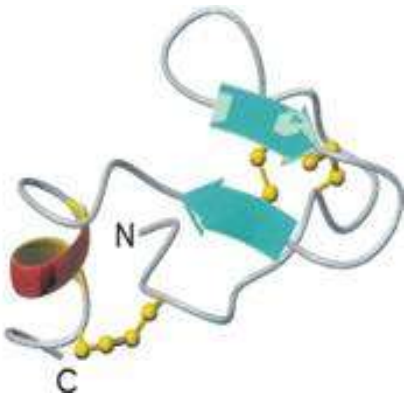

Figure 6. Ribbon representation of kissper solution structure. The three disulfide bridges are shown in yellow

Functional characterization has shown that kissper is able to form voltage-gated ion channels preferentially transporting anions, such as Cl^- through model plasmatic membranes *(29)*. This property suggests a possible positive effect of this molecule in the treatment of pahtologies characterized by insufficient anion transport.

OBJECTIVES

Several factors may affect human health. In this context, food quality plays an important role, being sometimes a cause of positive or negative effects on human health and desease. For instance, some foods are a source of nutraceuticals, i.e. molecules providing health benefits, beyond the basic nutritional aspects. Nevertheless, some subjects cannot eat some foods because they are also sources of allergenic components.

The objectives of this thesis are in the framework of a research program focused on the study of allergens and nutraceuticals from plant foods and their possible effects on human health. Kiwifruit has been chosen as a food model, although for comparative purposes other foods are sometimes included in the study.

Identification, purification and characterization of allergenic proteins from plant food.

At present, the recommended treatment of allergic subjects is based on the avoidance of the allergen source. The implication is that an adeguate management of allergic subjects requires a highly reliable diagnosis in order to identify the molecules that each allergic subject has to avoid. However, the allergenic molecules identified so far are much less than those actually contained in the allergenic sources. In addition, the available test systems sometimes produce false results that can be due to several factors.

1. With the aim of contributing to the elucidation of allergomes of different allergenic foods, the identification and biochemical and immunological characterization of new allergens from different plant sources have been planned. In particular, we sought to identify and characterize the allergen Lipid Transfer Protein (LTP) from different allergenic sources, such as green and gold kiwifruit, black mulberry and tomato.

2. Allergenic LTPs have also been chosen as a model to investigate how heterogeneous is the immunological behavior of homologous allergenic proteins. At present, the concept "one allergenic molecule fits all the homologs" is widely applied in allergy diagnosis. To investigate the validity of this concept, a comparative analysis of the immunological properties of homologous LTPs from different sources was included in the aims of this study.

3. The protein nature of allergens makes these molecules sensitive to the physico-chemical features of the environment that can affect their structural properties. In this context, a further aim of this thesis was the study of the influence of different experimental conditions on the structural and immunological properties of the allergen kiwellin (Act d 5).

Characterization of the bioactive peptide kissper, isolated from kiwifruit.

A peptide, named kissper, able to form voltage-gated ion channels, preferentially transporting anions, such as Cl^- ions, through model plasmatic membranes was identified in kiwifruit. The funtional properties of this food molecule prompted the investigation of the effect of kissper on human intestinal tissues following appropriate markers of inflammation.

MATERIALS AND METHODS

MATERIALS

Black mulberry (*Morus nigra*), gold kiwifruit (*Actinidia chinensis*), green kiwifruit (*Actinidia deliciosa*), tomato (*Solanum lycopersicum*) used for the purification of the allergenic LTPs, Mor n 3, Act c 10, Act d 10 and Lyc e 7kDa, respectively, were purchased in local markets. Peach fruit (*Prunus persica* cv Stark Saturn) used for the purification of Pru p 3 was kindly provided by the CRA-FRC Fruit farming Research Unit, Caserta, Italy.

METHODS

1. Protein extracts

1.1.Preparation of the protein extracts from whole kiwifruit

Gold kiwifruit (*Actinidia chinensis*) and green kiwifruit (*Actinidia deliciosa*), were peeled, homogenized in a household blender after addition of 1M NaCl (1:1, w/v) and stirred a 4°C for 2 h. After centrifugation at 12500 × g for 60

min, the supernatant representing the total protein extract, was collected, dialyzed against water, aliquoted and stored at -20°C until used.

1.2. Preparation of the protein extracts from pulp and seeds of kiwifruit

Seeds of gold and green kiwifruits were manually separated from the pulp. Pulp samples were homogenized in a blender after addition of 1M NaCl (1:1, w/v) and stirred at 4 °C for 2 h. Seeds were crushed by pestle in a mortar until a smooth powder was obtained. Seed proteins were extracted in 0.5 M NaCl at 4 °C for 2 h. After protein extraction, pulp and seed samples were centrifugated at 12500 × g for 60 min and the supernatants were collected.

1.3. Estimation of the protein concentration

The protein concentration in the extracts and in the purified protein preparations was estimated by the BIO-RAD Protein Assay (Bio-Rad, Milan, Italy), using calibration curves made with bovine serum albumin.

1.4. Fractionation of protein extracts by RP-HPLC

RP-HPLC of extracts was performed on a Vydac (Deerfield, IL, USA) C_8 column (0.21 × 25 cm), using a Beckman Gold Apparatus (Fullerton, CA, USA). Elution was carried out by a multistep linear gradient of eluent B (0.08 % TFA in acetonitrile) in eluent A (0.1 % TFA in water) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The separated fractions were manually collected and analyzed.

2. Proteins and peptide purification

2.1. Purification of LTP from black mulberry.

The black mulberry fruit was homogenized in water (1:1, w/v) and centrifuged at 10400 x g for 30 min. The supernatant was discarded and the pellet, containing the cell wall fraction, was collect and homogenized again after addition of 0.5 M NaCl. After a 60 min extraction followed by centrifugation, the supernatant was collected, dialyzed against 10 mM Tris-HCl, pH 7.2, and loaded on a DE52 (Whatman, Brentford, UK) column, equilibrated in the same buffer. LTP was eluted in the column flow-through. The

sample was then adjusted to pH 5.0 and loaded on a SP-Sepharose (Amersham Biosciences, Uppsala, Sweden) column, equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing LTP were identified following the analysis by RP-HPLC using a Vydac (Deerfield, IL, USA) C_8 column and a Beckman System Gold apparatus (Fullerton, CA, USA). LTP was further purified by RP-HPLC. Elution was obtained by a linear gradient of eluent B (0.08% TFA in acetonitrile) in eluent A (0.1% TFA). The LTP eluted by RP-HPLC was manually collected and dried in a rotary vacuum centrifuge.

After several washing with water, the protein samples were dried and stored at -20 °C until required. Protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm of 3480 M^{-1} cm^{-1}. Purity of the protein preparation was checked by SDS-PAGE, RP-HLPC and N-terminal amino acid sequencing.

2.2. Purification of LTP from green and gold kiwifruit and from tomato

LTPs from kiwifruit seeds, peach peel and tomato seeds were purified using the procedure described above for black mulberry LTP. Purity of the protein preparations were checked by SDS-PAGE, RP-HLPC and N-terminal amino acid sequencing.

2.3. Purification of kiwellin (Act d 5) from green kiwifruit

Kiwi fruit were homogenized in water. After centrifugation, the supernatant, representing the soluble fraction (SF), was discarded. The pellet, containing the cell wall fraction, was resuspended in 0.5 M NaCl, adjusted to pH 8.3 by addition of NaOH, and homogenized again. After centrifugation, the supernatant (cell wall salt extract, CW) was collected. A total protein extract was obtained homogenizing kiwi fruit in 0.5 M NaCl, and collecting the supernatant obtained after centrifugation. The CW fraction, dialyzed against 10 mM Tris-HCl, pH 7.5, was loaded on a DE52 column equilibrated in the same buffer. The protein was eluted in the column

flow-through, which was adjusted to pH 5.0 and loaded on a SP-Sepharose column, equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing the 28 kDa band (kiwellin) upon SDS-PAGE were pooled, dialyzed against 10 mM sodium acetate, pH 5.0, and purified by FPLC ion-exchange chromatography carried out on a Mono-S HR 10/10 column (Amersham-Pharmacia), equilibrated in the same buffer (buffer A). Flow-rate was 3 ml/min. Elution was carried out increasing the concentration of buffer B, as above. The absorbance was recorded at 280 nm.

2.4. Purification of the peptide kissper

Kissper was purified following the already described procedure *(29)* with few modifications. Briefly, ripe kiwifruits (*Actinidia deliciosa*) were homogenized in water (1:1, w/v) by a household blender. After centrifugation, the supernatant was dialyzed against 10 mM Tris-HCl, pH 8.0, and loaded on a DE52 (Whatman, Brentford, UK) column equilibrated in

the same buffer. The column was eluted with 0.5 M NaCl in the equilibrating buffer, and aliquots of the collected fractions were analyzed by RP-HPLC. The fractions containing kissper were dialyzed against 10 mM Tris-HCl, pH 8.0, and then loaded on a Mono-Q HR 10/10 column, equilibrated in the same buffer. The column was eluted by a linear gradient from zero to 0.3 M NaCl. The fractions from Mono-Q were analyzed by RP-HPLC, and those containing kissper were concentrated by ultrafiltration.

3. Structural characterization of purified proteins

3.1. N-terminal amino acid sequencing

Amino acid sequencing of the N-terminal region of intact proteins under investigation or of peptides obtained by proteolytic digestion was performed with an Applied Biosystems Procise 492 automatic sequencer (Applied Biosystem Foster City, CA), equipped with on-line detection of phenylthiohydantoin amino acids.

3.2. Mor n 3 primary structure dermination and analysis

Denaturation and alkylation of the LTP sulfhydryl groups with 4-vynilpyridine was carried out as already described *(30)*. Denatured LTP was divided into three aliquots and subjected to the proteolytic cleavage by either trypsin, Asp-N and Arg-C, following manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Separation of peptides obtained by proteolytic cleavages was performed by RP-HPLC on a Vydac C_8 column. Elution was carried out by a linear gradient of eluent B (0.08% trifluoracetic acid in acetonitrile) in eluent A (0.1% trifluoracetic acid) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The separated fractions were manually collected and subjected to automated amino acid sequencing as described at the paragraph 3.1.

3.3. Act d 10 primary structure determination and analysis

Denaturation and alkylation of the LTP sulfhydryl groups with 4-vynilpyridine was carried out as already described *(30)*. Denaturated Act d 10 was divided in two aliquots and

subjected to proteolytic cleavage to either trypsin and Asp-N, following manufacturer's instructions (Roche Diagnostics GmbH). Due to the large size, a peptide deriving from digestion with Asp-N was sub-digested with chymotrypsin (Roche Diagnostics GmbH). Separation of peptides obtained by proteolytic cleavages and amino acid sequencing were performed as described above (paragraph 3.2).

3.4. Bioinformatic analysis

Homology search and protein sequence analysis were performed using softwares available on the ExPASy Proteomics Server (www.expasy.org).

3.5. Molecular mass determination by mass spectrometry of purified LTPs

The molecular mass of purified proteins was estimated by MALDI-TOF mass spectrometry measurements carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager-DE Biospectrometry Workstation. Analysis were performed on pre-mixed solutions prepared by diluting

samples (final concentration 5 pmol/ml) in 4 volumes of matrix, namely 10 mg/ml α-ciano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3 % TFA.

3.6. Circular dichroism studies on kiwellin

Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter using a quartz cell of 0.1 cm path length. Spectra were recorded, at 25 °C, over the wavelength range 260-190 nm with a bandwidth of 2.0 nm and a time constant of 8.0 s. Spectra were baseline corrected for the contribution of the solvent. A protein concentration of 0.15 mg/ml was used. CD spectra were acquired in pure water and in mixtures containing two different fluorinated alcohols, hexafluoroisopropanol (HFIP) and 2,2,2-trifluoroethanol (TFE), corresponding to 20/80 and 80/20 (v/v) water/alcohol composition. To obtain the same protein concentration in all samples, two stock protein solutions of equal concentration were prepared and adjusted at either pH 4.5 or pH 7.5 with HCl or NaOH, respectively. Identical aliquots of these stock solutions were diluted by addition of

the same volume of water or preformed mixtures of water with an appropriate amount of fluorinated alcohol.

4. Stability to the gastric and intestinal digestion

4.1. Treatment of purified LTPs with SGF

In vitro simulated gastric digestion (SGF) of Actd 10 and Pru p 3 was performed as described by Bublin et al. *(31)*. LTPs were subjected to pepsin digestion (Roche Diagnostics GmbH, Germany) using an enzime/substrate ratio 1:20 w/w. The incubation was performed in SGF conditions (0.15 M NaCl adjusted with 1 M HCl to pH 2.0) at 37°C. Aliquots were taken at 0 and 120 min and analyzed by SDS-PAGE and RP-HPLC. The digestion was stopped by raising the pH to 7.4 by addition 50 mM sodium phosphate pH 7.4 and the samples were stored at -20°C. LTP samples were subjected to trypsin digestion (Roche Diagnostics GmbH, Germany) using enzime/substrate ratio of 1:20 w/w. The incubation was performed in 1% ammonium bicarbonate at 37°C. Aliquots were taken at 0 and 120 min and analyzed by SDS-PAGE and RP-HPLC. The digestion was stopped by boiling the samples

for 2 min. The samples were dried, washed three times with water to remove traces of ammonium bicarbonate, solubilized in PBS and stored at -20°C.

4.2. Kissper stability in SGF and SIF

In vitro gastric digestion of the purified peptide was performed as described by Moreno *et al*. Kissper was solubilized in Simulated Gastric Fluid (SGF) (0.15 M NaCl adjusted to pH 2 with 1 M HCl) before the addition of pepsin (porcine pepsin, Roche Diagnostics GmbH, Germany) at the enzyme/substrate ratio of 1: 20 (w/w). The digestion was performed at 37°C. Aliquots were taken at 0 and 120 minutes and analyzed by RP-HPLC. The digestion was stopped by raising the pH to 7.4 by addition of 50 mM Na-Phosphate buffer pH 7.4.

For intestinal digestion porcine trypsin and bovine chymotrypsin were used. Simulated Intestinal Fluid (SIF) was prepared as described in the *United States Pharmacopeia*, and consists in a mixture of trypsin and chymotrypsin in 0.05 M

K-Phosphate buffer pH 6.8. Digestions were performed at 37°C, at an enzyme to protein ratio of 1:50 (w/w), both for trypsin and chymotrypsin. Aliquots of the digest were withdrawn at 0 and 120 minutes and then loaded on a Vydac (Deerfield , Il, USA) C_8 column for RP-HPLC analysis, using a Beckman System Gold apparatus (Fullerton, CA, USA).

5. Immunological characterization

Immunological and clinical studies, including patients' selection, Skin Prick Tests and tests on the ISAC microarray, were carried out in collaboration with Dr Adriano Mari and his research group at the Center for Molecular allergology, IDI-IRCCS, Rome.

5.1. Biotinylation of kiwifruit LTPs

Purified Act d 10 and Act c 10 preparations (1 mg/ml in sodium carbonate buffer pH 8.7) were incubated with 5 times molar excess biotin N-hydroxysuccunimmide estere (Sigma-Aldrich, Milano, Italy) dissolved in DMSO (Sigma-Aldrich).

After 3.5 hours at room temperature, the excess of reagents were removed by gel-filtration chromatography using a PD10 column (Amersham Biosciences, Uppsala, Sweden) equilibrated in PBS. The biotinylated preparations were then used for coupling with streptavidin-CAP.

5.2. Preparation of protein solutions for SPT

Dialyzed protein extracts and purified LTP samples were solubilized in deionized water and mixed with sterile glycerin in a 1:1 ratio. The final proteins concentration was 0.5 mg/ml. The LTP solutions were sterilized by membrane filtration through a 0.22-μm filter (Millex, Millipore, Bedford, MA, USA), in a sterile horizontal laminar a flow hood.

5.3. Preparation of kiwellin (Act d 5) and kiwifruit extract solutions for SPT

For SPT, (i) the extract solution was mixed with sterile glycerin in a ratio 1:1 and the final protein concentration was 1 mg/ml; (ii) aliquots of lyophilized Act d 5 were solubilized

in water brought to pH 7.4 by the addition of NaOH, and then mixed with sterile glycerin in a ratio 1:1, and the final protein concentration was 0.5 mg/ml. Acidified solutions of kiwellin were obtained by solubilizing the protein in water brought to pH 4.5 by the addition of HCl, and the final protein concentration was 0.5 mg/ml. The value of pH 4.5 was chosen in order to test conditions as much similar to those of kiwi fruit as possible, trying to avoid too acidic pHs that could discomfort patients, whereas pH 7.4 was chosen as a reference condition, since commercial preparations for SPT have neutral pH values. Solutions for SPT were sterilized by membrane filtration through 0.22-µm filters (Millex, Millipore, Bedford, MA, USA), in a sterile horizontal laminar flow hood.

5.4. IgE immunoblotting of kiwellin (Act d 5)

Purified Act d 5 was subjected to 15% SDS-PAGE, transferred onto PVDF membrane and stained with ponceau red. Serum samples of patients that had shown a positive SPT were

selected and used in immunoblotting experiments. IgE immunoblotting was carried out by using the blocking solution and goat anti-human IgE of the Enzallergy Specific E kit (Bio Genetix, Fiumicino, Italy). After washing with the blocking solution, membranes were incubated with sera diluted 1 : 4 for 3 h. IgE–allergen complex was detected by incubation with secondary antibody, i.e. a 1 : 20 dilution of goat anti-human IgE conjugated to alkaline phosphatase, for 18 h, followed by incubation with BCIP/NBT solution prepared according to the manufacturer's instructions. Non-specific binding of the anti-IgE antibody conjugate was estimated in a similar blotting procedure omitting the incubation step with patient serum.

5.5. Measurement of IgE-allergen interaction by ISAC microarray

Allergen-specific IgE were detected by ISAC (Immuno Solid-phase Allergen chip) microarray. The microarrays containing the immobilized allergenic proteins were activated by washing with TBS (10 mM NaCl, 10 mM Tris-Cl and 0.5%

Tween 20, pH 8.0) for 60 min, followed by washing with deionized water for 10 min and then dried. Microarrays were then positioned in a humid chamber before adding 20 µl of serum on the reaction site. After incubation for 120 min at room temperature in the humid chamber, the microarrays were washed for 10 min with TBS-T and for 5 min with deionized water and then dried in a centrifuge. The presence of IgE specific for the immobilized allergens was detected using an anti-human IgE labeled with fluorescein (Pharmingen, San Diego, CA), diluted 1:1000 in TBS-T containing 5 % milk powder. The microarrays were then washed in TBS-T for 10 min, in deionized water for 5 min and then dried and stored in the dark. The fluorescence intensity was measured at 543 nm by a ProScanArray HT Microarray scanner (PerkinElmer).

5.6. Inhibition Assay of IgE allergen interaction by SPHIAa method

IgE inhibition experiments were performed applying the SPHIAa (Single Point Highest Inhibition Achievable assay) assay as already reported *(32)*. Briefly, 20 µl of patients'sera were incubated overnight, at room temperature, with 20 µl of a solution containing protein extracts or purified allergen preparations used as inhibitors. Then, IgE binding inhibition was evaluated by running the ISAC microarray, as described in the previous paragraph.

6. Treatment of intestinal tissues with the peptide kissper

6.1. Patiens and ex-vivo organ cultures

Biopsy specimens were taken from uninflamed mucosal areas immediately next to inflamed tissues of 14 patients suffering from Crohn's desease (CrD) (16-41 years). The primary site of involvement was ileal in 4 patients, ileocolonic in 4 patients and colonic in 5 patients. Normal controls (18-29 years) included mucosal samples taken from 4 patients. Informed consent was obtained from all individuals, and the study was

performed according to the local ethical committee. One specimen from each patient was used for diagnosis; the other samples were cultured *in vitro* for 4 and 24 h with medium alone, EC-LPS (1μg/ml *Escherichia coli* Lipopolysaccharide, Sigma-Aldrich, Milan, Italy) in the presence or absence of the peptide kissper 10μg/ml.

6.2. Immunolocalization in human tissue sections

Five μm thick cryostat sections were fixed in acetone for 10 min. The sections were individually incubated for 2 h at room temperature with the following antibodies: COX-2 (rabbit polyclonal, Cell Signaling Technology, Danvers, Massachusetts, USA) and ICAM-1 (rabbit polyclonal, SantaCruz Biotechnology, Santa Cruz, CA, USA). Antigen expression and distribution was visualized using donkey anti-rabbit IgGs conjugated to Alexa Fluor 488 for 60 min at room temperature. Data were analyzed under fluorescence examination using a LSM510 Zeiss confocal laser scanning unit (Carl Zeiss, Germany). COX-2 and ICAM-1 positive mononuclear cells (MNC) were counted per mm^2 of mucosa.

6.3. Detection of TNF-alfa by ELISA

TNF-alfa secretion was measured using the BD OptEIATM ELISA kit II (BD Biosciences) in accordance with the manufacturer's instructions. Protein concentrations of whole-cell lysates were measured using the BioRad Dc protein Assay (BioRad). TNF-alfa levels were normalized to standard protein concentrations.

RESULTS

1. Identification and biochemical and immunological characterization of allergenic LTPs from plant food (33, 34)

1.1. Purification of Mor n 3

Black mulberry LTP (Mor n 3) was purified from the fruit protein extract as described in the Materials and Methods Section.The recovery of the pure protein was 1 mg/100 gr of black mulberry. Protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm (3480 M^{-1} cm^{-1}). Protein preparation resulted pure upon analysis by SDS-PAGE and Coomassie staining, RP-HPLC and N-terminal amino acid sequencing.

1.2. Primary Structure Elucidation of Mor n 3

The first twenty-seven residues of Mor n 3 were elucidated by direct sequencing of the N-terminal region of the native protein. The complete primary structure was determined by automated sequencing of peptides deriving from enzymatic

digestion of denaturated and S-pyridylethilated protein. Most of the primary structure was elucidated by aligning the amino acid sequence of peptides from trypsin digestion. The sequence of the regions corresponding to the residues 40-44 and 86-91 were obtained by sequencing peptides from Arg-C and Asp-N digestions, respectively. In **Figure 7** only the peptides necessary to elucidate the

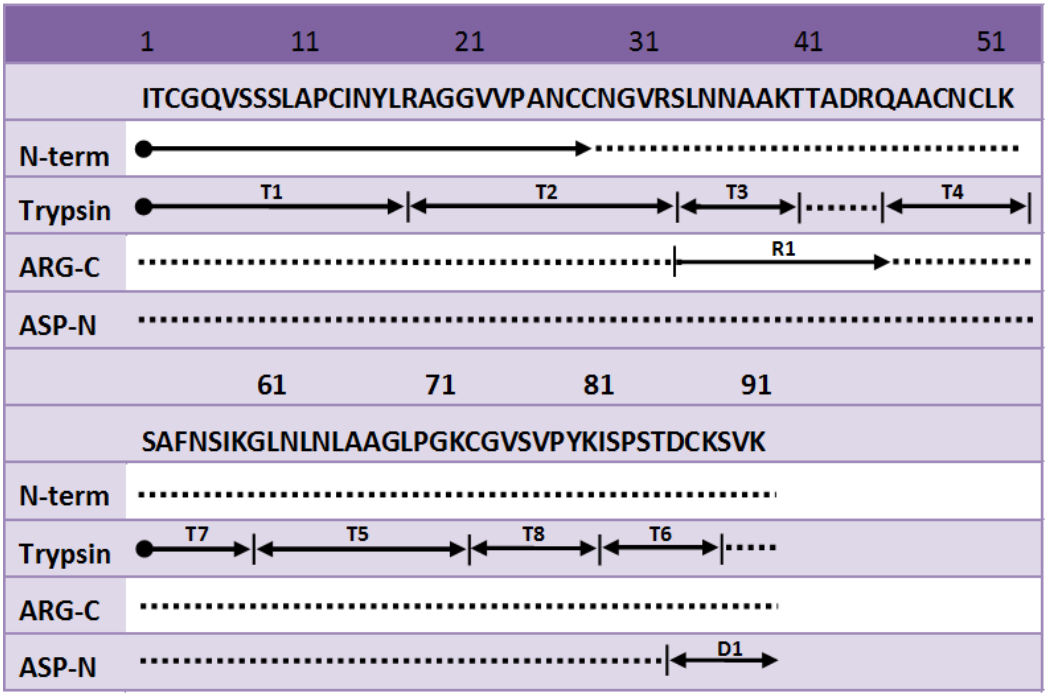

Figure 7. Amino acid sequence of black mulberry LTP, Mor n 3.
Arrows indicate fragments obtained by enzymatic digestion with trypsin (T), Arg-C (R) and Asp-N (D) . The amino acid sequence of the N-terminal region obtained by direct sequencing of the entire molecule is indicated by N-term.
Peptides are numbered according to their order in the sequence.

complete primary structure are indicated. The sequence of black mulberry LTP comprises 91 amino acids producing a molecular mass of 9,246 Da. This value is in good agreement with the value obtained by MALDI-TOF mass spectrometry (9,235± 20 Da).

1.3. Homology Search and structure analysis of Mor n 3

Homology search in Uniprot protein database performed using BLAST algorithm (www.expasy.org) has shown that the amino acid sequence of Mor n 3 displays high sequence identity with several LTPs. The sequence identities observed for some of them, showing values ranging from 62% to 75% are shown in **Table 1. Figure 8** shows the alignment of the amino acid sequence of Mor n 3 with those of some homologs, namely Fra a 3, Pru p 3, Vit v 1, Cor a 8, Art v 3 and Par j 2. In order to align Par j 2, displaying significant sequence differences, including the presence of 11 additional residues, it was necessary to insert two gaps in the LTP1 sequences. Nevertheless, 3D modelling (**Figure 9**) showed that, as for many other LTPs, the overall folding is conserved

Table 1. Amino acid sequence identity (%) among LTPs from different sources

	Mor n 3	Fra a 3	Cit s 3	Rub i 3	Pru d 3	Pru p 3	Pru av 3	Mal d 3	Pru du 8	Pru ar 3	Pyr c 3	Cor a 8
Mor n 3	100	75	73	72	70	70	70	70	68	68	64	62
Fra a 3		100	64	82	73	70	68	73	67	69	68	59
Cit s 3			100	64	63	68	62	70	64	69	61	59
Rub i 3				100	74	69	70	75	67	68	71	61
Pru d 3					100	88	87	82	91	91	77	59
Pru p 3						100	87	79	97	91	75	56
Pru av 3							100	83	89	85	79	59
Mal d 3								100	79	86	85	61
Pru du 8									100	89	78	62
Pru ar 3										100	78	60
Pyr c 3											100	56
Cor a 8												100

Figure 8. (A) Multlipe sequence alignment. Alignment of the amino acid sequences of mulberry (Mor n 3), strawberry (Fra a 3), peach (Pru p 3), grapes (Vit v 1), hazelnut (Cor a 8), artemisia (Art V 3), and Parietaria judaica (Par j 2) allergens.
Residues which have been shown to be relevant for IgE binding activity in Pru p 3 (green) and conserved in other sequences (pink) have been highlighted.

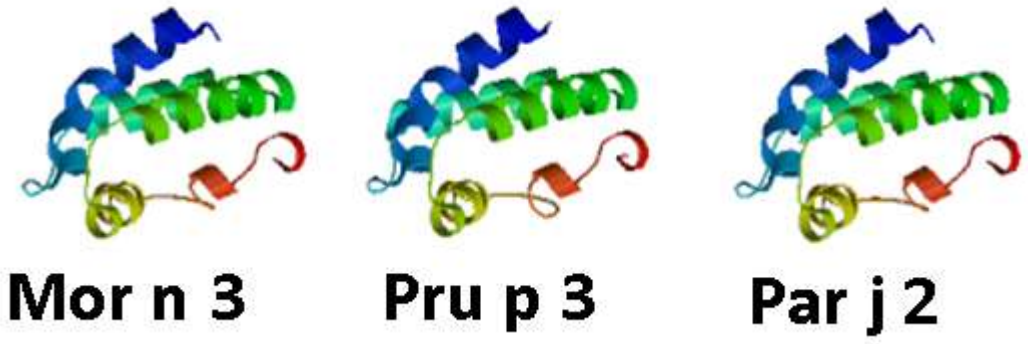

Figure 9. Molecular models of Mor n 3, Pru p 3 and Par j 2 allergens determined by using the services of the Swiss-Model Protein Modelling Server using the structure of a maize lipid transfer protein as a template.

in Pru p 3, Mor n 3 and Par j 2. In **Figure 8**, three regions comprising the residues 11-25, 31-45 and 71-80, reported to be relevant for IgE binding in Pru p 3, have been highlighted *(35)*. The Figure shows that the amino acid residues contained in these regions are only partially conserved in the aligned sequences. The highest conservation with respect to Pru p 3 is observed in the region comprising the amino acids 71-80, where only one residue out of ten is substituted in Mor n 3, Fra a 3, Vit v 1 and Cor a 8; two residues are substituted in Art v 3 and eight residues are substituted in Par j 2. In the other two regions, different patterns of substitutions can be

observed in each one of the analyzed LTPs. Par j 2 shows the highest number of replacements with respect to Pru p 3. The three residues R39, T40 and R44, reported to be involved in the IgE binding of Pru p 3, are all conserved in Cor a 8. In Mor n 3, Fra a 3, Vit v 1 and Art v 3, T40 and R44 are conserved, whereas the residue in position 39 is conservatively substitued (R39/K39).

1.4. Mor n 3 and Pru p 3 SPT in LTP sensitized subjects

Twenty-six subjects tested positive to at least one LTP on the ISAC system were selected for the analysis by Skin Prick Test (SPT). Almost all the selected subjects (96%) were sensitized to the peach LTP, Pru p 3. Only one subject IgE negative to Pru p 3 was enrolled because of sensitization to the hazelnut LTP (Cor a 8). Within this selected group, sensitizations to other LTPs were recorded as follows: Art v 3 46%; Cor a 8 50%; Par j 2 19%. One subject was sensitized to Art v 3 but not to Cor a 8, whereas two were sensitized to the hazelnut LTP but not to the artemisia LTP. Mor n 3 was positive in SPT in 23 out of 26 enrolled subjects (88.46%), exerting reactions

comparable to those of Pru p 3. The 25 subjects positive to Pru p 3 on the ISAC system, reacted to the Pru p 3 preparation by SPT. The subject with a negative ISAC result on Pru p 3, was confirmed negative by SPT, but was scored positive with the Mor n 3 preparation. In three Pru p 3^+ subjects, Mor n 3 was recorded negative by SPT. The prevalence of Mor n 3^+ subjects lacked significant difference when compared to Pru p 3^+ patients (n=25, 96.15%).

1.5. IgE-binding inhibition by Mor n 3 and Pru p 3 on LTPs spotted on the ISAC microarray

Immunological co-recognition of LTPs was studied by IgE inhibition assay, using the high throughput multiplex inhibition method SPHIAa assay on the ISAC system.. As reported in **Figure 10**, IgE inhibitions were performed by detecting residual IgE binding on LTPs available on the ISAC system by incubating single sera with Mor n 3 or Pru p 3. **Figure 10** shows that all the 12 Pru p 3^+ sera were fully

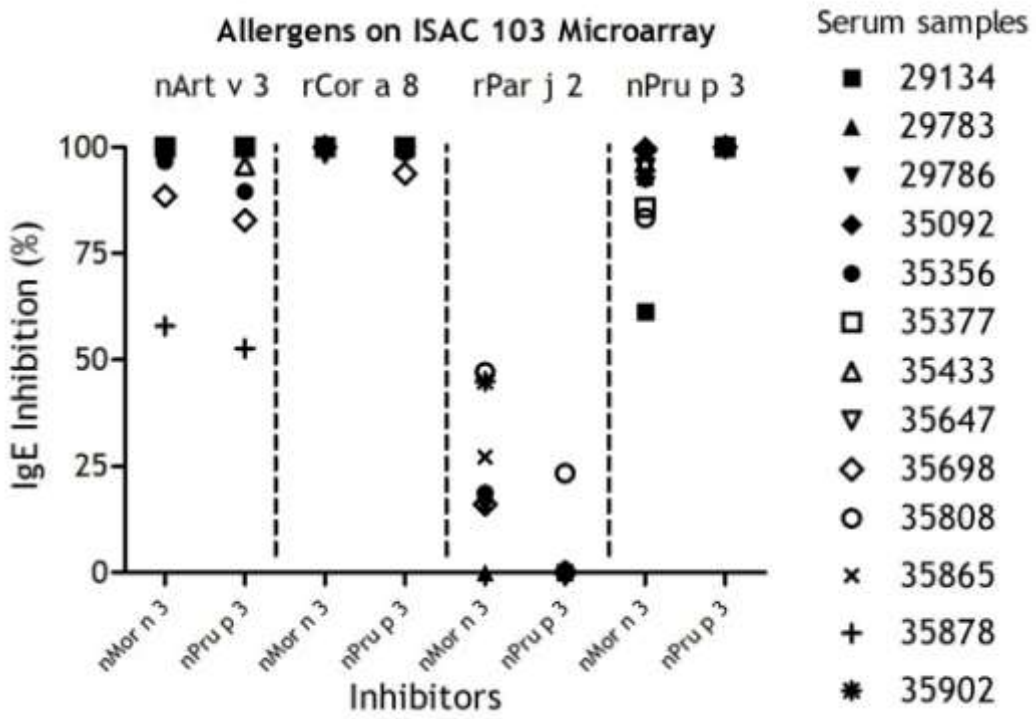

Figure 10. IgE cross-reactivity experiments. Single point highest inhibition achievable assay (SPHIAa) carried out using Mor n 3 and Pru p 3 as inhibitors of IgE binding on Art v 3, Cor a 8, Par j 2 and Pru p 3 immobilized on the ISAC microarray.

inhibited by the homologous natural preparation. Mor n 3 produced almost the same results on the same allergen with inhibition values ranging between 83% and 100%, except for one sample where a 62% inhibition value was obtained. Both Mor n 3 and Pru p 3 showed an almost full inhibition on all Cor a 8+ samples. IgE inhibition on seven Par j 2+ samples was negligible when Pru p 3 was used as inhibitor, whereas 6

out of 7 sera showed a slight inhibition using Mor n 3 preparation as inhibitor, with values ranging between 15% and 47%.

1.6. Detection of LTP in kiwifruit pulp and seeds

Seed and pulp extracts of green and gold kiwifruit were prepared as described in the Materials and Methods section, fractionated by RP-HPLC (**Figure 11-12, panels A-B**) and the separated protein components were manually collected and analyzed by N-terminal amino acid sequencing. The analysis of a peak eluted at a retention time very similar to that observed for Pru p 3 allowed the identification of LTP, namely Act c 10 and Act d 10, in the seed extracts from gold and green kiwifruit, respectively (**Figure 11**). Act c 10 was eluted as a single peak, whereas Act d 10 was eluted in three overlapping peaks suggesting the presence of some isoforms. Direct protein sequencing of the purified protein allowed the identification of two different isoforms of Act d 10 (see below). Possible additional isoforms were not identified probably because of the low yield.

Conversely, the LTP peak was absent in the RP-HPLC profiles obtained for the pulp extracts of the two kiwifruit

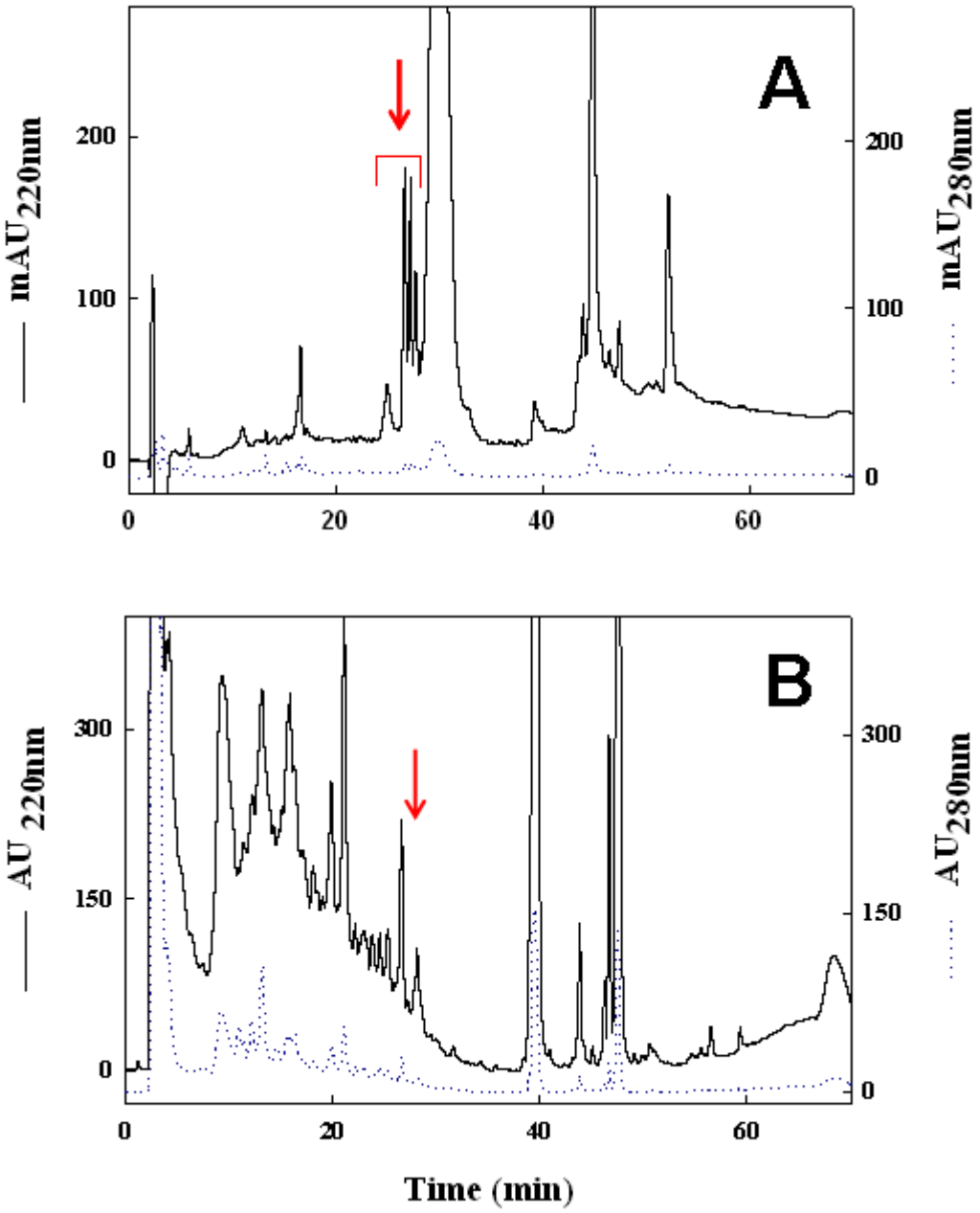

Figure 11. RP-HPLC profiles of the seed (A) and pulp (B) protein extracts of green kiwifruit tissues. The amount of loaded proteins was 1 mg. The arrow indicates the elution time of Act d 10.

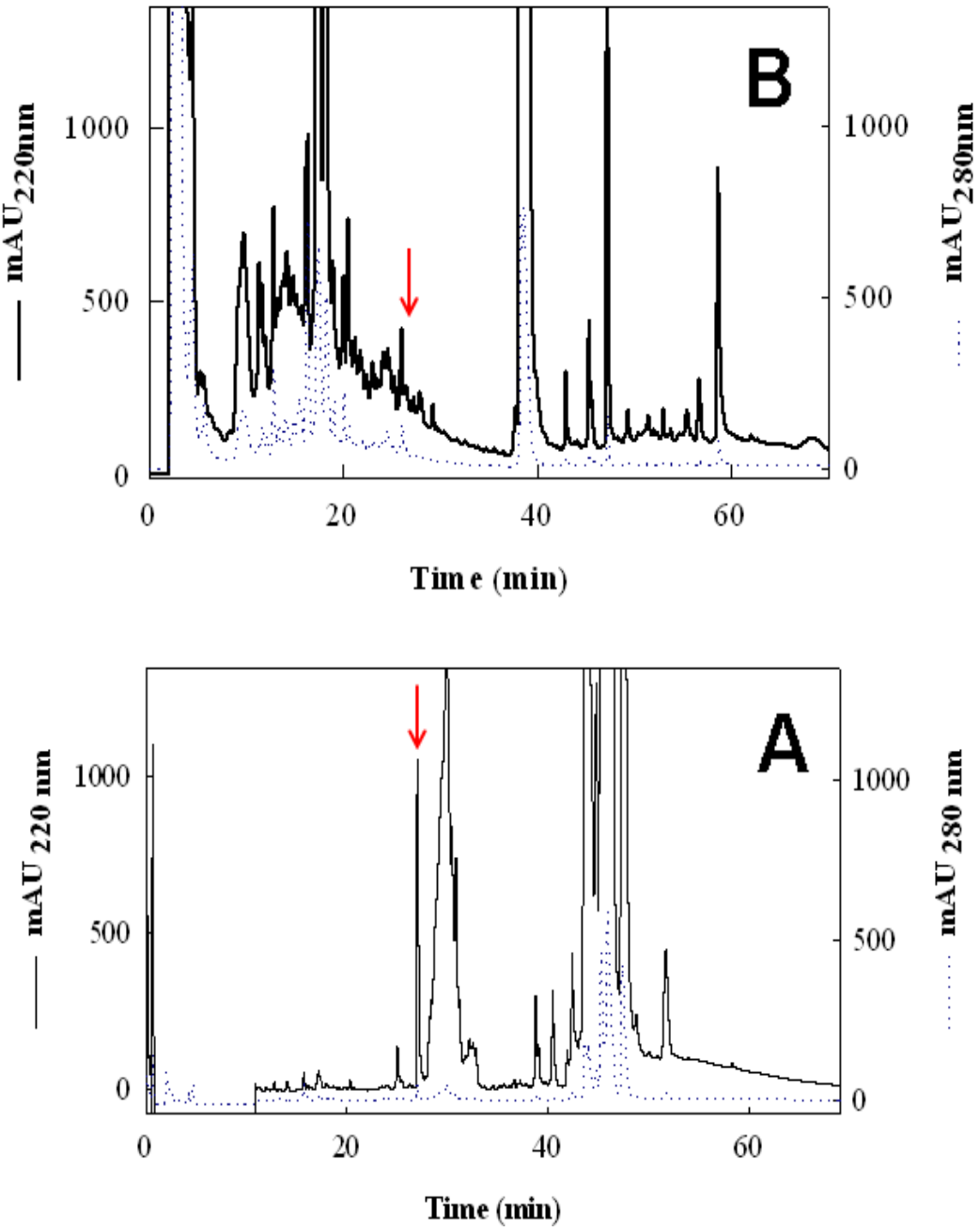

Figure 12. RP-HPLC profiles of the pulp (B) and seed (A) protein extracts of gold kiwifruit tissues. The amount of loaded proteins was 0.3 mg. The arrow indicates the elution time of Act c 10.

species (**Figure 11-12, panels B**). Nevertheless, the fractions of pulp extracts eluted at the retention time of Act d 10 and Act c 10 were collected and analyzed by N-terminal amino acid sequencing. The results obtained suggested the absence of detectable amounts of Act d 10 and Act c 10 in the pulp extracts.

1.7. Act d 10 and Act c 10 purification

Act c 10 and Act d 10 were purified from the seed extracts using the procedure described in the Materials and Methods Section for the purification of Mor n 3. The recovery of the pure protein was about 0.4 mg per gram of green and gold kiwifruit seeds. Protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm (3480 M^{-1} cm^{-1}). Protein preparation resulted pure upon analysis by SDS-PAGE and coomassie staining, RP-HPLC and N-terminal amino acid sequencing.

1.8. Primary structure of Act d 10

Direct sequencing of the N-terminal region of native Act d 10 provided the following sequence: AVSCGQVDTALTPCLTY (**Figure 13**).

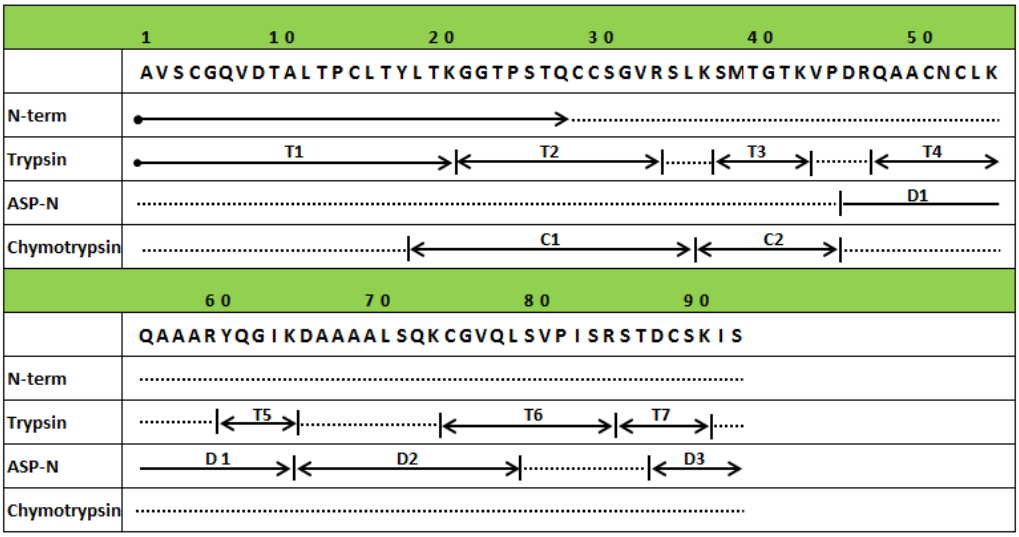

Figure 13. Amino acid sequence of green kiwifruit LTP, Act d 10. Arrows indicate fragments obtained by enzymatic digestion with trypsin (T), Asp-N (D) and chymotrypsin (C). The amino acid sequence of the N-terminal region obtained by direct sequencing of native Act d 10 is indicated by N-term. Peptides are numbered according to their order in the sequence.

A small fraction of the molecules contained the amino acid threonine (T) rather than alanine (A) as first residue. The N-terminal sequence of Act c 10 was identical to that of Act d 10

(AVSCGQVDTALTPCLTY), and no heterogeneity at first position was observed. The complete primary structure of Act d 10 was determined by sequencing of peptides obtained from the enzymatic digestion of denatureted and alkylated protein. Most of the amino acid sequence was obtained by aligning the sequence of peptides produced by trypsin and Asp-N digestions, whereas the regions corresponding to the residues 18-35 and 36-42 were elucidated by sequencing fragments from chymotrypsin digestion. **Figure 13** shows only the peptides necessary to elucidate the complete amino acid sequence. Act d 10 comprises 92 residues producing a molecular mass of 9,458 Da for the most abundant isoform, having the alanine at the N-terminus. The 100% sequence identity in the N-terminal region, together with the observation that Act d 10 and Act c 10 show the same chromatographic behavior and very similar molecular masses as estimated by mass spectrometry analyses (see below), suggested a very high structural similarity between the two proteins. Therefore, only the full Act d 10 primary structure was elucidated. The amino acid sequence of the Act d 10

isoforms having alanine (A) or threonine (T) as N-terminal residue, and the partial N-terminal sequence of Act c 10, were registered in the Uniprot knowledgbase with the accession numbers P85205, P85206, and P85204, respectively.

1.9. Estimation of the molecular mass of kiwifruit LTPs by mass spectrometry

The analyses by MALDI-TOF mass spectrometry of purified Act d 10 provided two values, 9,464 and 9,484 kDa. These values are in good agreement with the molecular masses deduced from the amino acid sequence of the two isoforms having alanine (9,458 kDa) or threonine (9,488 kDa) as N-terminal residue, respectively. A molecular mass of 9,460 kDa was obtained by mass spectrometry for Act c 10.

1.10. Evaluation of the resistance to proteolysis

The analysis by SDS-PAGE and RP-HPLC of Act c 10 and Act d 10 subjected to digestion by SGF or trypsin as described in the Materials and Methods section showed that, they both are resistant to proteolysis.

1.11. Analysis of Act d 10 primary structure

Homology search in Uniprot protein database carried out using BLAST algorithm showed the sequence identity between Act d 10 and other already known allergenic LTPs to be not very high, ranging from 55% to 35% with Ara h 9 and Par j 2, respectively. Identities among the amino acid sequence of the six allergenic LTPs under study (Act d 10, Ara h 9, Art v 3, Cor a 8, Mor n 3 and Pru p 3) are comprised in the range 42-70% (**Table 2**). The identities between Act d 10 and other LTPs, such as Api g 2 (celery stalk), Cit s 3

Table 2. Amino acid sequence identity (%) among LTPs from different sources

Accession Number	Allergen	Act d 10	Ara h 9	Art v 3	Cor a 8	Mor n 3	Pru p 3
P85205	Act d 10	100	55	43	42	48	46
B6CG41	Ara h 9		100	46	54	64	61
C4MGH2	Ar t v 3			100	50	56	45
Q9ATH2	Cor a 8				100	62	59
P85894	Mor n 3					100	70
P81402	Pru p 3						100

(orange), Fra a 3 (strawberry), Lac s 1 (lettuce), Len c 3 (lentil), Lyc e 3 (tomato), Mal d 3 (apple), Ori s 14 (rice), Pla or 3 (plane tree pollen), Pru du 3 (almond), Pyr c 3 (pear), Sin a 3 (mustard), Tri a 14 (wheat), Vit v 1 (grape) and Zea m 14 (maize) are comprised in the narrow range of 40-55% (data not shown). The multiple alignment of the amino acid sequence of Act d 10 with some other LTPs, namely Ara h 9, Art v 3, Cor a 8, Mor n 3 and Pru p 3 (**Figure 14**) underlines that the best conserved region is a stretch of 10 contiguous residues (positions 45-54, Act d 10 numbering).

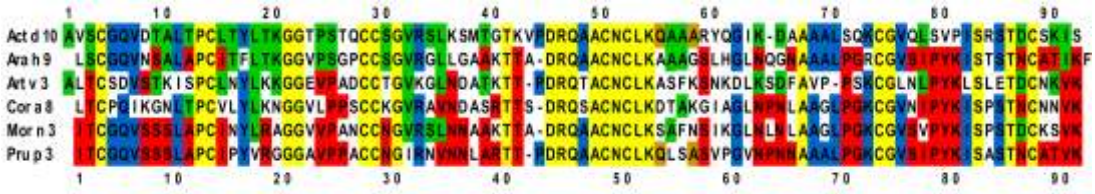

Figure 14. Sequence alignments of Act d 10, Ara h 9, Art v 3, Cor a 8, Mor n 3 and Pru p 3. Identical amino acid residues are highlighted in yellow; in blue are identical amino acids in Act d 10 and Pru p 3 and some other LTP sequences; in green are amino acids of Act d 10 substituted in Pru p 3, but conserved in at least one of the other sequences; in red are amino acids of Pru p 3 substituted in Act d 10, but conserved in at least one of the other sequences; in brown are identical amino acids only in Act d 10 and Pru p 3; in white are residues of Act d 10 not conserved in any other sequence.

Twenty-four amino acid (26%) (yellow background) are conserved in all the six aligned sequences, including the eight cysteine residues. Act d 10 shares 18 additional residues with Pru p 3: two of them (brown background) are only conserved in Act d 10 and Pru p 3, whereas the remaining 16 shared residues (blue background) can be found also in other aligned sequences but not all of them. Twenty-one residues (23%) of Act d 10 are substituted in Pru p 3, whereas they are conserved in at least one of the aligned sequences (green background). A higher number of residues, that is 35 amino acids of Pru p 3 (38%), are substituted in Act d 10, but they are conserved in at least 1 of the other sequences (red background). Act d 10 and Pru p 3 do not share identical residues with any of the other aligned sequences in 27 and 14 sequence positions, respectively (white background).

1.12. Analysis of a group of kiwifruit allergic patients sensitized to LTPs

Patients selected for this investigation reported a reliable clinical history of reactions when eating kiwifruit. Their diagnostic profile is reported in **Table 3**. The IgE screening performed using the ISAC system showed that six out of seven subjects were not sensitized to other kiwifruit allergens, and only one subject was recorded positive to the kiwifruit allergen Act d 1, but all were positive to Pru p 3, the homologous peach LTP.

Results obtained by testing the patients with a commercial kiwifruit extract both by SPT and CAP were sometime negative (**Table 3**). All patients underwent DBPCFC and showed symptoms following the ingestion of kiwifruit. They also showed a positive reaction following in vivo testing by SPT. Detection of IgE in the sera of the selected subjects was carried out using the biotinylated kiwifruit LTPs coupled with streptavidin-CAP. Results obtained showed that all the subjects had IgE specific for Act d 10 and Act c 10, whereas they were negative in 10 allergic subjects used as controls.

Table 3. Diagnostic profiles of seven selected kiwifruit allergic patients enrolled for the initial characterization of kiwifruit LTPs

Subjects	Age	Gender	Symptoms	Skin test		CAP IgE	DBPCFC	ISAC 103 IgE	
				Commercial extract	Prick-Prick	Commercial extract		Act d 1	Pru p 3
1	26	M	URT	18	Neg	Neg	URT	Neg	1,07
2	40	M	URT	Neg	Neg	4,1	URT	Neg	1,01
3	37	F	OAS	43,69	89,05	1,24	URT	Neg	3,61
4	31	F	GI-URT	Neg	29,39	1,02	GI-URT	Neg	1,89
5	16	M	GI	Neg	60,06	3,4	URT	4,34	9,35
6	28	M	GI-URT	56	109,11	0,84	GI-URT	Neg	2,7
7	27	F	OAS	17,34	23,76	7	URT	Neg	5,46

Subjects	Age	Gender	Skin test			CAP IgE		ISAC 103 IgE		
			Act d 10	Act c 10	Pru p 3	Act d 10	Act c 10	Act d 10	Act c 10	Pru p 3
1	26	M	15	100,4	78	0,74	0,93	0,7	0,43	2,59
2	40	M	27	36	12	25,90	65,9	9,91	10,41	6,62
3	37	F	90,53	25,51	98,3	4,7	4,13	1,58	1,5	2,85
4	31	F	67,82	40,16	57,68	8,68	12,3	12,64	13,99	5,14
5	16	M	74,98	69,5	129	26,8	30,2	4,12	6,29	17,02
6	28	M	97,07	98,69	98	1,02	1,2	0,49	0,6	4,37
7	27	F	95,5	85,4	72,57	88,4	100	22,84	15,65	21,51

1.13. Comparative analysis of reactivity towards different LTPs

A large population was analyzed using the routine testing approach by the ISAC microarray, in order to define any possible subsets related to different LTP IgE recognition. A comparative evaluation of seven LTPs was undertaken using the ISAC 103 and ISAC Exp96 microarrays in parallel. A population of 431 subjects positive to at least one LTP was

selected and analyzed. Act c 10, Act d 10, Ara h 9, Art v 3, Cor a 8, and Mor n 3 were positive when Pru p 3 was negative in 48, 41, 17, 56, 43, and 17 cases, respectively. The number of subjects positive to one LTP as listed above and negative to all others was relatively low, being 5, 0, 9, 25, 10, 9, 17. These prevalence were statistically different only when considering Art v 3 values *versus* kiwifruit LTPs, peanut and mulberry ones. Raw prevalence are reported in **Figure 15**, calculated on the sub-population of 431 patients tested positive to at least one of the seven LTPs.

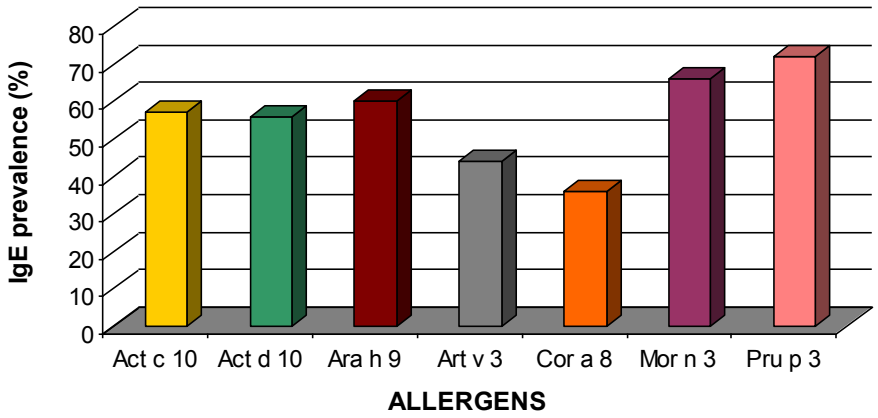

Figure 15. IgE prevalence for Act c 10, Act d 10, Ara h 9, Art v 3, Cor a 8, Mor n 3, Pru p 3 obtained by testing 431 sera from subjects positive to at least one LTP under study.

Pru p 3 was anyway the most prevalent sensitization with no difference only when compared to Mor n 3. IgE sensitization to Act c 10 and Act d 10 had significant higher prevalence than the mugwort and the hazelnut LTPs, but lower prevalence than Mor n 3 and Pru p 3. The less prevalent IgE reactivity have been recorded for the mugwort and the hazelnut LTPs being different from all other LTPs.

To visualize exclusive sensitization to one LTP compared to others, a series of Venn diagrams using IgE values obtained by microarray testing have been generated and reported in **Figure 16.** The first Venn diagram (**Figure 16, panel A**) shows almost overlapping results between the two kiwifruit LTPs, and non-overlapping results considering Act c 10 and Act d 10 opposed to Pru p 3. Due to the highly similar behavior of the two kiwifruit LTPs, **Figure 16** shows how each of the other four LTPs under study behaves compared to Act d 10 and Pru p 3.

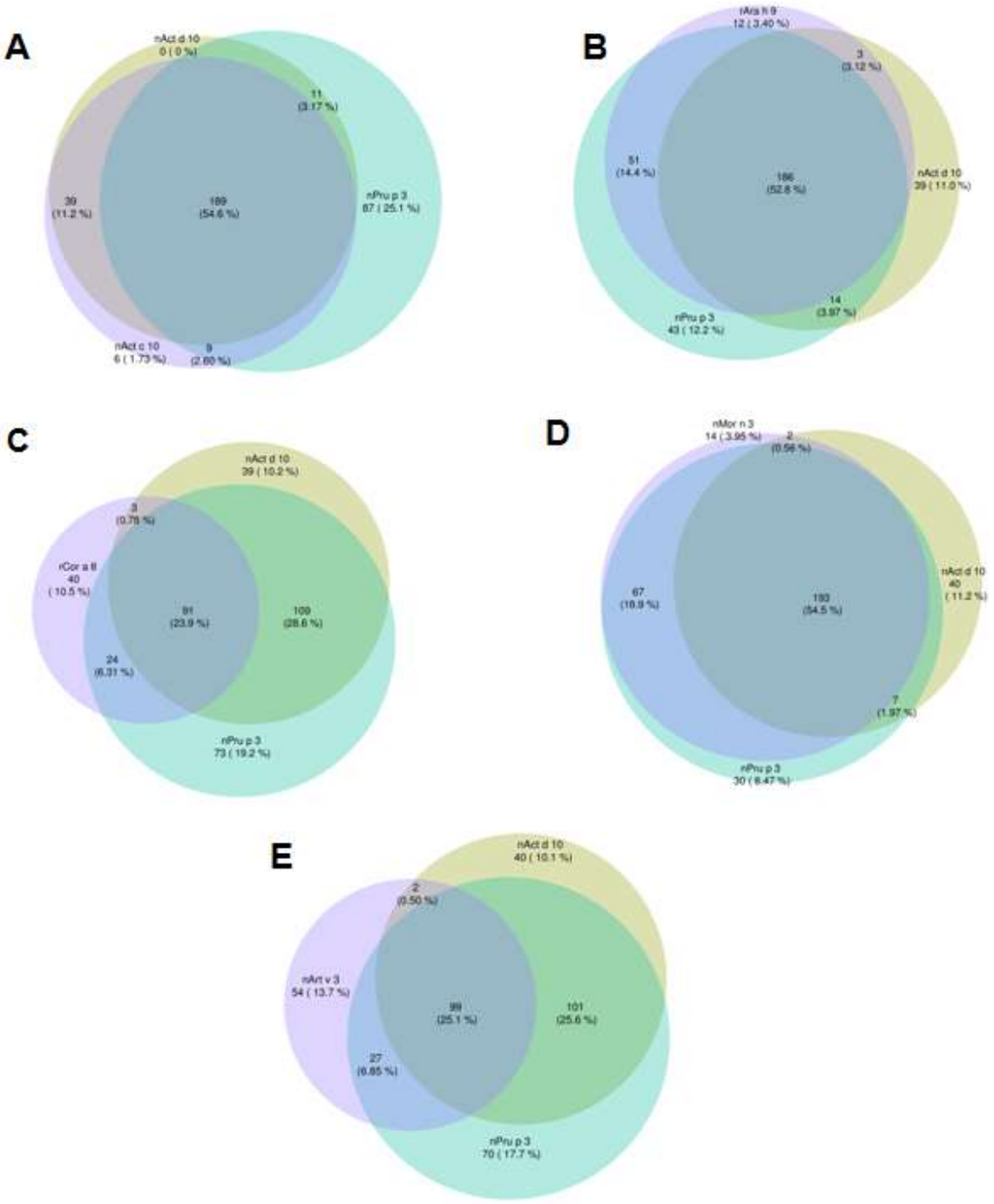

Figure 16. Venn diagrams obtained using specific IgE values for different LTPs. Specific IgE for Act d 10, Act c 10, Pru p 3, Mor n 3, Art v 3, Cor a 8, determined by the ISAC system, were used. Due to the highly similar behavior of the two kiwifruit LTPs, panel from B to E show how the other four LTPs behave compared to Act d 10 and Pru p 3. Absolute and relative IgE prevalence are given for each combination on graphs as follows: Panel A: Act c 10, Act d 10, and Pru p 3; Panel B: Act d 10, Pru p 3, Ara h 9; Panel C: Act d 10, Pru p 3, Art v 3; Panel D: Act d 10, Pru p 3, Cor a 8; Panel E: Act d 10, Pru p 3, Mor n 3.

Venn diagrams change depending on individual LTP, further showing heterogeneity of the members of this group of molecules. An index of the different reciprocal behavior of the LTPs is the changing number of isolated positivity to the three LTPs used in each graph depending on the third LTP used.

A cluster analysis performed using all the 431 subjects having IgE positive results to at least one of the seven LTPs produced the heat map shown in **Figure 17**.

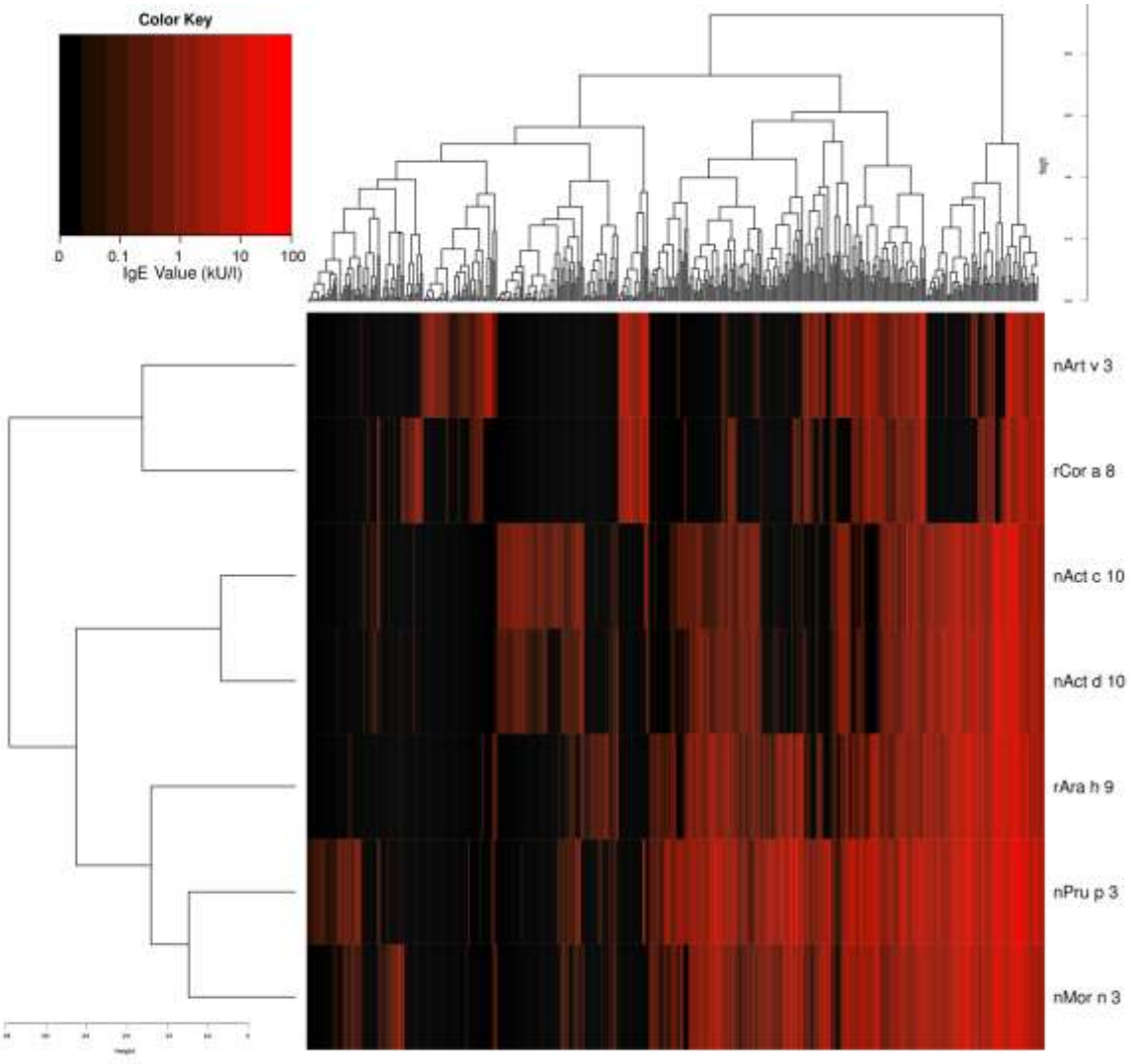

Fig 17. Clustering analysis of 431 subjects tested for IgE on seven LTPs. Logarithmic IgE value distribution has been used to generate the heat map. Subjects had at least one IgE-positive result to one LTP under study. LTPs and subjects are reported on the y-axis and x-axis, respectively. Black to dark red scale corresponds to IgE values from negative to strongly positive.

The picture shows the clustering heterogeneity of both allergens and patients. LTP IgE recognition by the sensitized subjects is well represented by the presence of the first two clusters segregating Art v 3 and Cor a 8 reactivity apart from the other LTPs. A second dichotomy is observed, separating the two kiwifruit LTPs from the others and showing, as expected, the two kiwifruit molecules to be the closest ones. The remaining three LTPs form an additional cluster further divided in two. In addition, subject clustering is dispersed in a great number of clusters, reflecting the individual heterogeneity of LTP IgE recognition.

1.14. Experiments of immunological co-recognition among different LTPs

To investigate immunological co-recognition among LTPs, the SPHIAa assay was chosen using Act c 10, Act d 10, and Pru p 3 as inhibitors of IgE binding on the seven LTPs. The assay was carried out using ten individual sera selected for being IgE positive to the three inhibitors as shown in **Figure 18**, where IgE values for any given inhibited LTP are given

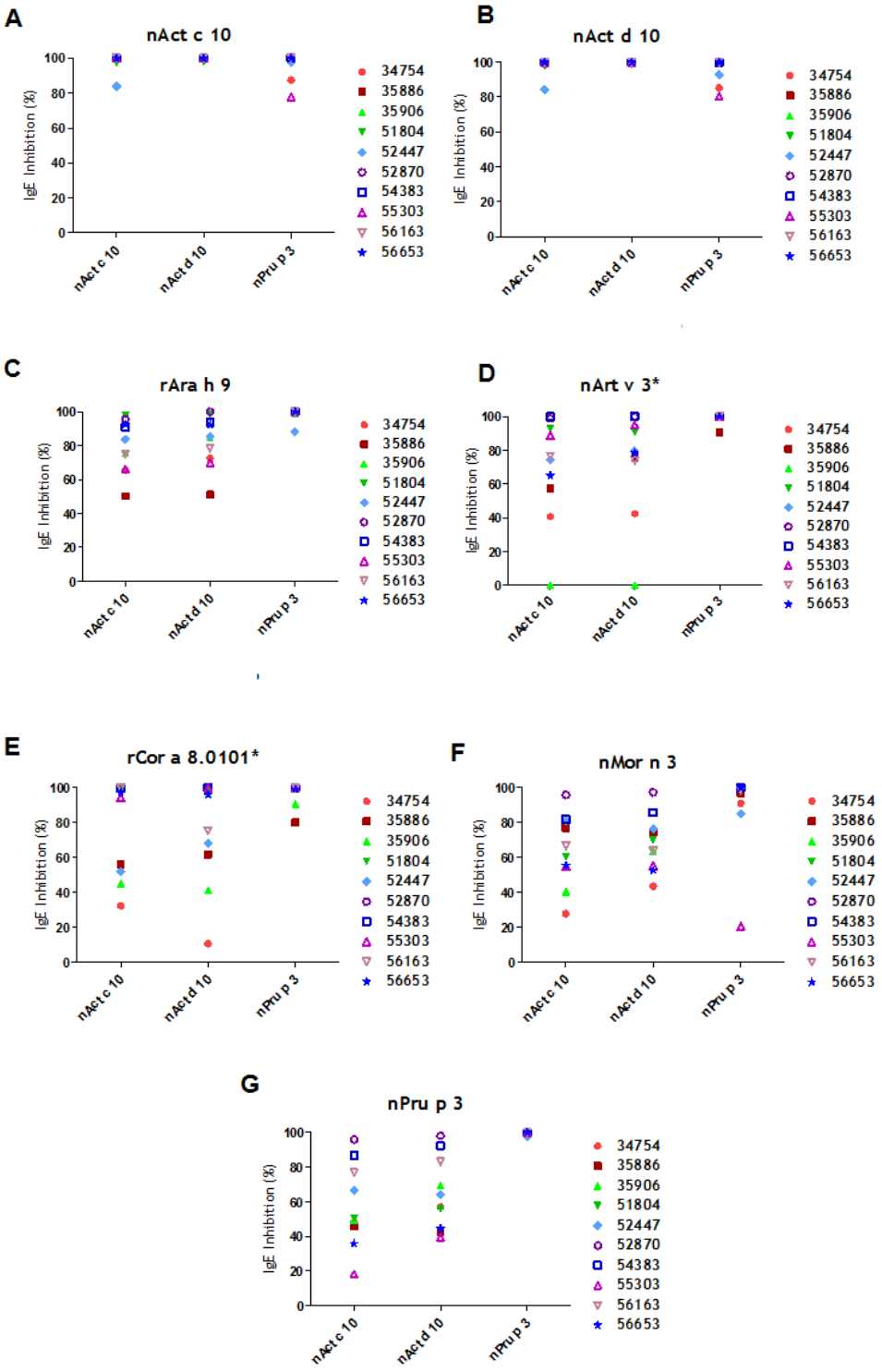

Fig 18. Single Point Highest Inhibition Achievable assay (SPHIAa) for LTP IgE inhibition (reported as percent inhibition). Inhibitors are on the X-axis. LTP immobilized on the microarray whose IgE binding was inhibited is on the top of graphs.

for each of the ten sera. Full autologous IgE inhibitions on the two kiwifruit LTPs were observed, as well as with the homologous Pru p 3 preparation (**Figure 18, panel A and B**). Pru p 3 fully inhibited all the other LTPs (panel C to F). The two kiwifruit LTPs showed IgE inhibition on the other LTPs as follows: greater than 50% on Ara h 9 for all samples; spread between 0 and 100% on Art v 3; separated in two subgroups, one achieving 100% the other staying below 70% on Cor a 8; hardly reaching 100%, and spread in a wide range, with the lowest value at 29% on Mor n 3; not reaching 100%, spread in a wider range, with the lowest value at 19% on Pru p 3. Therefore, the inhibition test shows minor differences between the two kiwifruit LTPs. Overall, also IgE inhibition experiments confirmed the immunochemical differences between the two kiwifruit LTPs and the peach homolog and a different behavior in terms of epitope distribution, recognition and inhibition by the three inhibitors.

1.15. Analysis of the LTP distribution within the kiwifruit tissues by immunological and clinical methods

A series of experiments were performed to define the LTP distribution within the kiwifruit tissues, namely the pulp and the seeds, based on immunochemical and clinical methods. As the biochemical findings reported above suggested the presence of LTP in kiwifruit seeds and its absence in the pulp, to increase the confidence with the negative data we approached the issue of LTP distribution in kiwifruit tissues using the SPHIAa as well. The inhibition was performed using the same pulp and seed preparations from the green and gold kiwifruits, along with whole kiwifruit extracts. A pool of sera having IgE for both the two kiwifruit LTPs was used as probe. As shown in **Figure 19**, full or almost full IgE inhibition results were obtained with the whole kiwifruit extracts and the seed ones, whereas the pulp gave either no inhibition (green kiwifruit pulp on Act c 10, **Figure 19,** lower panel) or inhibition values ranging between 18% and 28% (all other pulp/allergen combinations in **Figure 19,** upper panel). These slightly positive results were replicated and could

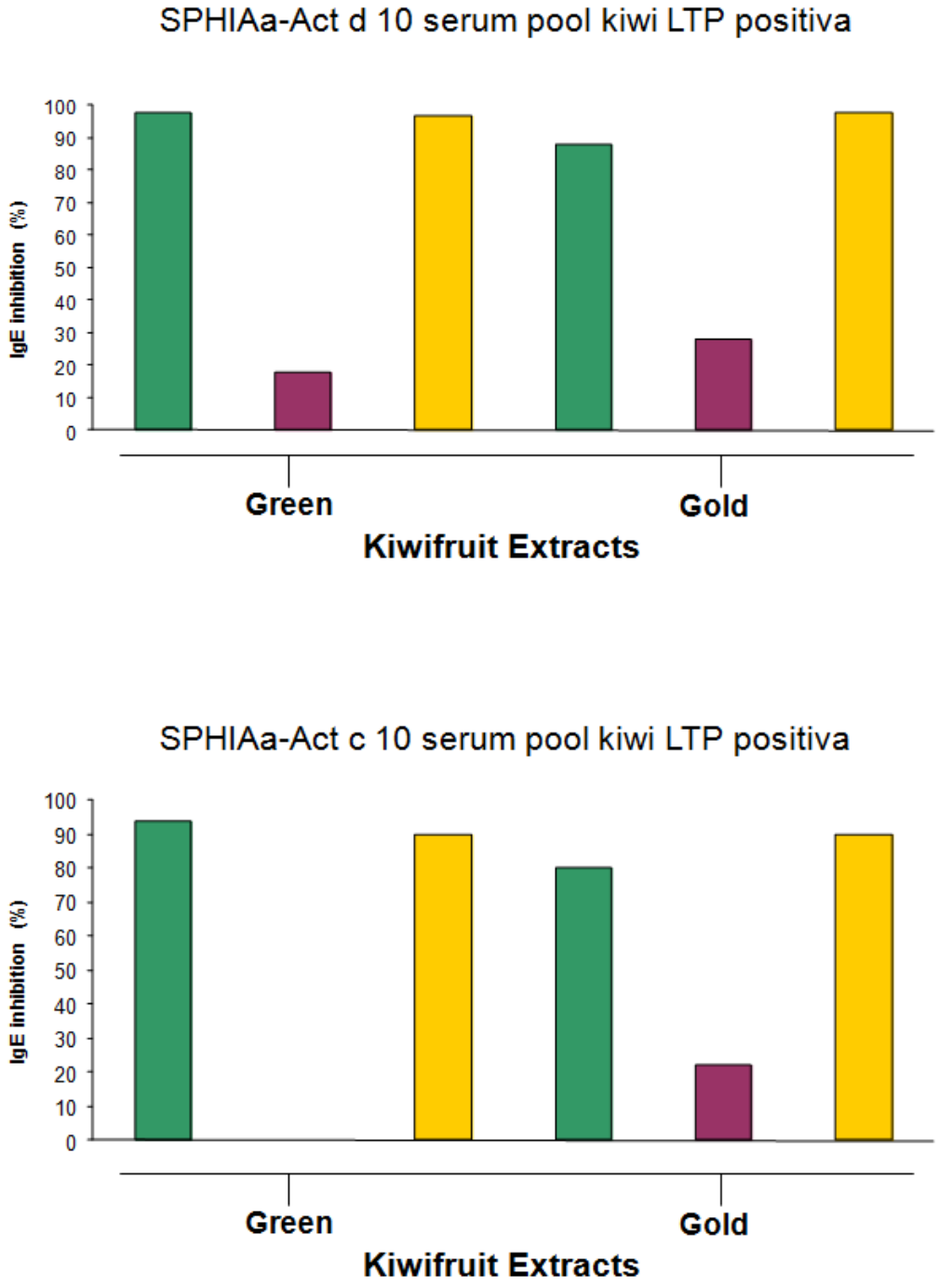

Figure 19. Immunochemical evaluation of LTP distribution in kiwifruit tissues. Single Point Highest Inhibition Achievable assay (SPHIAa) using pulp and seed extracts from green and gold kiwifruit. Green, violet and yellow bars show total kiwifruit, pulp and seeds extracts, respectively from green and gold kiwifruit, used as inhibitors on Act d 10 (upper panel) and Act c 10 (lower panel) immobylized on the ISAC microarray.

suggest the presence of very low amounts of LTP in the kiwifruit pulp not detected by the biochemical methods. The very low inhibition values were anyhow not considered conclusive, and needing a third discriminating proof. Therefore, *in vivo* SPT with green kiwifruit seed and pulp preparations in 21 selected subjects were performed. **Table 4** shows that some of the patients did react to the pulp.

Table 4. Diagnostic and clinical profiles of patients enrolled because of clinical allergy to peach and positive or negative kiwifruit LTP test results.

Subjects	Age	Gender	ISAC IgE			Skin test					DBPCF
			Act d 10	Act c 10	Pru p 3	Act c 10	Act d 10	Seed	Pulp	Pru p 3	Green Kiwi
1	37	M	Neg	Neg	9,92	Neg	Neg	41	74,8	315,3	NR
2	16	F	Neg	Neg	4,09	Neg	Neg	Neg	Neg	98,34	NR
3	38	F	Neg	Neg	30,54	Neg	Neg	Neg	Neg	7,4	NR
4	20	F	Neg	Neg	1,1	Neg	Neg	24	Neg	125	NR
5	18	F	Neg	Neg	4,42	Neg	Neg	9,2	6,6	24,5	NR
6	30	F	Neg	Neg	8,13	Neg	Neg	Neg	Neg	142	NR
7	33	F	Neg	Neg	0,75	5,7	Neg	3,2	4,4	97	NR
8	48	F	Neg	Neg	1,7	6,5	27,4	5,6	Neg	61,83	NR
9	45	F	Neg	Neg	4,91	11,5	10	ND	ND	139	NR
10	35	F	1,01	0.11	1,5	Neg	Neg	12,8	17,2	54,78	NR
11	38	M	0,16	0.53	0,22	Neg	Neg	14	10,4	49,2	NR
12	39	F	1,94	1.27	8,91	Neg	Neg	21,9	Neg	71,6	NR
13	20	F	5,4	7.16	29,08	Neg	Neg	Neg	Neg	53	NR
14	20	F	6,07	6.20	3,38	166,4	75,5	26	19	134,3	NR
15	38	F	0,95	1.31	1,19	213,8	148,4	184,8	20,2	30,4	NR
16	25	F	0,14	0.10	4,67	Neg	Neg	53,7	28,8	83,5	OAS
17	17	M	21,9	21.40	55,6	309	267,8	176,7	36,6	100,4	URT-ANG
18	15	M	0,26	0.46	4,06	76,3	65	80,8	52,3	52,6	OAS-ANG
19	27	F	0,33	10.57	8,79	194,6	256	88	25	215,9	OAS-ANG
20	32	M	37,12	34,57	12,31	102,2	146,5	156,2	41	73,8	GI-URT
21	22	F	0,13	1.15	4,94	29,5	36,5	25,7	24	67,7	GI-URT

As the key values were those obtained on patients having severe clinical reactions following kiwifruit ingestion, and high or very high SPT and IgE scores when tested with Act c 10 and Act d 10 (**Table** 4, lines 15-21), we concluded that tiny amounts of LTP are contained in kiwifruit pulp. Therefore, clinically allergic patients were not challanged with the kiwifruit pulp.

1.16. Analysis of the tolerability of the green kiwifruit by Pru p 3 positive, Act c 10/Act d 10 negative subjects

For initial experiments used to identify the new kiwifruit LTPs, patients with combined clinical and IgE reactivity to kiwifruit and peach had been enrolled. Then, experiments to define the *in vivo* clinical reactivity to green kiwifruit in well characterized peach allergic patients were planned. We thus recruited patients having different diagnostic profiles. All enrolled subjects were Pru p 3 positive with either a positive DBPCFC to peach or a recent severe generalized reaction following peach ingestion. All underwent the tests as reported in **Table 4**. Fifteen of them passed the DBPCFC

eating a full kiwifruit at the end, and six clearly showed symptoms after the challenge. The latter were all tested positive for almost all preparations either by *in vivo* or *in vitro* tests, whereas among the kiwifruit tolerant subjects in the majority of the cases the kiwifruit LTP were tested with negative results, but with exceptions, unless positive values were in the lowest range for both ISAC and SPT tests (**Table 4**). In the attempt to explain why the Act c 10 or Act d 10 positive results were sometimes not associated to clinical reactivity, we simulated physiological digestion of the two kiwifruit LTPs as reported above, and, using the SPHIAa, evaluated the retained capability to induce IgE inhibition. Pru p 3 was comparatively evaluated, as it is known to be resistant to digestion. The results showed that Act c 10 and Act d 10 subjected to digestion with SGF and tryspsin are still active in the IgE inhibition assay.

1.17. Identification, purification and biochemical and immunological characterization of Lyc e 7kDa from tomato

1.18. Detection and purification of Lyc e 7kDa

A protein extract from tomato (*Solanum lycopersicum*) seeds was prepared as described in the Materials and Methods Section, fractionated by RP-HPLC (**Figure 20**) and the separated protein components were manually collected and analyzed by amino acid sequencing. The analysis by N-terminal amino acid sequencing (see below) of a peak eluted at a retention time close to that observed for other LTPs, such as Act d 10 (see above), allowed the identification of a 7-kDa LTP (Lyc e 7kDa).

Lyc e 7kDa was purified from tomato seeds extract following the procedure used to purify Mor n 3 (see Materials and Methods Section). The recovery of the pure protein was 0.07 mg/1 gram of seeds. Protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm (1490 M^{-1} cm^{-1}). The purity of the protein preparation

was checked by SDS-PAGE and Coomassie staining, RP-HPLC and N-terminal amino acid sequencing.

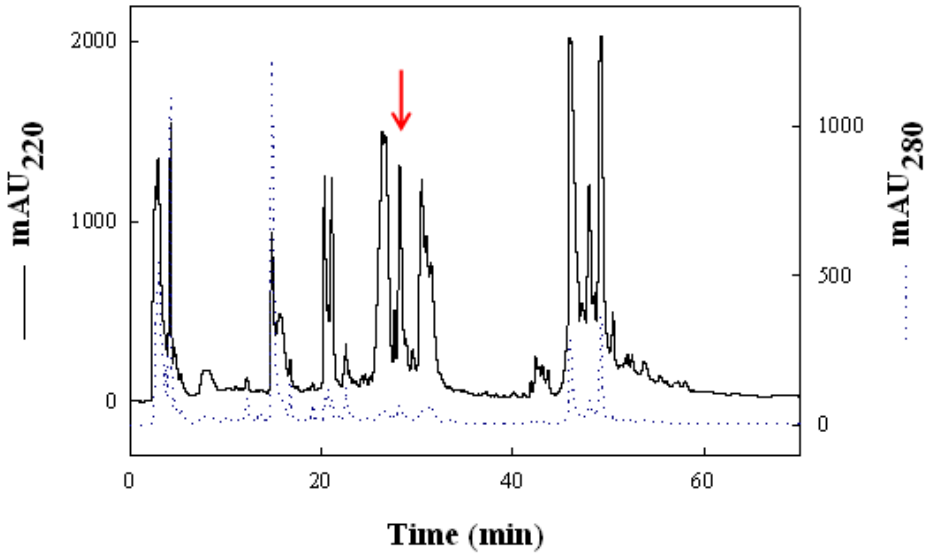

Figure 20. RP-HPLC profile of the protein extract of tomato seeds. The amount of loaded proteins was 1 mg. The arrow indicates the peak of Lyc e 7 kDa LTP.

1.19. Primary Structure of Lyc e 7kDa and homology search

The first 15 residues (ATCSASQLSPCLGAI) of the N-terminal region of Lyc e 7kDa were identified by direct sequencing of the native protein. The homology search in Uniprot Database revealed the absence of tomato LTP having the same N-terminus. Conversely, the homology search in the EST database using tblastn (www.expasy.org), at NCBI,

allowed the identification of a clone (BW690549) coding for the tomato 7-kDa LTP (**Figure 21)**. This protein comprises 68 amino acids producing a molecular mass of 7045 Da. This value is in good agreement with the experimental value obtained by MALDI-TOF mass spectrometry (7026 ± 20 Da) measurement carried out on the purified Lyc e 7kDa.

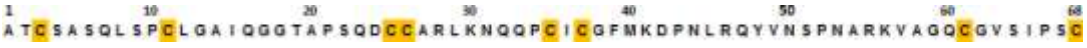

Figure 21. Primary structure of Lyc e 7kDa. The eight cysteine residues conserved in the homologous proteins are highlighted in yellow.

Homology search in Uniprot database carried out using BLAST (www.expasy.org) has shown that the amino acid sequence of Lyc e 7 kDa displays high sequence identity with several already known 7 kDa LTPs (LTP2). The values of sequence identity between some representative components of the family of 7-kDa LTPs, ranging from 43 to 82%, is shown in **Table 5**. For comparative purposes, the identity values between some LTP1 have also been reported

Table 5. Amino acid sequence identity (%) between LTPs from different sources. Sequence identities between LTP belonging to the LTP1 or LTP2 protein families are highlighted in orange and heavenly, respectively. White background has been used to highlight sequence identities between LTP1 and LTP2 proteins.

LTP	Act d 10	Ara h 9	Art v 3	Cor a 8	Mor n 3	Pru p 3	Lyc e 3	Lyc 7k-	HORVU	SOLTU	SOYBN	ORYSJ	MAIZE	WHEAT
Act d 10	100	55	43	42	48	46	45	23	22	28	26	20	24	23
Ara h 9		100	46	54	64	61	62	29	25	28	28	26	27	28
Art v 3			100	50	56	45	44	20	11	18	23	10	11	11
Cor a 8				100	62	59	50	27	16	18	20	13	14	23
Mor n 3					100	70	60	25	14	20	14	8	10	22
Pru p 3						100	50	35	23	26	28	22	22	22
Lyc e 3							100	29	20	20	21	20	18	23
Lyc 7k-LTP								100	55	60	54	56	55	47
HORVU									100	54	45	77	70	46
SOLTU										100	57	56	53	43
SOYBN											100	47	49	47
ORYSJ												100	82	49
MAIZE													100	46
WHEAT														100

in the Table. They fall in the range 43-82%. Conversely, low sequence identities were obtained when components of the family of LTP2 were compared to those of LTP1. The highest value (35%) was recorded by comparing the tomato 7-kDa sequence (Lyc e 7 kDa) and the peach LTP1 (Pru p 3).

Figure 22 shows the alignment of the amino acid sequence of Lyc e 7 kDa with that of homologs belonging to the LTP2 family and some components of the LTP1 family.

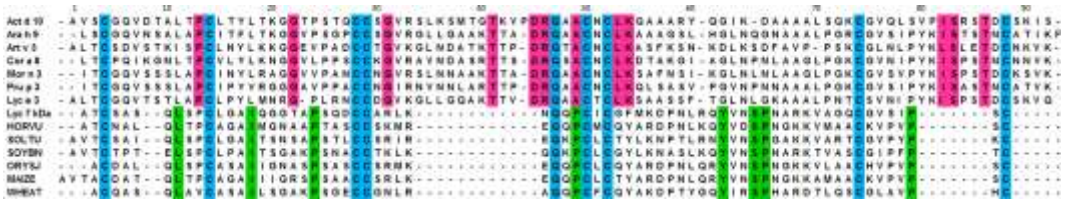

Figure 22. **Multiple sequence alignment of LTPs from different sources.** In blue are the residues conserved in all sequences, in green the residues conserved in the group of LTP2 and in pink the residues conserved in the group of LTP1.

In particular, the sequences of LTP2 from barley (Horvu), potato (Soltu), soy (Soybn), rice (Orysj), maize and wheat and allergenic LTP1 from kiwifruit (Act d 10), peanut (Ara h 9), artemisia (Art v 3), hazelnut (Cor a 8), mulberry (Mor n 3), peach (Pru p 3) and tomato (Lyc e 3) were aligned using the CLUSTAW algorithm (www.expasy.org). The multiple alignment **(Figure 22**) shows that to align LTP1 and LTP2 sequences, displaying significant differences at the primary structure level, it was necessary to insert two gaps in the LTP2 sequences. In addition to the eight cysteine, only Q31 is conserved in all the aligned sequences.

1.20. Immunological and clinical characterization of Lyc e 7kDa

Purified Lyc e 7kDa was immobylized on a customized ISAC microarray, where other allergens, including other allergenic LTP, were spotted. A population of 18 subjects allergic to tomato were selected and analyzed by the ISAC system. Symptoms reported following tomato ingestion were: OAS (9 subjects), dyspnea (4 subjects), gastritis (3 subjects), enteritis (1 subject), food-dependent exercise-induced anaphilaxis (FIDEIA, 1 subject). The analysis of these subjects revealed that 15 patients (83%) had IgE specific for Lyc e 7kDa. Positive results were also recorded for other spotted LTP. **Figure 23** shows the prevalence of subjects positive to Lyc e 7kDa compared to those recorded for other allergenic LTPs, such as Pru p 3, Mor n 3, Art v 3, Ara h 9, Act d 10 and Act c 10.

The analysis of a larger population (89 subjects), IgE positive to at least one LTP, showed that 45 subjects (50%) had IgE recognizing Lyc e 7kDa.

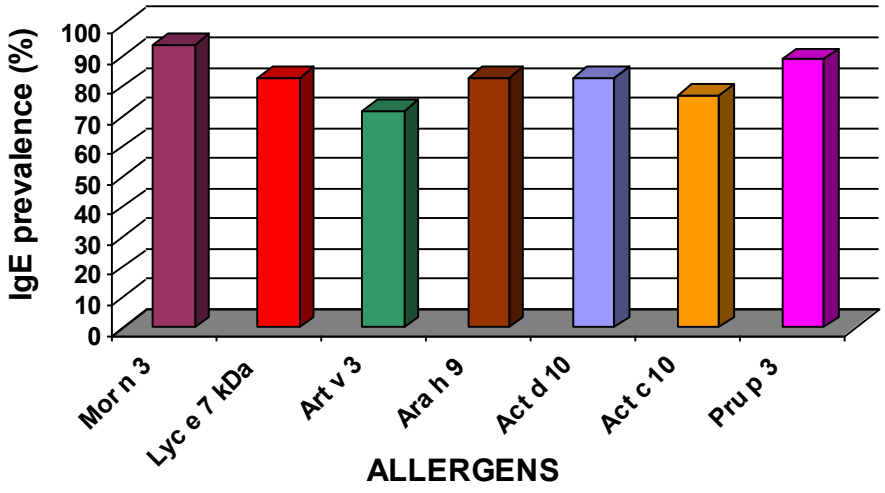

Figure 23. Prevalence of IgE specific for Lyc e 7kDa compared to those found for other allergenic LTPs belonging to the LTP1 family.

Thirty-four allergic subjects underwent *in vivo* testing by SPT. Eight of them (23%) gave a positive reaction to the purified protein.

2. Influence of chemico-physical features of the environment on the protein conformation and the IgE reactivity: kiwellin (Act d 5) as a model (36)

2.1. Skin Prick Test with kiwifruit extracts and purified Act d 5

Protein extracts from green kiwifruit and purified kiwellin (Act d 5) were prepared as described in Materials and Methods Section. Twenty-nine subjects were selected on the basis of a reliable history of allergic reactions to kiwifruit. Patients were tested with both commercial and in-house prepared green kiwifruit extract, and with Act d 5 in a neutral solution (pH 7.4), and in acidic water solution (pH 4.5).

Eleven subjects out of 29 (38%) reacted to the purified Act d 5 by SPT. Among them, 3 patients (10%) showed a reaction only to Act d 5 at pH 4.5, three (10%) showed a reaction only to the allergen in neutral environment and five (17%) showed a reaction to Act d 5 solubilized in both the environments. Some features of the eleven subjects reacting to the SPT, in at least one condition, are listed in **Table 6**.

Table 6. Clinical data of the population under study showing positive SPT to Act d 5

Patient	Age	Symptoms with kiwi	Specific Ig E (ISAC)	Commercial GrK extract	In-house GrK extract	Prick by prick Grk	SPT with Act d 5	
							pH 7.5	pH 4.5
1	15	A,D	0	+	++++	++++	NEG	+
2	23	O	0	+	+++	++	NEG	+
3	13	V	0	++	+++	+++	+	NEG
4	53	O	0	++	+++	+++	NEG	+
5	25	A,P	0	++	+++	+++	++	+
6	33	O	0	+	+	NEG	+/-	NEG
7	32	O	0	NEG	+	ND	+/-	NEG
8	55	A,U	0	++	+++	+++	+	+
9	34	O	0	+	+++	++	+	+
10	50	O	0	NEG	+++	++	++	+
11	57	E,O	0	+++	+++	+++	+	+

This population includes 7 female and 4 male. Six patients reported oral symptoms (O), one patient edema and oral symptoms (E, O), one patient vomiting (V). Three patients had asthma (A): one togheter with atopic dermatitis (D), one with urticaria (U) and another with skin itching (P).

All the 11 subjects with a positive SPT to purified Act d 5, were tested with both commercial and in-house kiwi extract by SPT. Two of them (7%) had no reaction to the commercial extract, whereas all patients reacted to the in-house prepared kiwifruit extract.

2.2. IgE binding experiments

Eleven subjects with positive SPT were also tested on Act d 5 immobilized on the ISAC system. As shown in **Table 6**, no one of the tested sera recognized the immobilized Act d 5. Ten patients out of 11 were tested for specific IgE to green kiwi extract using the CAP system, and seven of them showed high IgE levels. (**Table 6**).

Sera of 9 subjects among those showing a positive SPT were used to test Act d 5 by IgE immunoblotting experiments (**Figure 24**). Eight out of nine sera contained specific IgE recognizing Act d 5 from both reducing and non-reducing conditions. Nevertheless, a variation of the signal intensities was observed, and generally the signals shown by the non-reduced Act d 5 were weaker than those observed for the reduced allergen.

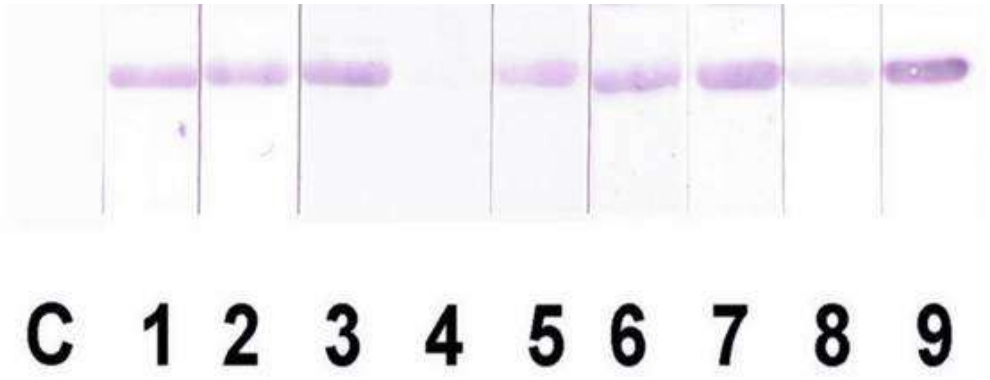

Figure 24. IgE immunoblotting of Act d 5. The allergen was probed with nine individual sera (lanes 1–9, Table 5) and with buffer as a negative control for the anti-human IgE antibody (lane C).

2.3. Circular dichroism studies

A study of the structural properties of Act d 5 has been performed by circular dichroism (CD) in different experimental conditions, to evaluate the effect of different environments on the protein conformation. Spectra have been acquired at pH 7.5, close to the human physiological value, and at pH 4.5, close to the acidic value of the kiwi fruit. Furthermore, the influence of lower polarity media has been investigated, trying to reproduce the chemico-physical features of membrane-like environments, where the interaction between Act d 5 and its target cell takes place. Water/fluorinated alcohol mixtures represent a handily

medium to simulate biological environments of low and medium polarity *(37)*, and have successfully been used to characterize the conformation of several bioactive peptides in membrane-like environments *(38-40)*. **Figure 25** shows the CD spectra obtained at pH 7.5 (upper panel) and pH 4.5 (lower panel) in the different experimental conditions explored. The analysis of the CD curves shows that all of them display the typical shape of a mainly helical structure, characterized by two minima around 222 and 206 nm, although the not-fully canonical shape indicates the contribution of other secondary structures, namely alfa-structures and/or random-coils. In addition, the comparison among the two panels reveals that the curves obtained at lower pH (lower panel) are in general more intense than the curves obtained around a neutral pH (upper panel), suggesting a higher degree of structure at acidic pH. Furthermore, at pH 4.5, the differences among the curves are well evident, and indicate that in the presence of both fluorinated alcohols the fraction of folded molecules

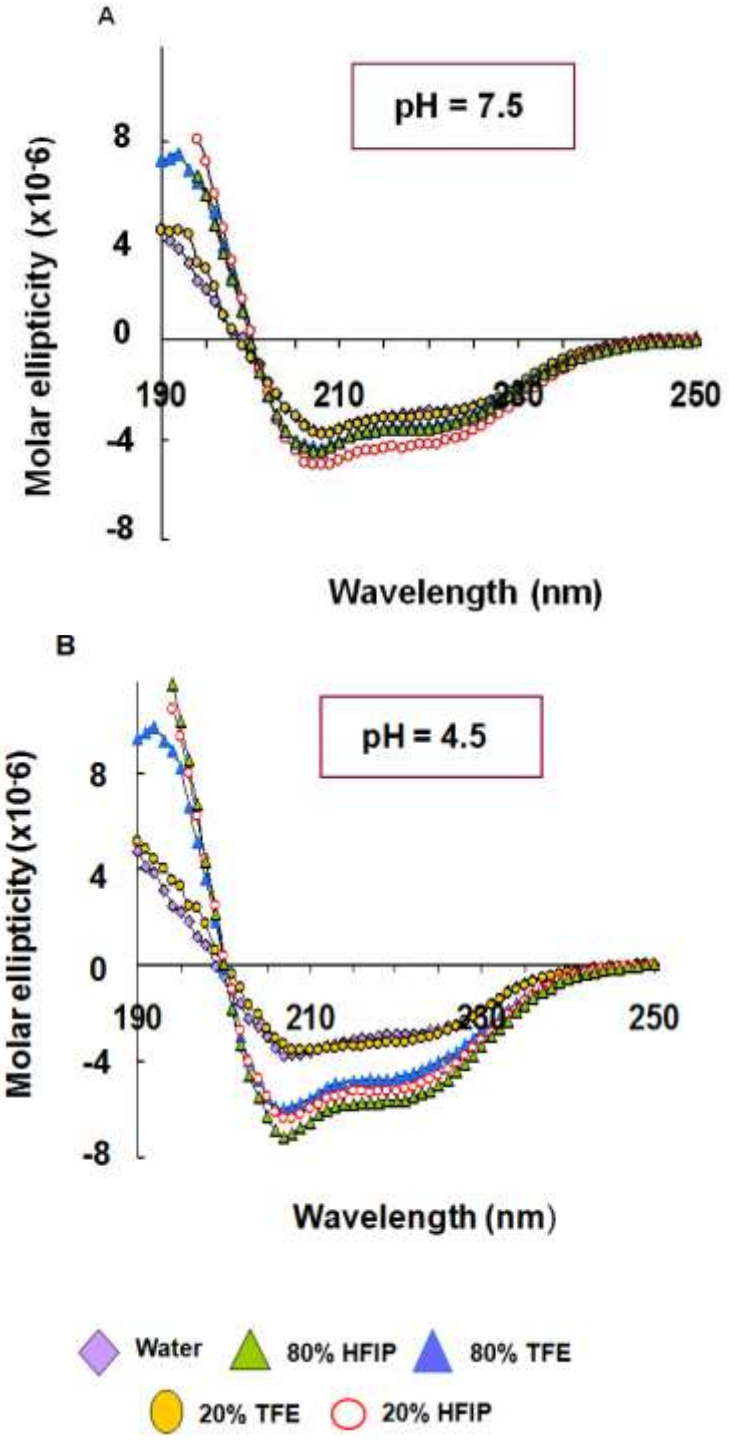

Figure 25. Structural characterisation. Circular dichroism spectra of Act d 5 at pH 7.5 and 4.5 and in mixtures of trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP).

increases. Conversely, at pH 7.5 all the curves are more or less superimposed.

Figure 26 underlines the effect of the experimental conditions on Act d 5 structure. It reportes the molar ellipticity at 206 nm, which represents one of the hallmarks of helical structures, in the different experimental conditions analyzed. In this representation it is well evident that in fully

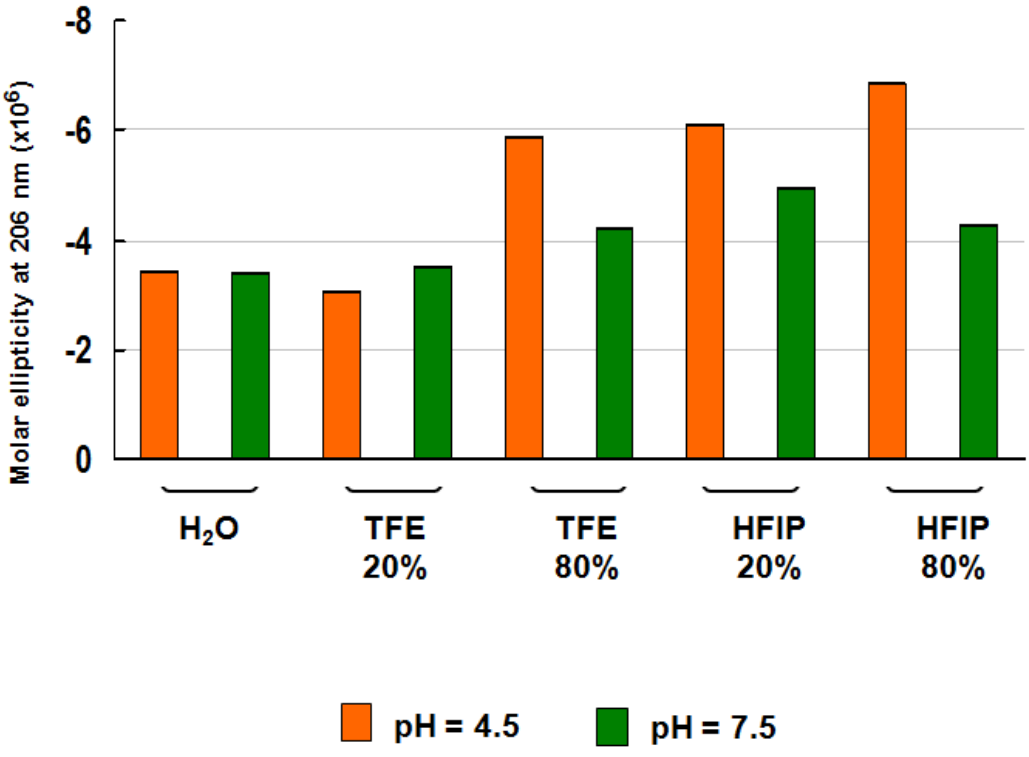

Figure 26. Helical content in Act d 5 under different experimental conditions.

polar media (represented by bulk water) the protein is less structured and there is no pH effect. This behavior is not affected by the addition of a low amount of TFE (20 % by volume), but becomes more pronounced as far as the medium polarity decreases, i.e. in 80 % TFE and in the presence of HFIP, both at 20 and 80% by volume. Overall, the data reported here indicate that the conformation of Act d 5 is strongly affected by the chemico-physical features of the experimental conditions, first of all by the solvent polarity. In general, Act d 5 appears more structured in low polarity media and at low pH.

3. Sensitivity to the gastrointestinal digestion and effect on human intestinal tissue of the kiwifruit peptide kissper

3.1. Kissper purification from green kiwifruit

Several ripe kiwifruit batches were analyzed in order to assess the concentration of the peptide kissper in the protein extracts. The analysis of the kissper content in the kiwifruit protein extracts was performed by RP-HPLC chromatographic separation. The fruits containing high amounts of peptide were selected and used as starting material for kissper purification, following the procedure described in the Materials and Methods Section. The purity of the preparation was checked by RP-HPLC analysis and N-terminal amino acid sequencing.

3.2. Treatment of the peptide kissper with gastrointestinal proteases

Treatment of the peptide kissper with SGF and SIF was performed as described in the Materials and Methods Section. The analysis by RP-HPLC of all the aliquots taken at 0 and 120 min of incubation showed a single peak eluted at

the retention time of the native peptide, thus indicating that kissper was resistant to the treatment with pepsin, trypsin and chymotrypsin. The absence of proteolytic cleavages was also confirmed by N-terminal amino acid sequencing analysis of the aliquots taken at 120 min of incubation, where, in fact, only the N-terminal sequence of the native peptide was obtained.

3.3. Influence of the peptide kissper on human colonic mucosal inflammation

To investigate a possible influence of the peptide kissper on the human colonic mocosa, biopsy specimens from subjects suffering from Crohn's desease (CrD) were analyzed by detection of the expression of some pro-inflammatory cytokines. In particular, the release of TNF-alfa, COX-2 and ICAM-1 after stimulatation by *Escherichia coli* lipopolysaccharide LPS (EC-LPS) was recorded. The immunohystochemical analysis of the tissue shows that the treatment with EC-LPS toxin induced a strong increase of the

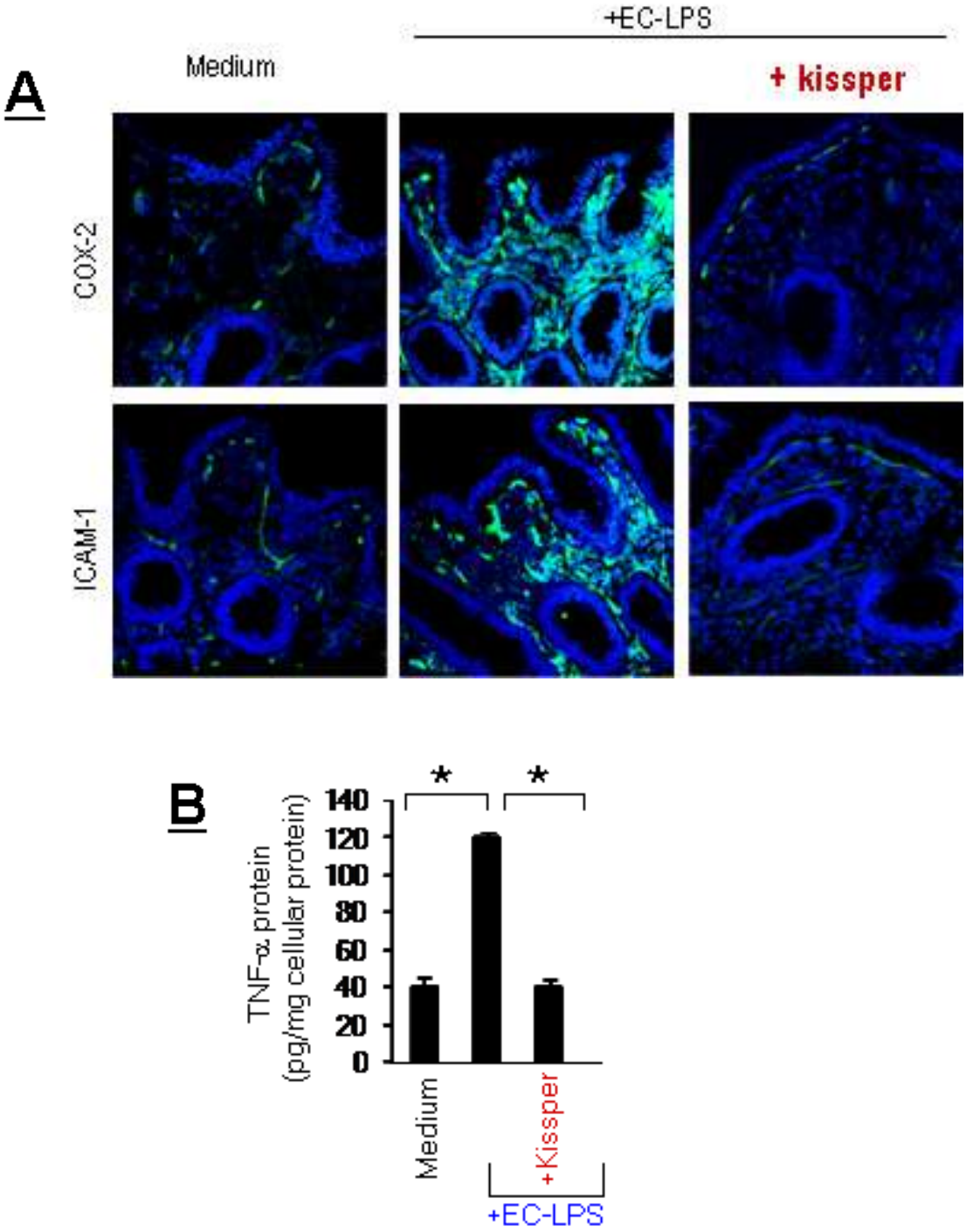

Figure 27. Effect of kissper on the expression of COX-2, ICAM-1 and TNF-alfa after incubation with EC-LPS for 4 h in intestinal biopsies from patients suffering from Chron disease.
(**A**) increased expression of COX-2 and ICAM-1 after challenge with EC-LPS. Pretreatment with kissper reduced their expression at the level observed in the tissue incubated with medium only.
(**B**) Pretreatment with kissper reduced the release of TNF-alfa by EC-LPS treatment.

cytokines expression. However, pre-treatment with the peptide kissper efficiently counteracted this effect by controlling the level of released COX-2 and ICAM-1, that was mantained as low as the control (**Figure 27A**). **Figure 27B** shows that the pre-treatment with kissper nullifies the effect of EC-LPS on the cellular TNF-alfa concentration. In fact, the increased concentration induced by EC-LPS was not observed in the presence of kissper.

DISCUSSION

The knowledge of molecules contained in foods and their properties allows the development of the best and safest diet for each subject. This is especially important for allergic subjects, that needs to avoid allergy sources. In addition, it is also very helpful for people suffering from dysfunctions or diseases that, in some cases, can find a significant improvement of their health by the consumption of specific functional foods or the use of nutraceuticals *(18-20)*. In this framework, the study described in this thesis provides new information in the field of allergology and nutraceutical molecules. In fact, results obtained include the identification and characterization of new allergens from plant foods and at least a partial characterization of a bioactive kiwifruit peptide as an anti-inflammatory molecule.

Kiwifruit has been chosen as a model of food endowed with beneficial effects on human health and also an important source of food allergy. In fact, both the properties have been widely described in literature *(21-23) (12, 26)*. However, the

molecules responsible of beneficial effects and the molecular mechanisms involved in their biological activity are still unknown, as well as not all the allergenic kiwifruit molecules have been described.

Lipid transfer protein (LTP) is an ubiquitous plant protein. It is also a very important allergen *(8, 41)*. For instance, LTP from peach fruit is well known as a molecule that can cause severe allergic reactions, such as the anaphylactic shock *(35)*. In kiwifruit, LTP had never been reported before. Here we describe for the first time the identification of LTP in kiwifruit and the characterization of its immunological properties. It has been identified both in green and gold kiwifruit and on the basis of immunological and clinical properties it has been included in the official list of allergens of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) with the allergen names Act d 10 and Act c 10. Kiwifruit LTP was found in high amounts in the seeds, whereas the pulp seems to contain a very low amount of this allergen. This result suggests that, at least subjects reacting with mild symptoms to kiwifruit

LTP ingestion, can eat this fruit if they care to remove the seeds. Moreover, the absence of good quantity of the LTP in the pulp raises doubts about the usefulness of the prick-prick test technique as reported negative in some subjects in the present study *(34)*.

LTP are small proteins divided in two sub-families on the basis of the molecular weight: the group of LTP1 (9-kDa) and the group of LTP2 (7-kDa) *(3)*. The kiwifruit LTP described in this thesis belongs to the sub-family of LTP1. Anyhow, all the allergenic LTP described so far belong to the LTP1 sub-family. Some allergenic homologs of kiwifruit LTP, that is included in the group of LTP1, have been selected and used for comparative studies. In addition, the study of an LTP belonging to the sub-family 2, isolated for tomato, has been undertaken.

A new allergenic LTP1, used for comparative analysis, has been identified in black mulberry *(33)*. Allergy to this fruit had already been reported in literature, but the responsible molecule(s) had not yet been described. We identified the

LTP1 in the fruit of mulberry. After immunological and clinical characterization it was included in the WHO/IUIS list of allergens with the name of Mor n 3 and it represents the first allergenic molecule reported for black mulberry. The analysis of the structural features of Mor n 3 reveals that it has a very high similarity with the well known allergenic peach LTP, Pru p 3. In fact, the sequence identity between Mor n 3 and Pru p 3 is high (70%). In line with the structural evidence, the immunological and clinical behavior is very similar. Conversely, the structural features of kiwifruit LTP, Act d 10, indicate that this LTP is not closely related to anyone of the already known allergenic LTPs, including Mor n 3 and Pru p 3. In fact, the sequence identities between Act d 10 and Mor n 3 and Pru p 3 are 48% and 46%, respectively. The alignment of Act d 10 sequence with the five LTPs included in this study (Ara h 9, Art v 3, Cor a 8, Mor n 3, Pru p 3) shows that depending on the compared sequences pair, unique combinations of conserved residues occurr. The absence of sequence regions conserved in all the LTP1 suggests that the existence of even one identical epitope,

shared by all the allergenic LTP1, is unlikely. It is coincevable that a complete or partial epitope sharing can be found only when closely related LTPs are compared. The observed sequence micro-heterogeneity, mainly found in distantly related LTPs, could be thus the main cause of heterogeneous epitope patterns producing inhomogeneous LTP IgE recognition as shown by the results obtained following inhibition tests using the ISAC microarray technology *(16, 42, 43)*. In fact some subjects were IgE positive to some LTPs and negative to some others. To the heterogeneous epitope pattern present in the different LTPs could also be ascribed the results obtained by *in vivo* tests on allergic subjects. In fact, in addition to subjects showing positive reaction to both peach and kiwifruit LTP, few subjects reacted only to LTP from one fruit and did not react to the other. The observation that some subjects have isolated IgE positivity to single LTP provides useful information on what to exclude but, most importantly, on what to leave in patient's diet. In addition, these results underline that the concept "one allergen fits all the homologs", that is sometimes applied *(44-46)*, may

produce some erroneous diagnosis. For instance, there is the trend to use the peach LTP, Pru p 3, to assess the allergy to all the plant LTP. In line with data recently reported for a kiwifruit Bet v 1-like allergen *(32)*, the results here reported clearly indicate that the biochemical grouping of allergens can be misleading in the allergy diagnosis and that an improved allergy diagnosis can be obtained by testing every single patient with the most comprehensive panel of available LTPs/allergens .

During the last decade, several efforts have been done to improve allergy diagnosis by integrating the traditional methods, based on protein extracts from natural raw materials, with components based on purified natural or recombinant allergens *(47-50)*.

Purified natural kiwellin (Act d 5) was used to perform different *in vitro* and *in vivo* tests. By testing a population of subjects allergic to kiwifruit using a standard protocol for SPT, 8 out of 29 (28%) had a positive reaction. However, the number of positive reactions to SPT increased when an additional modified protocol was used. In fact, 3 additional

positive reactions (10%) to SPT were observed using Act d 5 in acidic solution, that is an environment more similar to that of the natural source. The observation that some subjects had a positive reaction either at neutral or acidic pH values suggest that this allergen, depending on the environmental conditions, may expose different epitopes. Nevertheless, some subjects may have IgE antibodies recognising epitopes exposed in each of the two examined environmental conditions. The conformational analysis by CD measurements in different experimental conditions indicate that Act d 5 3D structure is modulated by the solvent pH and polarity *(36)*. In fact, the protein, having a mainly helical conformation, is more structured in low polarity media and at acidic pH value, rather than in neutral and fully polar media. Therefore, Act d 5 displays pH-driven conformational changes but this effect is more evident in a low-dielectric costant medium, that is representative of several cellular environments *(37)*, like for instance the interior of biological membranes. During the transit in the gastrointestinal system, Act d 5 can encounter environments characterized by

different pH values, ranging from the very acidic one of the stomach to values close to neutrality, and can meet hydrophobic environments, for instance when approaching cell membranes. Therefore, it may be hypothesized that, depending on the environments encountered, this allergen may undergo *in vivo* conformational changes and expose different epitopes, inducing the synthesis/interaction of different specific IgEs.

IgE-immunoblotting experiments indicate that most of the subjects displaying reactivity to Act d 5 by SPT also recognize sequence epitopes. In fact, eight out of nine subjects analysed by immunoblotting show IgEs reacting with denatured Act d 5. One subject did not recognize sequence epitopes and reacted to SPT only with Act d 5 at acidic pH, and not with Act d 5 in neutral condition. Therefore, this subject could have IgE antibodies recognizing only the conformational epitopes exposed in acidic conditions. These epitopes cannot be revealed by test systems using neutral pH conditions, such as those using the classical PBS buffer, and cannot be revealed by IgE immunoblotting. Probably, the only way to

detect the reactivity to Act d 5 of this subject is the prick by prick test, performed with the fresh kiwi fruit having its naturally acidic pH value. Otherwise, the SPT performed with the purified molecule in acidic conditions may represent a useful alternative.

On the basis of the results obtained by the characterization of Act d 5 it may be suggested that the environmental condition, such as the pH value of the allergen source, could be an additional factor affecting the sensitivity of allergy test performances. In fact, the structural features and, therefore, the ability of some allergens to interact with specific antibodies could be different in environments similar to those of the natural sources respect to other experimental conditions. This is an aspect to which usually very low or even no attention is paid. Similar to Act d 5, it can be imagined that, depending on the environmental conditions, other allergens may undergo conformational changes associated with the exposition of different combinations of epitopes recognized by the IgE antibodies of allergic subjects. This behavior may affect in a very significant way the results

of allergy testing, both *in vitro* and *in vivo*. Therefore, this is an additional factor to be considered in the evaluation of allergy test system reliability.

Therefore, this study on kiwellin, Act d 5, indicates that the 3D structure of this allergen is sensitive to the physico-chemical characteristics of the environment. The protein may undergo pH- and polarity-driven conformational changes that may affect the pattern of exposed antigenic epitopes. This behaviour may have an influence on the sensitivity of diagnostic tests, such as SPT. Therefore, a deep knowledge of the physico-chemical properties of allergenic proteins would be desirable and potentially useful for the improvement of diagnostic test systems.

The functional characterization of kissper *(28, 29)* has shown that this food peptide can have beneficial effects on human health. A previous study had shown that kissper is able to form ion channels and transport ions, preferentially anions, in synthetic plasmatic membranes. This property has suggested a potential farmaceutical use of this molecule in the treatment of pathologies involving an insufficient anion

transport, such as cystic fibrosis, constipation, etc. The exploitation of a molecule as a nutraceutical includes the analysis of several aspects. The study described in this thesis had the aim to investigate the influence of kissper on the intestinal tract in order to exclude possible negative effects. The results obtained on human colonic mucosa deriving from biopsy performed on subjects suffering from Crohn's desease clearly exclude an harmful effect of kissper on this tissue. Even better, immunohystochemical and ELISA experiments have shown the capacity of kissper to suppress the release of inflammatory cytokines, such as COX-2, ICAM-1 and TNF-alfa, after the induction of inflammation by the *E.coli* toxin EC-LPS. This result suggests a possible use of kissper as a nutraceutical molecule in the treatment of inflammation and prompts further studies in order to verify this potentiality and to investigate the mechanism by which the biological activity is reached.

In conclusion, the research work here described has led to the identification of four new allergens that have been characterized at the biochemical and immunological level.

The identification of these new allergens can be helpful to the allergologists in the formulation of a safe diet for individual allergic subjects. In addition, this information could contribute to the improvement of allergy diagnosis following the inclusion of these new allergens in the available diagnostic systems. The study performed on kiwellin has demonstrated that the physico-chemical features of the environment can affect the conformation and the IgE reactivity of Act d 5. Therefore, this result shows that the experimental conditions could affect the response of allergy diagnostic systems by modulating the pattern of exposed antigenic epitopes. The functional characterization of kissper, the peptide naturally occurring in green kiwifruit, indicates that the health-promoting effects reported for this fruit probably cannot be ascribed only to the usually mentioned vitamins and fibers content. Kissper seems to be a nutraceutical molecule the potential pharmaceutical use of which is worthy of being considered.

REFERENCES

1. Nicolaou, N.; Siddique, N.; Custovic, A. Allergic disease in urban and rural populations: increasing prevalence with increasing urbanization. *Allergy* 2005, *60* (11), 1357-1360.

2. Wright, R. J.; Cohen, R. T.; Cohen, S. The impact of stress on the development and expression of atopy. *Curr Opin Allergy Clin Immunol* 2005, *5* (1), 23-29.

3. Yeats, T. H.; Rose, J. K. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci* 2008, *17* (2), 191-198.

4. Garcia-Olmedo, F.; Molina, A.; Segura, A.; Moreno, M. The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol* 1995, *3* (2), 72-74.

5. Park, S. Y.; Lord, E. M. Expression studies of SCA in lily and confirmation of its role in pollen tube adhesion. *Plant Mol Biol* 2003, *51* (2), 183-189.

6. Edqvist, J.; Farbos, I. Characterization of germination-specific lipid transfer proteins from Euphorbia lagascae. *Planta* 2002, *215* (1), 41-50.

7. Hoffmann-Sommergruber, K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* 2000, *122* (3), 155-166.

8. Salcedo, G.; Sanchez-Monge, R.; az-Perales, A.; Garcia-Casado, G.; Barber, D. Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin Exp Allergy* 2004, *34* (9), 1336-1341.

9. Zuidmeer, L.; van, R. R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. *Curr Opin Allergy Clin Immunol* 2007, *7* (3), 269-273.

10. Nelson, H. S.; Ikle, D.; Buchmeier, A. Studies of allergen extract stability: the effects of dilution and mixing. *J Allergy Clin Immunol* 1996, *98* (2), 382-388.

11. Focke, M.; Marth, K.; Valenta, R. Molecular composition and biological activity of commercial birch pollen allergen extracts. *Eur J Clin Invest* 2009, *39* (5), 429-436.

12. Ciardiello, M. A.; Giangrieco, I.; Tuppo, L.; Tamburrini, M.; Buccheri, M.; Palazzo, P.; Bernardi, M. L.; Ferrara, R.; Mari, A. Influence of the natural ripening stage, cold storage, and ethylene treatment on the protein and IgE-binding profiles of green and gold kiwi fruit extracts. *J Agric Food Chem* 2009, *57* (4), 1565-1571.

13. Mari, A.; Alessandri, C.; Bernardi, M. L.; Ferrara, R.; Scala, E.; Zennaro, D. Microarrayed allergen molecules for the diagnosis of allergic diseases. *Curr Allergy Asthma Rep* 2010, *10* (5), 357-364.

14. Harwanegg, C.; Hiller, R. Protein microarrays for the diagnosis of allergic diseases: state-of-the-art and future development. *Clin Chem Lab Med* 2005, *43* (12), 1321-1326.

15. Harwanegg, C.; Hutter, S.; Hiller, R. Allergen microarrays for the diagnosis of specific IgE against components of cow's milk and hen's egg in a multiplex biochip-based immunoassay. *Methods Mol Biol* 2007, *385*, 145-157.

16. Scala, E.; Alessandri, C.; Bernardi, M. L.; Ferrara, R.; Palazzo, P.; Pomponi, D.; Quaratino, D.; Rasi, C.; Zaffiro, A.; Zennaro, D.;

Mari, A. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 2010.

17. Mari, A.; Ciardiello, M. A.; Tamburrini, M.; Rasi, C.; Palazzo, P. Proteomic analysis in the identification of allergenic molecules. *Expert Rev Proteomics* 2010, *7* (5), 723-734.

18. Roberfroid, M. B. Global view on functional foods: European perspectives. *Br J Nutr* 2002, *88 Suppl 2*, S133-S138.

19. Hartmann, R.; Wal, J. M.; Bernard, H.; Pentzien, A. K. Cytotoxic and allergenic potential of bioactive proteins and peptides. *Curr Pharm Des* 2007, *13* (9), 897-920.

20. Eastwood, M. A. A molecular biological basis for the nutritional and pharmacological benefits of dietary plants. *QJM* 2001, *94* (1), 45-48.

21. Motohashi, N.; Shirataki, Y.; Kawase, M.; Tani, S.; Sakagami, H.; Satoh, K.; Kurihara, T.; Nakashima, H.; Mucsi, I.; Varga, A.; Molnar, J. Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. *J Ethnopharmacol* 2002, *81* (3), 357-364.

22. Collins, A. R.; Harrington, V.; Drew, J.; Melvin, R. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* 2003, *24* (3), 511-515.

23. Jung, K. A.; Song, T. C.; Han, D.; Kim, I. H.; Kim, Y. E.; Lee, C. H. Cardiovascular protective properties of kiwifruit extracts in vitro. *Biol Pharm Bull* 2005, *28* (9), 1782-1785.

24. Rush, E. C.; Patel, M.; Plank, L. D.; Ferguson, L. R. Kiwifruit promotes laxation in the elderly. *Asia Pac J Clin Nutr* 2002, *11* (2), 164-168.

25. Gavrovic-Jankulovic, M.; Cirkovic, T.; Vuckovic, O.; tanaskovic-Markovic, M.; Petersen, A.; Gojgic, G.; Burazer, L.; Jankov, R. M. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J. Allergy Clin. Immunol.* 2002, *110* (5), 805-810.

26. Tamburrini, M.; Cerasuolo, I.; Carratore, V.; Stanziola, A. A.; Zofra, S.; Romano, L.; Camardella, L.; Ciardiello, M. A. Kiwellin, a novel protein from kiwi fruit. Purification, biochemical characterization and identification as an allergen*. *Protein J.* 2005, *24* (7-8), 423-429.

27. Pastorello, E. A.; Conti, A.; Pravettoni, V.; Farioli, L.; Rivolta, F.; Ansaloni, R.; Ispano, M.; Incorvaia, C.; Giuffrida, M. G.; Ortolani, C. Identification of actinidin as the major allergen of kiwi fruit. *J. Allergy Clin. Immunol.* 1998, *101* (4 Pt 1), 531-537.

28. Tuppo, L.; Giangrieco, I.; Palazzo, P.; Bernardi, M. L.; Scala, E.; Carratore, V.; Tamburrini, M.; Mari, A.; Ciardiello, M. A. Kiwellin, a modular protein from green and gold kiwi fruits: evidence of in vivo and in vitro processing and IgE binding. *J. Agric. Food Chem.* 2008, *56* (10), 3812-3817.

29. Ciardiello, M. A.; Meleleo, D.; Saviano, G.; Crescenzo, R.; Carratore, V.; Camardella, L.; Gallucci, E.; Micelli, S.; Tancredi, T.; Picone, D.; Tamburrini, M. Kissper, a kiwi fruit peptide with channel-like activity: structural and functional features. *J. Pept. Sci.* 2008, *14* (6), 742-754.

30. Ciardiello, M. A.; D'Avino, R.; Amoresano, A.; Tuppo, L.; Carpentieri, A.; Carratore, V.; Tamburrini, M.; Giovane, A.; Pucci, P.; Camardella, L. The peculiar structural features of kiwi fruit pectin methylesterase: amino acid sequence, oligosaccharides structure, and modeling of the interaction with its natural proteinaceous inhibitor. *Proteins* 2008, *71* (1), 195-206.

31. Bublin, M.; Radauer, C.; Knulst, A.; Wagner, S.; Scheiner, O.; Mackie, A. R.; Mills, E. N.; Breiteneder, H. Effects of gastrointestinal digestion and heating on the allergenicity of the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin-like protein. *Mol Nutr Food Res* 2008, *52* (10), 1130-1139.

32. D'Avino, R.; Bernardi, M. L.; Wallner, M.; Palazzo, P.; Camardella, L.; Tuppo, L.; Alessandri, C.; Breiteneder, H.; Ferreira, F.; Ciardiello, M. A.; Mari, A. Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen. *Allergy* 2011, *66* (7), 870-877.

33. Ciardiello, M. A.; Palazzo, P.; Bernardi, M. L.; Carratore, V.; Giangrieco, I.; Longo, V.; Melis, M.; Tamburrini, M.; Zennaro, D.; Mari, A.; Colombo, P. Biochemical, immunological and clinical characterization of a cross-reactive nonspecific lipid transfer protein 1 from mulberry. *Allergy* 2010, *65* (5), 597-605.

34. Bernardi, M. L.; Giangrieco, I.; Camardella, L.; Ferrara, R.; Palazzo, P.; Panico, M. R.; Crescenzo, R.; Carratore, V.; Zennaro, D.; Liso, M.; Santoro, M.; Zuzzi, S.; Tamburrini, M.; Ciardiello, M. A.; Mari, A. Allergenic lipid transfer proteins from plant-derived foods do not immunologically and clinically behave homogeneously: the kiwifruit LTP as a model. *PLoS One* 2011, *6* (11), e27856.

35. Garcia-Casado, G.; Pacios, L. F.; az-Perales, A.; Sanchez-Monge, R.; Lombardero, M.; Garcia-Selles, F. J.; Polo, F.; Barber, D.; Salcedo, G. Identification of IgE-binding epitopes of the major peach allergen Pru p 3. *J Allergy Clin Immunol* 2003, *112* (3), 599-605.

36. Bernardi, M. L.; Picone, D.; Tuppo, L.; Giangrieco, I.; Petrella, G.; Palazzo, P.; Ferrara, R.; Tamburrini, M.; Mari, A.; Ciardiello, M. A. Physico-chemical features of the environment affect the protein conformation and the immunoglobulin E reactivity of kiwellin (Act d 5). *Clin Exp Allergy* 2010, *40* (12), 1819-1826.

37. Tanizaki S; Clifford J; Connelly BD; Feig M Conformational sampling of peptides in cellular environments. *Biophys Journal* 2008, *94* (3), 747-759.

38. Crescenzi, O.; Tomaselli, S.; Guerrini, R.; Salvadori, S.; D'Ursi, A. M.; Temussi, P. A.; Picone, D. Solution structure of the Alzheimer amyloid beta-peptide (1-42) in an apolar microenvironment. Similarity with a virus fusion domain. *Eur J Biochem* 2002, *269* (22), 5642-5648.

39. Tomaselli, S.; Esposito, V.; Vangone, P.; van Nuland, N. A.; Bonvin, A. M.; Guerrini, R.; Tancredi, T.; Temussi, P. A.; Picone, D. The alpha-to-beta conformational transition of Alzheimer's Abeta-(1-42) peptide in aqueous media is reversible: a step by step conformational analysis suggests the location of beta conformation seeding. *Chembiochem.* 2006, *7* (2), 257-267.

40. Spadaccini, R.; D'Errico, G.; D'Alessio, V.; Notomista, E.; Bianchi, A.; Merola, M.; Picone, D. Structural characterization of the transmembrane proximal region of the hepatitis C virus E1 glycoprotein. *Biochim Biophys Acta* 2010, *1798* (3), 344-353.

41. Andersen, M. B.; Hall, S.; Dragsted, L. O. Identification of European Allergy Patterns to the Allergen Families PR-10, LTP, and Profilin from Rosaceae Fruits. *Clin Rev Allergy Immunol* 2009.

42. Hiller, R.; Laffer, S.; Harwanegg, C.; Huber, M.; Schmidt, W. M.; Twardosz, A.; Barletta, B.; Becker, W. M.; Blaser, K.; Breiteneder, H.; Chapman, M.; Crameri, R.; Duchene, M.; Ferreira, F.; Fiebig, H.; Hoffmann-Sommergruber, K.; King, T. P.; Kleber-Janke, T.; Kurup, V. P.; Lehrer, S. B.; Lidholm, J.; Muller, U.; Pini, C.; Reese, G.; Scheiner, O.; Scheynius, A.; Shen, H. D.; Spitzauer, S.; Suck, R.; Swoboda, I.; Thomas, W.; Tinghino, R.; Van Hage-Hamsten, M.; Virtanen, T.; Kraft, D.; Muller, M. W.; Valenta, R. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J* 2002, *16* (3), 414-416.

43. Bublin, M.; Dennstedt, S.; Buchegger, M.; Antonietta, C. M.; Bernardi, M. L.; Tuppo, L.; Harwanegg, C.; Hafner, C.; Ebner, C.; Ballmer-Weber, B. K.; Knulst, A.; Hoffmann-Sommergruber, K.; Radauer, C.; Mari, A.; Breiteneder, H. The performance of a component-based allergen microarray for the diagnosis of kiwifruit allergy. *Clin Exp Allergy* 2011, *41* (1), 129-136.

44. Asero, R.; Arena, A.; Cecchi, L.; Conte, M. E.; Crivellaro, M.; Emiliani, F.; Lodi, R. F.; Longo, R.; Minale, P.; Murzilli, F.; Musarra, A.; Nebiolo, F.; Quercia, O.; Ridolo, E.; Savi, E.; Senna, G. E.; Villalta, D. Are IgE levels to foods other than rosaceae predictive of allergy in lipid transfer protein-hypersensitive patients? *Int Arch Allergy Immunol* 2011, *155* (2), 149-154.

45. Asero, R. Lipid transfer protein cross-reactivity assessed in vivo and in vitro in the office: pros and cons. *J Investig Allergol Clin Immunol* 2011, *21* (2), 129-136.

46. Ruiz-Garcia, M.; Garcia Del, P. M.; Fernandez-Nieto, M.; Barber, D.; Jimeno-Nogales, L.; Sastre, J. Profilin: a relevant aeroallergen? *J Allergy Clin Immunol* 2011, *128* (2), 416-418.

47. Deinhofer, K.; Sevcik, H.; Balic, N.; Harwanegg, C.; Hiller, R.; Rumpold, H.; Mueller, M. W.; Spitzauer, S. Microarrayed allergens for IgE profiling. *Methods* 2004, *32* (3), 249-254.

48. Lidholm, J.; Ballmer-Weber, B. K.; Mari, A.; Vieths, S. Component-resolved diagnostics in food allergy. *Curr Opin Allergy Clin Immunol* 2006, *6* (3), 234-240.

49. Constantin, C.; Quirce, S.; Poorafshar, M.; Touraev, A.; Niggemann, B.; Mari, A.; Ebner, C.; Akerstrom, H.; Heberle-Bors, E.; Nystrand, M.; Valenta, R. Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis. *Allergy* 2009.

50. Bublin, M.; Pfister, M.; Radauer, C.; Oberhuber, C.; Bulley, S.; Dewitt, A. M.; Lidholm, J.; Reese, G.; Vieths, S.; Breiteneder, H.; Hoffmann-Sommergruber, K.; Ballmer-Weber, B. K. Component-resolved diagnosis of kiwifruit allergy with purified natural and recombinant kiwifruit allergens. *J Allergy Clin Immunol* 2010.

Part of the results described in this thesis have been reported in the following Publications or Congress Communications.

PUBLICATIONS

1. Tuppo L., **Giangrieco I.**, Palazzo P., Bernardi M.L., Scala E., Carratore V., Tamburrini M., Mari A., Ciardiello M.A. (2008) Kiwellin, a modular protein from green and gold kiwi fruits: evidence of in vivo and in vitro processing and IgE binding. *J. Agric. Food Chem.* **56**:3812–3817.

2. Ciardiello, M.A., **Giangrieco, I.**, Tuppo, L., Tamburrini, M., Buccheri, M., Palazzo, P., Bernardi, M.L., Ferrara, R., Mari, A. (2009) Influence of the natural ripening stage, cold storage, and ethylene treatment on the protein and IgE-binding profiles of green and gold kiwi fruit extracts. *J. Agric. Food Chem.* **57** (4), 1565-1571.

3. Ciardiello M.A., Palazzo P., Bernardi M.L., Carratore V., **Giangrieco I.**, Longo V., Melis M., Tamburrini M., Zennaro D., Mari A., Colombo P. (2010) Biochemical, immunological and clinical characterization of a cross-reactive nonspecific lipid transfer protein 1 from mulberry. *Allergy* **65** (5), 597-605.

4. Bernardi M.L., Picone D., Tuppo L., **Giangrieco I.**, Petrella G., Palazzo P., Ferrara R., Tamburrini M., Mari A., Ciardiello M.A. (2010) Chemico-Physical features of environment affect to the protein conformation and the IgE reactivity of kiwellin (Act d 5). *Clin. Exp. Allergy* **40** (12), 1819-26.

5. **Giangrieco I.**, Bernardi M.L., Camardella L., Ferrara R., Palazzo P., Panico M.R., Crescenzo R., Carratore V., Zennaro D., Liso M., Santoro M., Zuzzi S., Tamburrini M., Ciardiello M.A., Mari A. (2011) Allergenic lipid transfer proteins from plant-derived foods do not immunologically and clinically behave homogeneously: the kiwifruit LTP as a model. *PLoS ONE* **6** (11), e27856.

CONFERENCE PROCEEDINGS PUBLISHED IN JOURNALS

1. **Giangrieco, I.,** Tuppo, L., Palazzo P., Camardella, L., Bernardi, M., Scala, E., Mari, A., Ciardiello, M. (2007) Analysis of green and gold kiwi fruit protein pattern as a function of the ripening stage. *Allergy* **62**, (Supplemento 83), 341-341.

2. Bernardi, M., Palazzo, P., Tuppo, L., **Giangrieco, I.,** Camardella, L., Tamburrini, M., Scala, E., Giani, M., Ciardiello, M., Mari, A. (2008) Pectin methylesterase (Act d 7) and pectin methylesterase inhibitor (Act d 6): two new kiwi fruit allergens. *Allergy* **63** (Supplemento 88), 585-586.

COMMUNICATIONS TO CONGRESS

1. Palazzo P., **Giangrieco I.**, Bernardi M.L., Tamburrini M., Giani M., Ciardiello M.A., Mari A. (2007) IgE reactivity to kiwellin (Act d 28kD), actinidin (Act d 1) and thaumatin-like protein (Act d 2) from green kiwi fruit in kiwi allergic patients tested by SDS-PAGE arrayed molecules. XXVI Congress of the European Academy of

Allergology and Clinical Immunology, EAACI, June 9-13, Göteborg, Abstract No. 961

.

2. Palazzo P., **Giangrieco I**., Bernardi M.L., Tamburrini M., Giani M.,Ciardiello M.A., Mari A. (2007) Analysis of kiwi allergic patients sera by testing the IgE reactivity to Kiwellin (Act d 28kD), Actinidin (Act d 1) and Thaumatin-like protein (Act d 2) from green kiwi fruit. Abstracts of the 2nd International Symposium on Molecular Allergology "Allergens: from Identification to Therapy", Rome, April 22-24, Abstract No. 57.

3. Tuppo L., **Giangrieco I.,** Palazzo P., Carratore V., Camardella L., Bernardi M.L., Scala E., Mari A., Ciardiello M.A. (2007) Conditions affecting the protein and allergen patterns of green and gold kiwi fruit extracts. Abstracts of the "2nd International Symposium on Molecular Allergology", Rome, April 22-24, p. 49.

4. Meleleo D., Tamburrini M., Picone D., Saviano G., Tancredi T., **Giangrieco I**., Petrella G., Galluci E., Iovino P., Ciardiello MA., Micelli S. (2008) Effect of salts on the channel activity of kissper, a kiwi fruit peptide, incorporated into PLMs with composition similar to that of intestinal membrane. 59° Congresso Nazionale della Società Italiana di Fisiologia. Cagliari, 17-19 Settembre. Abstract P093.

5. Tuppo L., **Giangrieco I**., Palazzo P., Bernardi M.L., Scala E., Carratore V., Tamburrini M., Mari A., Ciardiello M.A. (2008) Kiwellin from green and gold kiwi fruit: proteolytic processing and IgE binding. 3rd International Symposium on Molecular Allergology, Salisburgo 18-20 april, Abstract No. 33, p. 70.

6. Ciardiello M.A., Tuppo L., **Giangrieco I.**, Panico M.R., Carratore V., Camardella L., D'Avino R., Tamburrini M. (2009) Allergens and nutraceuticals in plant food: kiwi fruit as a model. 1st Joint Retreat IGB - TIGEM – IBP, Roccaraso, 10-12 June, A15.

7. Ciardiello A., Camardella L., Tamburrini M., D'Avino R., Tuppo L., **Giangrieco I.,** Farina L., Buccheri M., Palazzo P., Ferrara R., Bernardi M.L., Mari A. (2009) Allergenic food proteins and conditions that may affect their concentration: kiwi and peach fruits as models. COST 928 Meeting, Belgrade 2-3 April, p. 17.

8. **Giangrieco I.,** Palazzo P., Bernardi M.L., Ciardiello M.A., Carratore V., Longo V., Melis M., Tamburrini M., Zennaro D., Mari A., Colombo P. (2010) Characterization of food allergens to improve diagnostic systems and desensitizing immunotherapies: a comparative study on Lipid Tranfer Proteins. Abstracts of the 4thA.It.U.N. Annual Meeting "Innovation in Pharmaceutics: a glimpse in the Biothech World", Naples, February 26-27, p. 60.

9. Ciardiello M.A., Tuppo L., **Giangrieco I.**, Panico M.R., Carratore V., Camardella L., D'Avino R., Tamburrini M. (2010) Allergens and nutraceuticals in kiwi fruit. Cost 928 Meeting, Naples, 2-4 March, Abstract P.13.

10. Ciardiello M., Palazzo P., Bernardi M., Carratore V., **Giangrieco I.,** Longo V., Melis M., Tamburrini M., Zennaro D., Mari A., Colombo P. (2010) Biochemical, immunological and clinical characterisation of a cross-reactive nonspecific lipid transfer protein 1 from mulberry. 29th Congress of the European Academy of Allergy and

Clinical Immunology, EAACI meeting, London 5-9 June, Abstract p. 453.

11. **Giangrieco I**., Camardella L., Bernardi M.L., Tamburrini M., Carratore V., Palazzo P., Zennaro D., Mari A., Ciardiello M.A. (2010) Identification and biochemical characterisation of nonspecific Lipid Transfer Protein (Act d 10) from green kiwifruit. 4th International Symposium on Molecular Allergology, Munich 29-31 October, Abstract No. 5, p. 28.

12. Tuppo L., Bernardi M.L., Picone D., **Giangrieco I**., Petrella G., Palazzo P., Ferrara R., Tamburrini M., Mari A., Ciardiello M.A. (2010) Influence of the chemico-physical features of the environment on the structure and the IgE reactivityof kiwellin (Act d 5). 4th International Symposium on Molecular Allergology, Munich 29-31 October, Abstract No. 16, p. 33.

13. Pomponi D., Scala E., Bernardi M.L., Tuppo L., **Giangrieco I.**, Ciardiello M.A., Mari A. (2010) Evaluating the IgE-based biological activity of a purified Pru p 3, the peach LTP, preparation. 4th International Symposium on Molecular Allergology, Munich 29-31 October, Abstract No. 17, p. 34.

14. Zennaro D., Scala E., **Giangrieco I.**, Tamburrini M., Palazzo P., Liso M., Colombo P., Ciardiello M.A., Mari A. (2010) Prevalence of IgE to Mor n 3, a mulberry LTP, in consecutive patients and comparison with Pru p 3 (peach) and Act d 10 (kiwifruit) prevalence. 4th International Symposium on Molecular Allergology, Munich 29-31 October, Abstract No. 18, p. 34.

15. **Giangrieco I**., Panico M.R., Bernardi M.L., Tamburrini M., Carratore V., Palazzo P., Zennaro D., Mari A., Ciardiello M.A. (2011) Identificazione e caratterizzazione biochimica della Lipid Transfer Protein (Act d 10) dal frutto di kiwi verde. 2° Congresso Nazionale IFIACI, 26° Congresso Nazionale SIAIC, Roma 25-28 Maggio, Abstract p. 273.

16. Tuppo L., Bernardi M.L., Picone D., **Giangrieco I**., Petrella G., Palazzo P., Ferrara R., Tamburrini M., Mari A., Ciardiello M.A. (2011) Influenza delle caratteristiche chimico-fisiche dell'ambiente sulla conformazione della kiwellina(Act d 5) e sulla sua interazione con gli anticorpi IgE. 2° Congresso Nazionale IFIACI, 26° Congresso Nazionale SIAIC, Roma 25-28 Maggio, Abstract p. 302.

17. **Giangrieco I**., Panico M.R., Bernardi M.L., Tamburrini M., Carratore V., Palazzo P., Zennaro D., Mari A., Ciardiello M.A. (2011) Identification and biochemical characterisation of Act d 10 and Act c 10, the nonspecific Lipid Transfer Proteins from green and gold kiwifruit. 30th Congress of European Academy Of Allergy and Clinical Immunology, Istanbul 11-15 June, Abstract No. 331, p. 89.