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Serological Investigations of Fruit and Pollen Allergens

Diploma Thesis

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Abstract

Fruit and pollen allergies are a theme of uprising interest for populations in the Northern Hemisphere. The number of individuals suffering from immediate hypersensitivity increases rapidly. Although primary sensitization is assumed currently to occur through pollen exposure, there are only few investigations of pollen from woody crop species.

The purpose of this work was to provide an overview of the IgE-reaction pattern to several plant species common in the Northern hemisphere both at the fruit and the pollen level. The major accent was set on two apple allergens, Mal d 1 and Mal d 3. Their homologues in fruit and pollen of species from the families *Rosaceae, Caprifoliaceae, Ericaceae, Rutaceae, Apiaceae, Vitaceae, Cornaceae, Moraceae, Oleaceae, Lythraceae and Poaceae* detected by relying on the cross-reactivity of polyclonal antibodies raised against Mal d 1 and Mal d 3, using SDS-PAGE and Western blotting.

Homologues of the major apple allergen Mal d 1 were present in all analyzed fruit and pollen extracts. The presence of homologues of Mal d 3 was also confirmed in all extracts, except for members of the *Poaceae*. It was possible to discover allergens even in species, where no homologues have been reported yet, e.g. Mal d 3: in carrot pollen extract detected band in the carrot root extract - not detected, which could be due to an organ specific expression pattern. A comparable situation occurred with elderberry: polyclonal antibodies detected Mal d 1 in the pollen extract, but not in the fruit extract.

This work confirmed the existence of cross-reactivity with purified Mal d 1 and Mal d 3 in Inhibition ELISA tests with peach pollen as representative model. IgE antibodies in serum of patient with no reported sensitivity against peach before could be inhibited, an indication that the inhibition ELISA can be another alternative method to determine sensitization that has not provoked a severe allergic reaction yet, but is a possible threat for the patient’s health.

Finally, additional proteins in fruits of blackberry, blueberry, elderberry, pollen of quince, rowan and pomegranate, interacting with IgEs of allergic patients - not characterized so far - were detected, and require further analyses.

Zusammenfassung

Frucht- und Pollenallergie ist ein Thema von steigender Bedeutung für die Bevölkerung der Nordhalbkugel. Die Patienten leiden unter dem sogenannten oralen Allergiesyndrom (OAS) – einer IgE vermittelten Reaktion. Obwohl angenommen wird, dass die primäre Sensibilisierung durch Pollen verursacht wird, sind die meisten Obstbaumpollen kaum erforscht.

Ziel dieser Arbeit war, eine Übersicht von möglichen IgE Reaktionen auf die häufigsten Früchte und Pollen der Nordhalbkugel zu verschaffen. Zwei der vier Hauptallergene in Apfel, Mal d 1 und Mal d 3, standen im Mittelpunkt und wurden aufgrund der erwarteten Kreuzreaktivität mit polyklonalen anti Mal d 1 und Mal d 3 Antiseren mittels SDS PAGE und Western blot detektiert. Tatsächlich konnten Homologe in Frucht- und Pollenextakten von Arten der Familie *Rosaceae* (Apfel, Pfirsich, Kirsche, Marille, Erdbeere, Himbeere, Brombeere, Quitte, Mispel, Birne, Sauerkirsche, Rose), *Caprifoliaceae* (Holunder), *Ericaceae* (Heidelbeere), *Rutaceae* (Orange), *Apiaceae* (Karotte), *Vitaceae* (Weintraube), *Cornaceae* (Kornelkirsche), *Moraceae* (Maulbeere), *Oleaceae* (Olive), *Lythraceae* (Granatapfel) and *Poaceae* (gewöhnlicher Glatthafer, Wiesen-Rispengras, Wiesen-Knäuelgras, Mäusegerste, Weiche Trespe, Deutsches Weidelgras und Roggen) gefunden werden.

Mal d 3 Homologe wurden in allen untersuchten Arten mit Ausnahme der Gräser detektiert, auch in Spezies, wo sie noch nicht berichtet wurden, z.B. in Karottenpollen, aber nicht in den Wurzeln. Mal d 1 Homologe wurden in Holunderpollen, aber nicht in Holunderbeeren detektiert. Das sind eindeutige Hinweise auf organspezifische Unterschiede in der Allergenexpression.

Die Kreuzreaktivität wurde mittels Inhibitions-ELISA mit Pfirsichpollen als repräsentativem Modell untersucht und mit gereinigtem Mal d 1 und Mal d 3 bestätigt. IgE Antikörper in Patientenseren ohne Pfirsich Anamnese konnten mit gereinigtem Mal d 3 inhibiert werden. Dieser Umstand zeigt, dass Inhibitions-ELISA eine alternative Methode zur Detektion einer Sensibilisierung sein kann, die noch keine klinische Reaktion verursacht.

Weitere noch nicht charakterisierte IgE-reaktive Proteine wurden in Früchten von Brombeere, Heidelbeere, Holunder und Pollen von Quitte, Eberesche und Granatapfel gefunden. Diese Tatsache verdient Beachtung und erfordert weitere Analysen.

1. Introduction

The World Allergy Organization estimates that allergies are affecting about 22% of the world population and could be as high as 45% in some regions. All around the world, allergies are on the increase and even doubling by some statistics, in the last 25 years (www.allergy.com). One important problem is the increasing allergic reactions in patients of all ages, however with a clear prevalence in young and female patients (Jensen-Jarolim et al. 2008). Per definition allergy is the hypersensitivity of the organism against a defined allergen (antigen) at an immunological level. True food allergies are estimated to affect less than 2 percent of adults, but 4 to 8 percent of young children and infants. The clinical importance of food allergies increases every year (www.allergy.com) with symtoms ranging from pruritus, swelling of the lips, tongue and oral mucosa (often accompanied by mild laryngeal aymptoms as a sensation of tightness), itching, cough and pruritus of the ear canals to gastrointestinal symptoms, rhinitis, asthma, cutaneous reactions and systematic anaphylaxis (Hoffmann-Sommergruber 2002). These factors can significantly influence both the lifestyle and quality of affected patients. The past two decades have witnessed exciting advances in the field of food allergy research. There has been a wealth of reliable information published related to food allergy, including information about the cross-reactivity of food allergens, the evaluation of potential new therapies and the practical application of new diagnostic methods and management strategies. Today we know that sensitization to a particular allergen occurs due to direct exposure or due to cross-reactivity among allergens – the reaction appears because of the similarity in the protein structure and function of related allergens. This phenomenon is in first place explained with the degree of relationship within the plant families. In this context, the capacity of fruit tree pollen - insect- carried, heavy and of large size - to sensibilise and cause allergic reactions has been underestimated so far. The airborn grass pollen are also an important factor in the allergic epidemiology.

1.1. Pollen allergens

Pollen allergens are water-soluble proteins and glycoproteins with a molecular weight between 9 and 70 kDa (Knox et al. 1996 a), and cause a Type I allergy. The reaction occurs several minutes after contacting the allergen molecule by sensitized individuals. Most often it is caused by wind-carried pollen from species of the families *Poaceae*, *Betulaceae* and other Angiosperms (Breiteneder et al. 1989). Up to date are *Betula* species with their eight allergens and homologues one of the the best analyzed pollen allergy agents together with the *Poaceae* family (allergome.org). The role of insect-

carried pollen from species of the families *Rosaceae, Caprifoliaceae, Vitaceae, Cornaceae* and *Moraceae* so far was poorly studied and considered.

A typical pollen grain corresponds to a three cell reduced male gametophyte from an angiosperm plant. It derives from a pollen mother cell and contains one vegetative and one generative nuclei. The last one divides during the second pollen mitosis in two sperm cells. This can happen in the anthers leading to three-cell pollen grains, or after leaving the anthers, giving rise to two-cell pollen grain.

Typically, the pollen grain is covered by a wall (sporoderm) of two layers (Fig. 1). The inner layer (intine), built of polysaccharides like cellulose, pectin-like substances and hemicellulose is in contact with the cytoplasm (Heslop-Harrison et al. 1968). From here the pollen tube develops through apertures in the outermost layer, the exine (Fig. 1), a complex polyether of cross linked polymerized unsaturated fatty acids and phenols (Ahlers et al.1999).

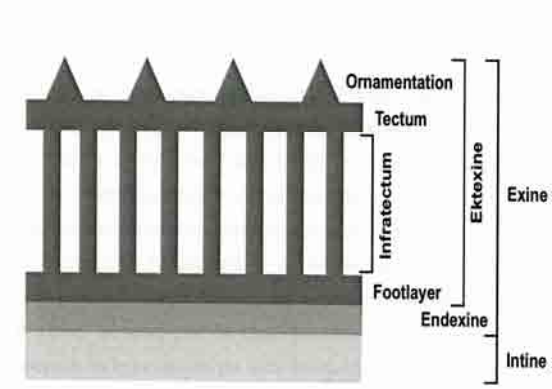

Fig 1. Pollen wall structure of an angiosperm plant (Diethart 2005)

There are various studies on the localisation of allergens in pollen grains. The birch and grass pollen allergens are mostly localized in the starch grains in cytoplasm and oncus, a thickening of the intine in the aperture region (El-Ghazaly et al. 1996, Marquez et al. 1997). The allergenic potential is mostly associated with the pollen-stigma recognition (Diethart 2005).

Before leaving the anthers, pollen grains are dehydrated. Grass pollen looses almost 35% of its water content. By contacting the stigma, pollen grains rehydrate and excrete the proteins responsible for recognition of the stigma from the same species. If pollen drops on a stigma of an alien species, a defence reaction occurs and a callus is built, so that the pollen can not contact the stigma. This reaction occurs very fast – within 25 seconds in grasses and 2 minutes in angiosperms. The same reaction is also supposed to cause allergic reactions (Knox et al. 1996 b). Additionally other proteins like expansins, which cause the loosening of the cell walls during the elongation, or profilins, as a part of the actin skeleton, can cause allergic reactions (Radauer et al. 2005). Also pathogenesis

related proteins that have the function in the defence the plant against pathogens can cause allergic reactions (e.g. PR proteins).

Mammalian nose mucosa offers similar conditions for the pollen grain as the stigma. The released recognition proteins cause a histamine reaction in sensitised individuals. Pollen grains are too large to reach the lung. There are great amounts of pollen dust in the air, originating from starch grains in pollen. Birch pollen grains for example swell when they land on a wet leaf. Grass pollen burst after fast rehydration during rain, because of the osmotic shock (Suphioglu et al. 1992, Diethart 2005).

Lipid transfer proteins (LTPs) in the pollen are involved in pollen tube adhesion. A 9 kDa protein with pI 8,71 has been isolated and sequenced from lily (*Lilium longiflorum*) (Park et al. 2000). The sequence contains a typical hydrophobic signal peptide at the N terminus and eight conserved cysteine residues. Adhesion of the pollen tube to the transmitting tract of the epidermal cells may facilitate the effective movement of the pollen tube and sperm cells to the ovary. Alternatively, the lipid transfer-like proteins may indirectly function in adhesion, even as a carrier of lipophilic compounds that act as signal molecules (Park et al. 2000).

1.2. Fruit allergens

The pathogenesis-related proteins PR-10 (Bet v 1 homologues) and PR-14 (nsLTPs) are responsible for most cases of fruit-related allergy. Interestingly, most of the plant food allergens belong to very few protein families (Breiteneder et al. 2004). Sensitisation seems to be caused by the common influence of three factors:

1. the genetic make-up of the exposed person
2. the structure of the allergen and
3. the biochemical and physico-chemical properties of the protein (Breiteneder et al. 2005).

PR-10 proteins were described to be steroid-carrier proteins, based on the high structural similarity between Bet v 1, the major birch allergen, and the steroid binding domain of the human MLN64 protein, which is supposed to take part in the process of steroidogenesis of placenta and brain (Tsujishita et al. 2000). The hydrophobic cavity of Pru av 1, a Bet v 1 homologue from cherry, is large enough to encapsulate two phytosteroid molecules (Neudecker et al. 2001). Proteins with ribonuclease functions also belong to the group of the intracellular pathogenesis related proteins (Breiteneder 2000, Hoffmann-Sommergruber 2002, Bufe 1996).

Birch pollen-related allergens are labile after heating and digestion. Bet v 1 related fruit allergy - usually occuring only as OAS, is caused by thermo-labile and protease-sensitive allergens. They cannot therefore, reach the effector cells (mast cells) in the gastrointestinal epithelium to trigger a

severe allergic response. In acid proteolytic environment in the stomach hydrolyses these allergens are rapidly degrated (Vieths et al. 2002). The apple allergen Mal d 1 is not stabile and therefore has little sensitizing capacity. However, there are also reports of allergic reactions to labile allergens, without primary sensitization to Bet v 1. The reason could be an elevated pH ratio in the stomach and an inhibition of the protease precuors pepsinogens, due to different reasons (Jensen-Jarolim 2006). It has also been observed that IgE binding to fruit homologues can be completely inhibited by Bet v 1, but not vice versa (Moneo et al. 1999).

Mal d 1 is a member of the pathogenesis-induced proteins PR-10 family. It is an intracellular protein with unknown function. Up to now no signal peptide was found for these proteins. Due to their sequence and size similarities, they are grouped together into PR-10 family (Hoffmann-Sommergruber et al. 2002). Mal d 1 is the first Bet v 1–related fruit allergen to be cloned and produced as a recombinant allergen (Vanek-Krebitz et al. 1995). Induction of Mal d 1 by pathogen and abiotic factors was shown by Pühringer et al. (2000). A number of Bet v 1–related allergen sequences have been isolated and cloned from fruits, including cDNA encoding Pru av 1 (sweet cherry) (Scheurer et al. 1997), Pru ar 1 (apricot), Pyr c 1 (pear) (Karamloo et al. 2001), Api g 1 (celery) (Breiteneder et al. 1995) and Dau c 1 (carrot) (Hoffmann-Sommergruber et al. 1999). They can be digested by pepsin within seconds and rapidly loose their allergenicity upon disruption of the tissue (Bjorksten et al. 1980).

The **LTPs** are reported as allergens in *Rosaceae* (Diaz-Perales et al. 2002, Pastorello et al. 1999, Pastorello et al. 2001), *Vitaceae* (Pastorello et al. 2003a) and many other plant species. Due to their stable physico-chemical features, like resistance against thermal and chemical denaturation and enzymatic digestion (Asero et al. 2000, Lindorff-Larsen et al. 2001, Pastorello et al. 2003b, Vassilopoulou et al. 2006) they reach the intestine in an immunogenic state. Their ability to sensitize via the gastrointestinal tract is well known, but the mechanism is not yet fully understood. LTPs are generally able to bind lipids in a tunnel lined with hydrophobic residues running through the protein. These two lipid molecules are lying side by side in the tunnel. In plants they transport lipids for the synthesis of waxy cutin and suberin layers in superficial plant tissues in seeds and pollen. The three dimensional structure of the LTPs is characteristic for the prolamin superfamily, related to parvalbumin from fish and casein from milk. They bind Ca^{2+} ions and seem to remain more stable after binding (Breiteneder et al. 2005, Van Do et al. 2005). In the case of LTP, peptides bind to

phosphatidylcholine, a physiological surfactant that is secreted by gastric mucosa and also occurs in bile. An additional enzymatic protection was shown, by slowing down the breakdown of the grape LTP (Vassilopoulou et al. 2006, Carvalho et al. 2007). These calcium-binding proteins (CBPs) containing two EF-hands (polcalcins) were detected and characterized in pollen from various trees, grasses and weeds. Although there is cross-reactivity described within the subfamilies of calcium-

binding allergens, there are no strong indications for IgE cross-reactivity between CBPs from plants, fish and humans (Wopfner et al. 2007, van Ree et al. 2002).

Pru p 3 was the first identified low-molecular mass allergen in the peach which lately turned out to be a LTP belonging to PR-14 protein family (Lleonart et al. 1992). These proteins are widely distributed throughout the plant kingdom (Kader 1996). Recently, an increasing number of LTP-homologous proteins with more than 80% similarity was identified in several plant foods, particularly in stone fruits, e.g. Pru p 3 (peach), Pru a 3 (apricot), Pru av 3 (cherry), but also in small fruits like blueberry, and raspberry, grape, and vegetables like carrot (Marzban et al. 2005).

Carbohydrate-specific IgE antibodies of patients with pollen allergy can cross-react with virtually all plant-based food allergens without triggering clinical symptoms (van der Veen et al. 1997, Altmann 2007). Besides the immunological relevance, the N-glycosylation has shown as structure stabilizer for proteins (Wormald et al. 1999, Breiteneder et al. 2005).

1.3. Serological detection methods

Since allergens are protein molecules, methods for their detection are the common used ones for protein analysis – their separation (one- or two- dimensional) by means of electrophoresis, and after transfer to a membrane – Western blotting and final visualizing via marked antibodies – immunodetection.

1.3.1. Protein separation by electrophoresis

Electrophoresis is a commonly applied methodology for protein analysis, based on the principle of the migrating charged particles (a molecule with high molecular weight, as protein, DNA, e.g.) under the influence of electric field. Under conditions of constant velocity the driving force of the particle is the product of the effective charge on the particle Q and the potential gradient E, and this is balanced by the functional resistance f of the medium. Since the dissociation constants (pK values) of the zwitterion groups will differ widely, the net charge on such a molecule will depend upon the pH of its environment, so that its mobility will be influenced. The ionic strength determines the electrokinetic potential, which reduces the net charge to the effective charge and it is found that the

mobility of the charged particle is approximately inversely proportional to the square root of the ionic strength. Low ionic strengths permit high rates of migration, while high ionic strengths give slower rates, but in practice sharper zones of separation than low ionic strength buffers (Maurer 1971). The higher the ionic strength of the buffer the greater is the conductivity and the amount of heat generated. Increasing temperature causes an increase of the diffusion rate of of the ions and also an increase in the ionic mobility amounting to about 2.4 % per degree Celsius rise in temperature. At the same time the viscosity of the medium falls with rising temperature. Heating therefore causes variations both in the current and voltage, and in order to minimize these fluctuations it is usual to carry out electrophoresis with power supplies which can be regulated to provide an output at constant voltage or constant current.

The electrophoresis is implemented on a supporting medium, e.g. polyacrylamide gels (PAGE). The purpose of the supporting medium is to cut down convection currents and diffusion so that the separated components remain as sharp zones with maximum resolution. It should be chemically inert during the separation process. Polyacrylamide gels can be reproducibly prepared and the composition modified in a controlled way to achieve the best conditions for the problems in hand.
The gel-electrophoresis can be performed under restrictive and non-restrictive conditions. Restrictive gel-systems counteract the diffusion; the zones get sharper and the sensitivity higher.
The SDS-PAGE is an electrophoresis with restrictive medium which separates the probe compounds only depending on the molecular weight. The SDS (sodium dodecyl sulphate) is an anionic detergent that overlays the molecules in the probes with negative charge. Sodium dodecyl sulphate molecules generate micelles with constant net negative charge and encapselate proteins with different sizes (Shapiro et al. 1967). Since the tertiary and secondary structures are destroyed by strongly negative chaged protein surface, the different molecular forms can be equalized; the H-bounds are also disrupted and the molecules unfolded. If necessary, the sulphur bounds can be splitted by reducing reagents like β-mercaptoethanol or dithiothreitol. The SH-groups can be later on protected by subsequent alkylation with iodacetamide, iodoacetic acid or vinylpyridine (Lane et al. 1978). The unfolded amino acid chains bound to SDS, form ellipsoids with identical central axes. During electrophoresis in restrictive polyacrylamide gels containing 0, 1% SDS there is a linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle. This relationship is only valid for a certain interval which is determined by the ratio of the molecular size to the pore diameter. Gels with a pore gradient offer sharper bands and minimal diffusion.

There are commercial available molecular marker proteins for various molecular weight intervals. SDS can be carried out in a continuous phosphate buffer system (Weber et al. 1969) or in a discontinuous system (Lämmli et al. 1970), as used in this work. The discontinuities in pH value are in the most of the cases not necessary. The protein-SDS micelles have very high negative charges; the mobility of glycine is lower than that of the proteins in the stacking gel at the beginning of the electrophoresis, even at pH 8.8. It does not bind SDS. During stacking no field strength gradients results, since there are no charge differences within the sample and no low ionic strength is necessary. The SDS disc electrophoresis can be cast in one step: glycerol is added to the resolving gel an then the stacking gel, which contains the same buffer but no glycerol, is directly cast over it.

The run time is shorter, since the separation starts more quickly. The resolution of peptides below 14 kDa is not sufficient in conventional Tris-Glycine-HCl system. This problem has been solved by introducing an additional spacing gel, increasing the molarity of the buffer and using tricine as terminating ion instead of glycine (Schägger et al. 1987).

The “high resolution 2-DE” (O`Farrell et al. 1975) is currently the state of the art in electrophoretic methodologies. Two-dimensional electrophoresis (2-DE) is a method that can resolve thousands of proteins in a single separation procedure. Using the physico-chemical properties of the proteins, like pI and molecular weight, a complex mixture of proteins can be completely resolved in single protein spots.

The first-dimension are carried out in gel rods or strips and loaded onto the second-dimension. A flat-bed gel can also be cut into strips after the first separation and transferred onto the second gel. The iso-electric focussing gel strip must be equilibrated in an appropriate buffer allowing the protein separation in a second dimension.

The first dimension is generally carried out by isoelectric focussing in presence of oversaturated urea and a non-ionic detergent such as CHAPS or Nonidet NP-40. The second dimension is an SDS electrophoresis and separation. The separation parameter of the first-dimension, the pI, is independent of the molecular weight, which is the separation parameter of the second-dimension. Denaturing conditions for the first dimension are prerequisite to prevent intermolecular interactions, keep hydrophobic proteins in solution and avoid different conformations of one protein. The result of the separation is a pattern of spots (Fig. 2). According to the Cartesian coordinate system, from the left to right we find increasing pI, from bottom to top- increasing molecular weight. The number of detectable proteins can be increased by lengthening the distance of separation, the use of thinner gels and the development of more sensitive detection method. The sample preparation and the type

of sample application also have a noticeable influence and are directly linked with the techniques in the first dimension.

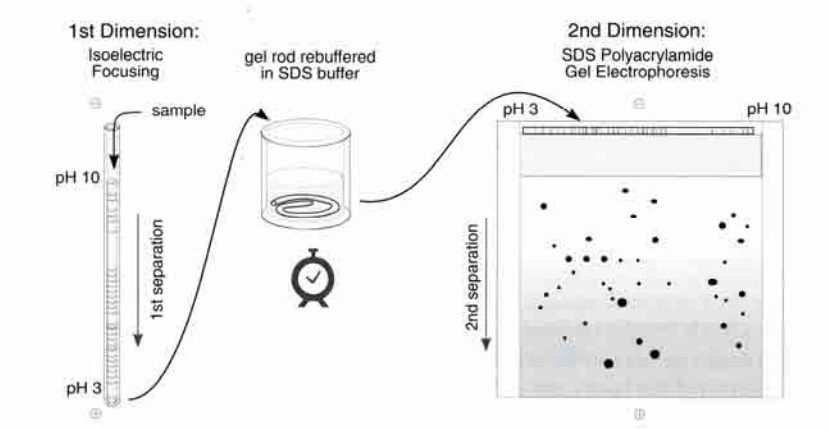

Fig. 2. The principle of the classical high-resolution 2-DE according to O'Farrell (1975) and Klose (1975) After separation of the proteins depending on their pI by isoelectric focusing (1st dimension), the strip is rebuffered and the proteins are separated again depending on their molecular weight (2nd dimension) by means of SDS-PAGE.

The use of immobilized pH gradients (IPG) for separation in the first-dimension allows a considerable increased reproducibility independent of the separation time and the kind of buffer, thus also allows the detection of extremely basic proteins. It has been called the "IPG-Dalt" method and the so-called "Iso-Dalt". Even extremely wide immobilized pH gradients, e.g. pH 2,5 to 11, can be used in order to separate nearly all possible cellular products in a single two dimensional map (Sinha et al. 1992, Westermeier "Electrophoresis in Practice").

Coomassie Blue R250 and Fast Green FCF as protein stains have been introduced by Wilson et al. (1979). The Coomassie staining is a quick method for protein staining on the gel. Coomassie Brilliant Blue R250 forms electrostatic bonds with NH_3^+ groups and non-covalent bonds with non-polar regions in the proteins. This kind of staining is suitable for densitometry, and used for quantitative measurements in gels having widely differing amounts of protein in the various stained spots (Andrews et al. 2001).

1.3.2. Western blot with IgG and IgE

The **Western blot** (alternatively, **immunoblot**) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D

structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

Different types of papers and membranes have been used for protein blotting, depending on the purpose. Nitrocellulose paper, a film of nitric acid esterified cellulose, is the most frequently used membrane. There are also improvements of different types of polyvinylidene fluoride membranes, e.g. a Teflon-type polymer $(-CH_2 -CF_2 -)_n$. Proteins interact with the polymer non-covalently through bipolar and hydrophobic interactions. They are resistant against harsh chemical conditions, in which nitrocellulose membranes dissolve or decompose. A PVDF membrane (polyvinilydenfluorid) (Immobilon™ Transfer Membranes 0, 45 µm, MILLIPORE) was used for the IgG-Western blot and nitrocellulose membrane – for IgE-Western blot.

The transfer buffer influences the protein transfer significantly. The original protocol by Erlich et al. (1979) uses a buffer containing methanol, added to counteract swelling of the gel. It also decreases gel pore size, removes SDS from proteins, and may precipitate the protein within the gel (Bers et al. 1985). However it also increases the capacity and the affinity of nitrocellulose for proteins.

Nitrocellulose membranes possess a high binding capacity for proteins: 80-100µg/cm². This absorbance capacity is important at the time of transferring the proteins. The quenching of the membrane is also important – all the unoccupied binding sites on the filter have to be blocked. The quenching agent is also used during probing to avoid non-specific interactions of the ligand with other proteins or with the filter. This has to be an "inert" protein. Here is BSA (bovine serum albumin) used for nitrocellulose membrane and non-fat milk powder for PVDF membrane. Of course, there is no absolutely "inert" protein. For example, milk is not recommended with lectins or with antibodies that recognize carbohydrate parts since milk contains large amounts of sugar that may block binding. Non-ionic detergents, such as Tween-20, are also used to reduce the background, because they interfere with the binding of proteins to the nitrocellulose membrane. But they may interfere with protein-ligand interactions and may remove proteins from the membrane, especially if their concentration is too high (above 0, 5%).

The detection of proteins involves different enzyme-linked antibodies e.g. horseradish peroxidase (HRP) or alkaline phosphatase. The ligand is modified by a reaction with another protein which possesses an intrinsic enzymatic activity. Generally, the modification occurs via generation of aldehyde groups in one of the molecules (oxidation of carbohydrates) and reaction with amino groups on the other protein (Schiff-base reaction). The binding of the enzyme-conjugated ligand to the target on the blot is detected by a chromogenic reaction. The advantage of this method is that it is

less hazardous than radioactivity and the probe can be stored for long periods without loosing its activity. The disadvantages are that the reactive bands are difficult to quantitate, the modifications on the ligand can change its activitycausing higher backgrounds. The activity of the interaction of the

chemo-luminescent substrate, here of HRP, is determined by a chemiluminescent reaction, detectable on a film (Durrant et al. 1990).

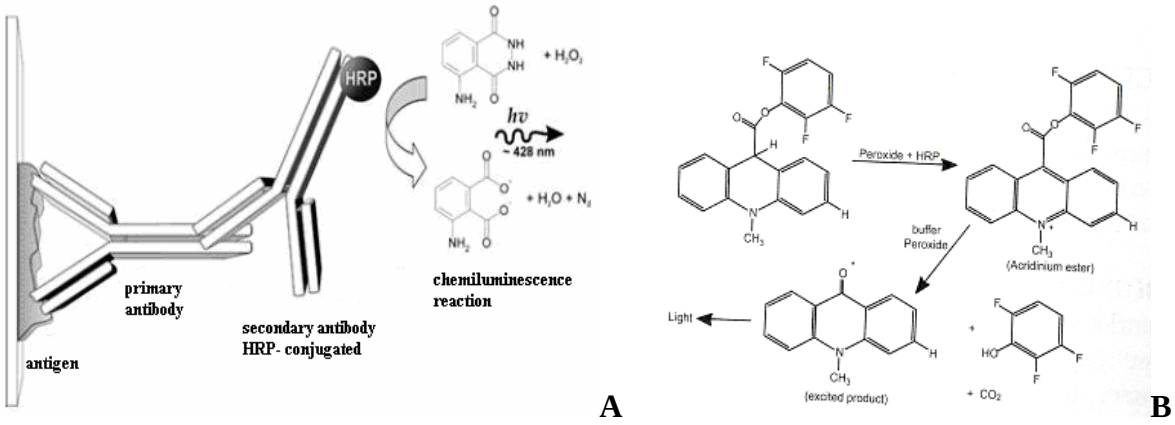

Fig. 3. Chemiluminescent reaction of Lumigen PS-3 with horseradish peroxidase: A) mechanism of the immunodetection and B) chemiluminescence reaction

The reaction is based on the oxidation the cyclic diacylhydrazide (luminol). The enzymatic generation of an acridinum ester produces a more intense light emission of longer duration. Combined HRP and peroxidase catalyzed oxidation of the substrate, Lumigen PS-3 Acridon, generate thousands of acridinum ester intermediates per minute. These react with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at a wavelength of 430 nm.

Alkaline phosphatase is a non-specific metalloenzyme which hydrolyzes many types of phosphate esters at an alkaline pH in the presence of zinc and magnesium ions (Rickwood 1998).

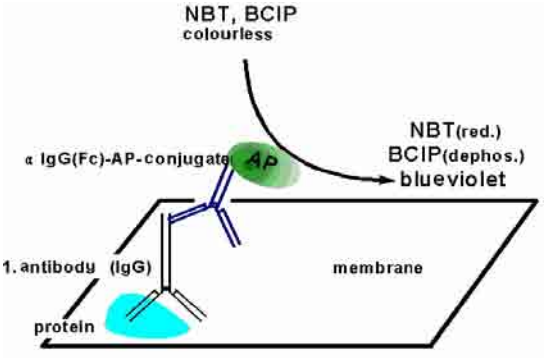

Fig 4. Chromogenic reaction of alcaline phosphatase with NBT and BCIP dying solution. After the 1st antibody (specific to the protein of interest) recognizes the antigene, the 2nd antibody, alkaline phosphatase (AP) labeled, binds the 1st one. The 5-Bromo-4-Chloro-3-Indolyl-phosphate (BCIP) as the chromogenic substrate is oxidized by AP into an indigo colored compound. The nitroblue-tetrazoliumchloride (NBT) is a redox dye, which turns blue after reduction and dimer formation.

1.3.3. Inhibition ELISA

The term ELISA (enzyme-linked immunosorbent assay) is introduced by Engvall and Perlmann (1971) to describe a subset of widely used immunoassay techniques. ELISAs are distinguished from other immunoassays such as RIA by the use of an enzyme label linked either to the antigene or the antibody. The label in conjunction with a suitable substrate produces the assay signal. ELISAs are distinguished from other enzyme immunoassay (EIA) methods by the fact that one of the reagents is bound on a solid phase, usually a 96-well microtiter plate, which provide handling of many different samples and facilitates the automatizaion. The ELISA technique has principally been applied to the determination of proteins. Many different enzymes have been used as tracers in ELISAs (Gosling et al. 1990, Porstmann et al. 1992), including urease, alkaline phosphatase, horseradish peroxidase (HRP) and β-galactosidase. Each of these can be employed with a number of substrates to generate an assay signal which usually takes the form of a coloured dye. There are many different ways of configuring ELISAs and the range of assay formats that are available can be bewildering.

Inhibition ELISA is a common method for confirming the cross-reactivity between two antigens: antibodies produced to one allergen can bind to epitopes of another antigen with a similar affinity (de Leon et al. 2005). To determine the cross-reactivity, an indirect ELISA is used as a relatively simple assay (Kemeny 1991). Microtiter plates are coated with antigen, mostly a purified allergen. The patient serum is inhibited by a homologous antigen and the bound antibodies can be detected by addition of an enzyme-labelled antibody specific for the detecting IgE antibodies. If the IgE molecules in the serum are successfully inhibited by the homologous antigen (e.g. allergens from pollen extracts), they cannot bind to the plate surface, coated with the original antigen. Their antigen binding epitopes are already occupied by a non-covalent complex with the inhibiting antigen. Thus the final reading will yield less signal strenght for succsessful ihibition (Fig. 5).

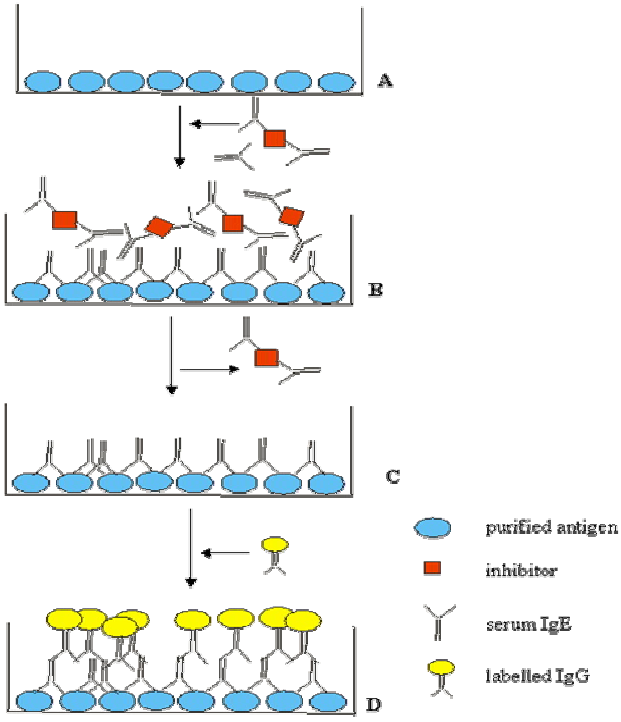

Fig. 5: Model of the inhibition sandwich ELISA displaying the different working steps: **A** - precoating; **B** – incubation with the inhibited serum; **C** – plate with the residual non inhibited antibodies; **D** - incubation with the detecting reagent and detection.

2. Objectives

Allergens are proteins that are an inevitable part of our life – due to occupation, climatic and domestic conditions. Usually the sensitized patients have been exposed or in contact with a particular fruit and the pollen level. For this purpose, fruit tree and grass pollen of the most common families grown in the Northern hemisphere will be characterised for their allergen content and allergen. Since the fruit tree pollen are insect-carried, heavier and of larger size than anemogamous pollen, their role in sensitization of susceptible individuals has been underestimated. Cross-reactivity of antibodies causes allergic reactions in patients sensitised to other fruit or pollen species, e.g. birch pollen allergy increases the possibility of allergic reactions to Rosaceous pollen and fruits. Further unexpected reactions to other foodstuffs and fruits also can occur.

Aim of this work was to investigate selected allergens in annual and perennial crops like fruit trees and grasses, accessible due to a potential cross-reactivity of specific antisera or patient sera. The accent was set on two fruit major allergens in apple, Mal d 1 and Mal d 3, and their homologues in different plant species of the families *Rosaceae, Caprifoliaceae, Ericaceae, Rutaceae, Vitaceae, Cornaceae, Moraceae and Poaceae.* Allergenicity of the different fruits and pollen is to be shown by the detection of IgE antibodies in patient sera with different anamnesis.

Protein extraction methods are crucial for extracts both from fruit and from pollen, and have to be optimized correspondingly. The changes of the protein structure during the sample preparation – under reducing and non reducing conditions, and the subsequent change in the reactivity of the IgE antibodies will also be presented. Polyclonal antibodies against Mal d 1 and Mal d 3 are applied as control in the serological tests. Methods to be employed comprize SDS-PAGE and Western blot with polyclonal ABs and patient sera. Inhibition ELISA with purified Mal d 1 and Mal d 3 against proteins from fruit and peach pollen extracts will be used to confirm the cross-reactivity between the allergens.

3. Materials and methods

3.1. Plant material

3.1.1. Fruits

The fruit material was harvested from trees at the Pomological Institute of the BOKU University in Vienna. The strawberry, raspberry blackberry, blueberry and oranges were purchased from the local supermarket.

Fruits belonged to the following five ***Dicotyledonae*** families;

- ***Rosaceae***

  Maloideae: apple[1] *(Malus domestica),*

  Amygdaloideae: apricot[2] *(Prunus armeniaca),* cherry[3] *(Prunus avium),* peach[4] *(Prunus persica)*

  Rosoideae: strawberry[5] *(Fragaria ananassa),* raspberry[6] *(Rubus idaeus),* blackberry[7] (*Rubus fruticosus)*

- ***Ericaceae***: blueberry[8] (*Vaccinium corymbosum)*
- ***Caprifoliaceae*****:** elder[9] *(Sambucus nigra)*
- ***Rutaceae:*** orange[10] (*Citrus sinensis)*
- ***Apiaceae***: carrot[11] *(Daucus carota)*

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3.1.2. Pollen

Flowers were collected from the orchards of the Pomology Institute BOKU in Vienna during the species specific flowering time between January and June 2006. Pollen material from fruit species and grasses was separated manually, weight and stored at -80˚C until protein extraction. The following pollen were collected and analyzed:

***Dicotyledonae*:**

- ***Rosaceae***

Maloideae: apple[1] *(Malus domestica)*, quince[2] *(Cydonia oblonga)*, medlar[3] (*Mespilus germanica)*, pear[4] *(Pyrus communis)*

Amygdaloideae: apricot[5] *(Prunus armeniaca)*, sour cherry[6] *(Prunus cerasifera)*, peach[7] *(Prunus persica)*, cherry[8] *(Prunus avium)*


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Rosoideae: strawberry[9] *(Fragaria ananassa)*, raspberry[10] *(Rubus idaeus)*, rosa[11] *(Rosa canina)*, rowan[12] *(Sorbus aucuparia)*, hawthorn[13] *(Crataegus monogyna)*, spirea[14] *(Spiraea vanhouttei)*


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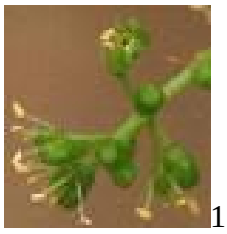
- ***Vitaceae:*** grape[15] *(Vitis vinifera)*
- ***Cornaceae:*** cornel cherry[16] *(Cornus mas)*
- ***Caprifoliaceae*** : elder[17] *(Sambucus nigra)*
- ***Moraceae***: mulberry[18] *(Morus alba)*

- ***Oleaceae*:** olive[19] (*Olea europaea)*
- ***Lythraceae:*** pomegranate[20] (*Punica granatum)*
- ***Apiaceae***: carrot[21] *(Daucus carota)*

 15  16  17  18

 19  20  21

Monocotyledonae:

- ***Poaceae****:*

false oat, French rye[22] *(Arrhenatius elatius),*

kentucky blue grass[23] *(Poa pratensis),*

cocksfoot[24] *(Dactylis glomerata),*

mouse barley[25] *(Hordeum murinum),*

soft chess[26] *(Bromus mollis),*

ryegrass[27] *(Lolium perenne),*

cereal rye[28] *(Secale cereale).*

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 25  26  27

3.2. Patient sera

The sera from allergic patients – provided by the Floridsdorf Allergy Center (FAZ, Vienna, Austria) - were kept for 24 hours at RT before centrifugation at 2400 rpm for 30 minutes by Allegra 21R Centrifuge, Beckman, Germany at 4˚C. Finally sera were stored in 400µl aliquots at -20˚C.

Table 1: List of the used patient sera

Patient	Sex	Anamnesis		
P2	F	Apple		
P3	M		Birch	
P4	F	Apple		
P5	M	Apple	Cherry, nuts	
P6	F	Unknown		
P9	F	Apple		
P10	F	Unknown		
P11	F	Apple		
P12	F	Peach		
P13	F	Strawberry		
P14	F	Apple		No birch pollen
P15	M	Apple		
P16	M	Apple	nuts	
P17	M	Apple	birch	
P18	F	Apple		
P19	F	Apple		
288	F Mal d 1		peach, kiwi, orange positive	birch negative
1105	F LTP patient		kiwi, strawberry, citrus fruits	SPT birch positive
3508	F LTP patient	Apple, only peel	peach, cherry	systematical OAS, urticaria, angiooedema, collapse, birch neg. (LTP)
PII_Mal d 1	F Mal d 1 patient	Apple	peach, carrot, kiwi, fig, nuts	
3705	F LTP patient	Apple	peach, cherry, apricot, kiwi nuts, fig, banana, tomato, watermelon, grape and weaker OAS orange, pistachio	
PI_Mal d 1	F, Mal d 1 patient	Apple	peach, cherry, apricot, nuts, fig, avocado, mango, birch	Bet v 2 negative
PIII_Mal d 1	F, Mal d 1 patient	Birch	apple, peach, carrot, kiwi, hazelnut, soy	SPT positive strawberry
PIV_Mal d 3	F LTP patient		(LTP) birch, apple, banana-OAS; tomato- urticaria, also coocked, peach, salad, pepper, aubergine, zucchini,	Bet v 2 and latex negative, SPT almost all pollen
3819	F LTP patient	Apple	(LTP) peach, carrot, banana, citrus fruits, melon	SPT birch negative
3913	F LTP patient	Apple	peach, cherry, apricot, nuts, banana, tomato, pepper, stones from stone fruits, salad –OAS, urticaria, dyspnoea , latex positive (Hev b 6)	SPT birch negative, systematic reactions after apple, only the peel is positive in SPT test , kiwi potato, grape, salad, tomato, pepper, banana
PV_Mal d 3	F LTP patient		orange, lime, OAS lid swelling and rhinitis after citrus fruits,	SPT negative for birch SPTpositive for, raspberry and blueberry and apples PAGO-peach sirup, banana, tomato, grape, pepper
4050	F LTP patient	Apple	(LTP) peach, cherry, apricot	angiooedema after peach and apricot jam, OAS after apple, pip fruits and stone fruits

F – female, M – male, red numbers – patient sera for Inhibition ELISA assay

3.3. Protein extraction

Protein extraction from fruits:

Extraction buffer: 10 mM K_2HPO_4
10 mM KH_2PO_4
13,3 mM DIECA (2,28 g/L)
(Sodium diethyldithiocarbamate trihydrate, Merck)
0,27 mM EDTA (300 mg/L)
2% PVPP (20 g/L)
Polyvinylpolypyrrolidone, Fluka
pH 7, 0

- All steps were carried out at 4˚C
- Fruits were carefully washed with hot water an dried with a paper towel
- Fruits were sliced, weighted and homogenized with precooled extraction buffer (1:2 w/v) in a blender (Osterizer, cycle blend) at maximum speed for 3 minutes
- The mixture was stirred for 2 hours or at least 30 minutes and subsequently filtrated through 2 layer Miracloth (Calbiochem) to remove fruit derbies
- After filtration the extract was centrifuged in Beckman centrifuge Allegra™ 21R, swing out rotor S4180, at maximum speed 5500 rpm for 30 minutes at 4˚C.
- Supernatants were aliquoted in 2 ml tubes and immediately frozen at -20˚C.
- If a concentration from the extracts was necessary, in case that the protein concentration in the extracts was too low to be detected with human sera (< 0,4 mg/L), the extracts were lyophilized (Björksten et al. 1980, Marzban et al. 2006).

Protein extraction from pollen:

1. Water extraction

- 0,1 g pollen material was frozen in liquid nitrogen and grinded.
- The powder was shaken on ice for 60 minutes in 1 ml aqua dest.
- After 10 minutes centrifugation at 4˚C at 10000 rpm, the supernatant was aliquoted in 2 ml tubes and stored at -20˚C.

2. Protein precipitation according to Wang et al 2006.

- 0,1 g of the sample was ground into a fine powder into a mortal and pestle under liquid nitrogen

- The powder was transferred into a tube (2g into a 15ml tube), the tube was filled up with 10% TCA/acetone, mixed well by shaking on a Vortex, centrifuged (4˚C, 5 minutes, 5500rpm), and the supernatant was removed.
- The procedure was repeated with 0, 1 M ammonium acetate in 80% methanol.
- The procedure was repeated with 80% acetone.
- The pellet was air dried (-20˚C).
- SDS-buffer (0,1 M Tris-HCl, 30% sucrose, 2% SDS, pH 8.0) and phenol solution (Sigma pH 8,0) were added in a ratio 1:1, mixed for at least 15 minutes at RT and centrifuged (4˚C, 5 minutes, 5500rpm).
- The phenol phase (upper phase) was transferred in a new tube and 5x V of 0,1 M ammonium acetate in methanol were added approximately and precipitated overnight at -20˚C.
- The pellet was centrifuged and washed once with 100% methanol and once with 80% acetone.
- The pellet was air dried at -20˚C.
- Then resolved in a suitable buffer

All solutions were stored at least 1 hour at -20˚C before the experiment was started.

The pellets were resolved in SDS Sample buffer without bromphenolblue.

3.3.1. Protein determination and buffer exchange

Buffer exchange: To eliminate the compounds from the fruit extracts that interfere with the proteins and disturb the total protein determination, a buffer exchange was performed as follows:

Elution buffer: PBS, pH 7,4

Na_2HPO_4	1.15g
KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8.0 g
ad 1000 ml aqua dest	

- PD-10 Columns (Amersham Bioscience) were equilibrated 5 times using 3,5 ml elution buffer.
- 2,5 ml of the sample was applied to the column. After the last drop 3,5 ml of elution buffer were added and the solution from the column end was gathered.
- During the buffer exchange a dilution factor of estimated 1,4 times was expected.

Protein determination: Total soluble protein was determined using the Micro BCA™ Protein Assay Kit (Pierce) in microtiter format.

- 150 µl PBS 1x buffer were pipetted in each well of the microtiter plate (NUNC), accept the H line
- 300µl of the blanc, BSA standard (40 mg/ml) and the sample were pipetted in the H line
- Dilution steps were made
- 150µl of the working reagent (25:24:1, reagent MA:MB:MC) were added at each well and the plate was mixed thoroughly on a plate shaker for 30 seconds
- The plate was covered and incubated at 37°C for 2 hours.
- The plate was tempered at RT
- The absorbance was measured at 562 nm on a plate reader.

3.3.2. Allergen purification

For the further analysis controls of Mal d 1 and Mal d 3 were prepared as purified allergens.

- The lyophilized apple extracts were resolved in 30 ml aqua dest and centrifuged in Beckman centrifuge Allegra™ 21R, swing out rotor S4180, maximum speed 5500 rpm for 30 minutes at 4˚C.
- The supernatant was filtered with Minisart High Flow Sartorius (16532 030903) and the buffer was exchanged.

Proteins were separated by IEX (Anion Exchange Chromatography). Chromatographic purification was performed on an AEKTA 100 explorer chromatography system (GE Healthcare, Uppsala, Sweden). A Cationexchange Mono S column (GE Healthcare) was used for purification (Herndl et al. 2007).

Elution buffer: 20 mM NaH_2PO_4/ 1M NaCl pH 7, 5

- SDS PAGE and Western blotting (polyclonal antibodies Mal d 1 and Mal d 3) were performed, in order to check which elution contains the most of the protein of interest.
- Total protein was determined using the BCA-Protein Assay (Pierce) in microtiter format.

3.4. Allergen analytics

3.4.1. SDS PAGE-Electrophoresis

The SDS PAGE electrophoresis was performed in a Novex – Gel apparatus (X Cell Sure Lock™, Invitrogen) using precasted 4-20% Tris/Glycine acrylamide gels (Invitrogen).

The running conditions were constant 125V and current from 27 mA on the beginning to 17 mA at the end for two hours.

3.4.1.1. Sample preparation

The samples were prepared both at reducing and non-reducing conditions to screen possible influence of the reducing agent on the molecule properties, caused by the dissolving of the disulfide bounds.

Non-reducing conditions: The extracts were mixed with sample buffer (2x) in relation 1:1 and denaturized for 60 minutes at RT.

Reducing conditions: The extracts were mixed with sample buffer (2x) in relation 1:1 and reduced with 5% β-mercaptoethanol for 10 minutes at 85˚C.

3.4.1.2. Buffer preparation

Sample buffer (2x) for Tris Glycine, SDS PAGE, denaturing, non reducing:

20 ml buffer

0,5M Tris HCl, pH 6,8	5,0 ml
Glycine	4,0 ml
10% (w/v) SDS	8,0 ml
0,1% bromphenol blue	1,0 ml

ad aqua dest 20 ml

pH ~ 8, 3

Stored at 4˚C

Running buffer (10x) for Tris-Glycine, SDS PAGE gel

Tris Base, SIGMA	29 g
Glycine, SIGMA	144 g
SDS, SIGMA	10 g

Add aqua dest to 1L

pH should be 8,3

Stored at 4˚C

3.4.2. 2D-Electrophoresis

3.4.2.1. Sample preparation (protein precipitation)

- To 2 ml of Topaz pollen extract were added 0,6 g sucrose (30%) for the phase inversion and vortexed until the sucrose was resolved.
- 1x V Phenol Tris buffered, pH 8.0 was added and stirred for at least 15 minutes at 4˚C.
- The solution was centrifuged for 10 minutes at 5500 rpm, swing out rotor S4180, Allegra 2R, Beckmann centrifuge at 4˚C.
- The upper phase was mixed with 10x V 100mM ammonium acetate in methanol (-20˚C) and precipitated ON at -20˚C.
- The solution was centrifuged for 30 minutes at 5500 rpm, swing out rotor S4180, Allegra 2R, Beckmann centrifuge at 4˚C.
- The pellet was washed two times with 100 mM ammonium acetate in methanol (-20˚C) and two times in acetone (-20˚C).
- The pellet was air dried for about one hour at -20˚C and resolved in 8 M urea (100µl).
- Stored at -20˚C.

3.4.2.2. Total protein quantification

Total soluble protein was determined using the BCA-Protein Assay (Pierce) in microtiter format, described in 3.3.1.

Total soluble protein 1256,56 µg protein /ml solution

In the remaining 60 µl there were 7,236 µg proteins. The whole amount was loaded on the strip.

3.4.2.3. Isoelectrical focussing

Rehydration solution:

2 ml reswelling solution

6 mg DTT (Dithiothreitol), SIGMA

2 µl bromphenol blue

3% IPG buffer (pH 3-10 for reswelling of Immobiline™ Dry Strip, Amersham Biosciences)

IPG strip rehydration: The IEF-strip (Immobiline™ Dry Strip pH 3-10, 7 cm, (GE Healthcare Bio Sciences) is rehydrated for 10 hours at RT in 125 µl mixture of 60 µl protein solution and 90 µl rehydration solution.

- The apparatus (Multiphore 2, Pharmacia) was precooled at 20˚C and poured with 10 ml petrol oil
- The red (anodic) side of the tray was placed at the top of the plate, large bubbles have to be removed.
- The tray was filled with 10 ml paraffin and an aligner was put on it, avoiding air bubbles.
- The strip was washed with aqua dest; the anodic side was on the positive pole of the tray. The both ends of the gel were covered with filter paper strips, soaked with aqua dest and attached to the electrode strips.
- The strips were covered with paraffin.
- Gradient voltage programme : 200V/ 30 minutes
  500V/ 30 minutes
  1000V/ 30 minutes
  2000V/ 30 minutes
  3500V/ 1,5 hours

3.4.2.4. Second dimension electrophoresis

The well of a ZOOM® gel, Invitrogen - was filled with 0,5% agarose in running buffer. The strip was cut on the both ends to the gel and put with the positive side to the marker Page Ruler™ Prestained Protein Ladder Plus (Fermentas) into the well. SDS PAGE electrophoresis was run at constant 125 V for 1,5 hours, inside 0,5 L 2x running buffer and outside 1x running buffer.

3.5. IgG- and IgE-Western blot

The western blot was performed in a Novex – Gel apparatus (X Cell Sure Lock™, Invitrogen) using PVDF (polyvinilydenfluorid) membranes (Immobilon™ Transfer Membranes 0,45 µm, MILLIPORE) for the **IgG-Western blot**. These membranes need pre-conditioning prior to the procedure of electro-blotting. In order to activate the membranes and increase the binding capacity to proteins they must be soaked in 100% methanol for 1 second, then in distilled water and incubated in blotting buffer before starting with the blotting procedure.

The Chromatography filter paper (3mm Chr, Whatman®, Schleicher& Schuell) in the same size as the blotting membrane (8 cm x 7 cm) was incubated in electro-blotting buffer. Three pieces of paper for each side of the gel are needed. The sponge layers were washed in distilled water and soaked in

electro blotting buffer. Two sponge layers were put on the cathode electrode of the Novex-cassette, then three filter paper layers, then the gel. The membrane must be laid on the gel without air bubbles and it should not be slipped on the gel. The air bubbles must be gently removed with the spatula. The membranes were covered with three layers filter paper and the rest of the cassette was filled with sponges. The anode electrode was put over. After fixing in the cell the cassette was filled with blotting buffer and the rest of the cell - with distilled water for cooling the system. The running time was 2 hours, constant current 200 mA for one gel and 400 mA for two gels. The determination of the transfer time depends on the concentration of acrylamide in the gel and the electroblotting chamber employed.

Electro blotting buffer (Novex): 50 mM $Na_2B_4O_7.H_2O$ 19g
0,1% SDS 1g
20% methanol 200ml
ad 1000 ml aqua dest
pH should be 9,2, do not adjust
stored at 4˚C

After finishing the membrane was washed in 100% methanol for fixing the proteins and air dried. The following buffers were necessary for the incubation:

Washing buffer: Na_2HPO_4 1,15g
KCl 0,2g
KH_2PO_4 0,2g
NaCl 8,0g
Tween 20 1,0ml
ad 1000 ml aqua dest
stored at 4˚C.

Blocking solution: 3% Skim Milk Powder in washing buffer

Dilution buffer: 1% Skim Milk Powder in washing buffer

Staining buffer for Alkaline Phosphatase:

100 mM NaCl 5,8 g/l
5 mM MgCl 1,09 g/l

100 mM Tris/HCl 12, 1 g/l
pH 9,5, do not adjust

Staining solution: 20 ml staining buffer + 24 µl NBT + 24 µl BCIP
5-Bromo-4-Chloro-3-Indolyl-phosphate (Fluka)
Nitrotetrazolium Blue Chloride (Fluka)

BCIP stock solution: 70 mg/ml in dimethylformamid, stored at -20˚C.

NBT stock solution: 70 mg/ml in 70% dimethylformamid, stored at -20˚C.

Polyclonal and monoclonal antibodies can be used for immuno-blot staining. The titre had to be determined empirically. The IgE-antibodies were generally diluted 1:50, nevertheless the titer of IgE`s in the different sera. The incubation time was also variable: from one hour to overnight. The longer the incubation time, the higher the background will be. Each protocol had to be optimized empirically.

The choice of the second antibody depends on the nature of the detecting antibody. Here were used anti rabbit IgG antibodies, alkaline phosphatase labeled and anti human IgE antibodies, HRP labeled with horseradish peroxidase, both commercially available.

In order to obtain the optimal signal/background ratio, the washcycles had to be optimized. The pH should be between 7 and 8. The non-ionic detergent concentrations, here Tween 20, should be in the rage of 0,5 to 1%. The membranes were usually washed three times for 10 minutes on a shaker.

Development of the blot:

- The blot was blocked overnight in blocking solution at 4˚C.
- Then incubated with the first antibody either anti Mal d 1 or anti Mal d 3 1:2000 diluted in dilution buffer for 2 hours, shaken at RT.
- Then washed 3 x 10 minutes at RT with washing buffer.
- Then incubated with the second antibody 1:2000 diluted in dilution buffer for 2 hours, shaken at RT. The antibody was anti-rabbit IgG (whole molecule) alkaline phosphatase conjugated, developed in goat, SIGMA.
- Afterwards washed 3 x 10 minutes at RT with washing buffer.
- Incubated with staining buffer 5 minutes at RT to adjust pH.
- Incubated with staining solution until the bands are visualized.
- Stop the reaction by washing with distilled water for 5 minutes.

The stained blot was air dried and stored in a foil.

For the IgE-western blotting a nitrocellulose membrane was used. This sort of membrane does not need a pre-treatment and must be only soaked in blotting buffer. The transfer procedure is the same as described above. At the end the membrane must be only air-dried, without soaking in methanol.
Development of the blot:

- The membrane was blocked in solution (3% BSA in washing buffer) for 4 hours at 37˚C. Then incubated with human serum containing the IgE antibody of interest, 1:25000 diluted in 1%BSA/washing solution buffer ON at 4˚C on a shaker.

- Then washed 3 x 10 minutes at RT with washing buffer.
- Then incubated with the second antibody 1:1000 diluted in dilution buffer for 2 hours, shaken at RT. The antibody was HRP conjugated sheep anti-human IgE, Immunology Consultants Laboratory.
- Then washed 3 x 10 minutes at RT with washing buffer.
- At last stained with Amersham ECL Plus Western Blotting Detection System, GE Healthcare.
- Chemo luminescent detection with Lumi-Imager™, Boehringer Mannheim.

3.6. Gel staining with Coomassie Blue

Staining solution: 0,25 g Coomassie® Brilliant Blue R250, SERVA in 250 ml destaining solution
Destining solution: 40% ethanol, 10% acetic acid (96-100%) in 1000 ml distilled water
The procedure: staining for 10 minutes at 95˚C degrees in staining solution, incubation of the gel in destaining solution at RT until the bands are visualized. No shaking is necessary. After the destaining the gel must be incubated in distilled water overnight.

3.7. Inhibition ELISA

Mal d 1 and Mal d 3 allergens were purified and used for the inhibition ELISA in the following concentrations:
Mal d 1: 0,500µg/ml and **Mal d 3:** 0,479 mg/ml
In order to optimize the following inhibition protocol (kindly provided by Dr. Hemmer, FAZ, Vienna), two sera, containing anti Mal d 1 or anti Mal d 3 IgEs, were first inhibited with apple pollen extract and then with peach pollen extract.

- Maxi Sorp ELISA plate was precoated with 5µg/ml purified antigen in coating buffer, incubated 30 minutes at RT, and overnight at 4˚C. Plates can be stored for 1 month at 4˚C, 100 µl/well
- Washed 5 times with PBS/0,05% Tween, 200µl/well

- Blocking with 1% BSA in PBS + 0, 05%Tween at least 4 hours at RT, 200µl/well.
- In the meantime the human sera were incubated with the inhibitor. Sera were diluted 1:20 in 1% BSA in PBS + 0, 05% Tween. The inhibitor was diluted in 1:2 steps. Incubated in Eppendorf tubes for at least 4 hours at RT, 100µl/well. The autoinhibition and the negative control were prepared in the same manner.
- Washed once with PBS + 0.05%Tween, 200µl/well.
- The tube content was transferred on the precoated plate and incubated at 4°C overnight.
- Washed 3 times with PBS/0.05%Tween, 200 µl/well.
- Incubated with anti human IgE-AP conjugated, diluted 1:2500 in 1% BSA in PBS + 0,05% Tween for 1 hour at RT, 100 µl/well.
- Washed 3 times with PBS + 0.05%Tween, 200 µl/well.
- Incubated with staining solution for alkaline Phosphatase until the yellow reaction occurs (about 2 hours). The plate was kept in darkness.
- The absorbance was measured at 405/620 nm.

Coating buffer: 4,2 g $NaHCO_3$
2,0 g Na_2CO_3
ad 500 ml aqua dest; pH adjust to 9,6 - 9,8
Stored at 4°C for 1 month

Staining buffer: coating buffer including 5 mM MgCl2 and 10 mM PNPP (p-nitrophenylphosphate)

4. Results

4.1. Detection of immunoreactive proteins

In this study the fruit extracts from apple, apricot, blackberry, blueberry, cherry, elderberry, raspberry, strawberry, orange and carrot were compared for their content of proteins and in particular of allergens.

4.1.1. Coomassie staining of SDS PAGE gel with fruit and vegetable extracts

The Coomassie staining of different fruit extracts separated by SDS-PAGE allowed a first insight into the protein composition and their distribution in molecular weight ranges (Fig. 6).

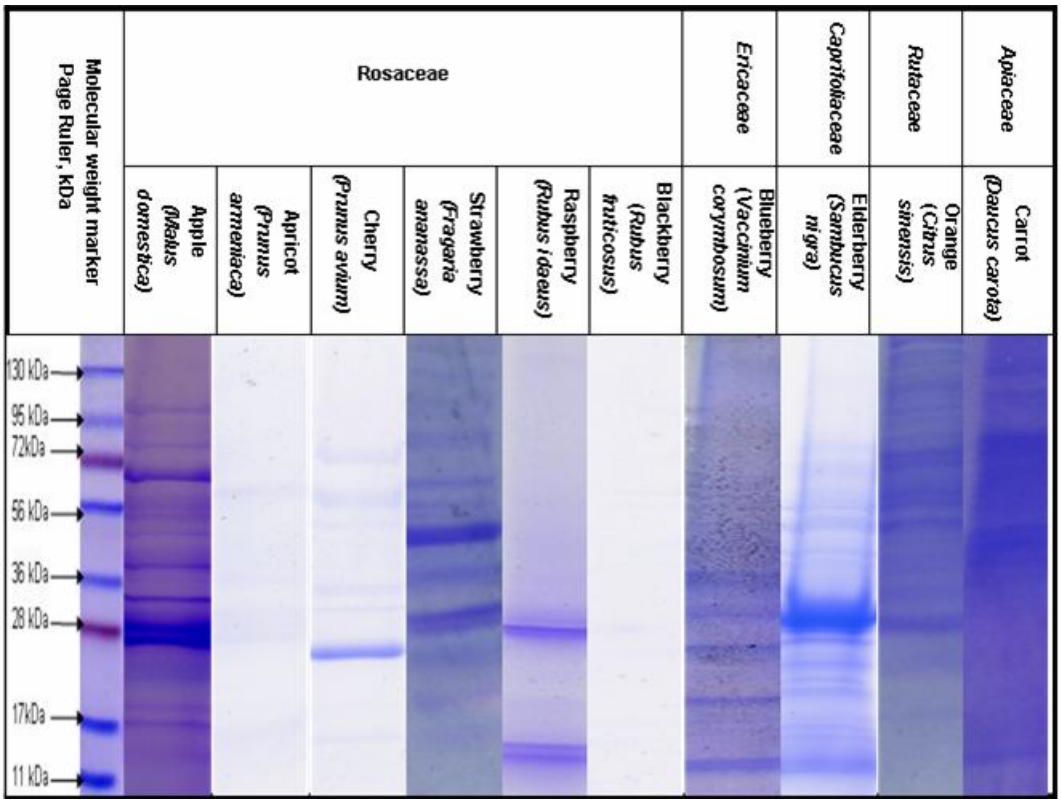


Fig 6. Coomassie stained SDS gel electrophoresis of protein extracts of fruits and vegetables. The samples were prepared under reducing conditions: 10 min. at 85˚C with β-mercaptoethanol.

The analysed fruit extracts from apple, strawberry, raspberry, blueberry, elderberry and orange were rich in proteins, indicating that the method for protein extraction was appropriate for these fruits. It should be mentioned, that in the case of orange the extract was made from peel, since the protein content of juice and pulp - which are commonly consumed - was too low to be detected by Coomassie staining. The extracts of apricot, cherry, blackberry and carrot show a lower protein concentration. Proteins with a MW lower than 11 kDa could not be extracted in a quantity required for visualization with Coomassie staining; nevertheless it was possible to detect some proteins with a

low molecular weight with serological methods, e.g. by employing polyclonal antibodies against the 9 kDa protein Mal d 3 (see below).

4.1.2. Western Blotting with polyclonal antisera against Mal d 1 and Mal d 3

SDS-PAGE followed by IgG-Western blotting with polyclonal anti Mal d 1 and Mal d 3 antibodies was employed for the detection of the presence of homologous proteins in extracts of fruit and vegetables.

4.1.2.1. Detection of immunoreactive proteins in fruit extracts with polyclonal anti Mal d 1 antibody

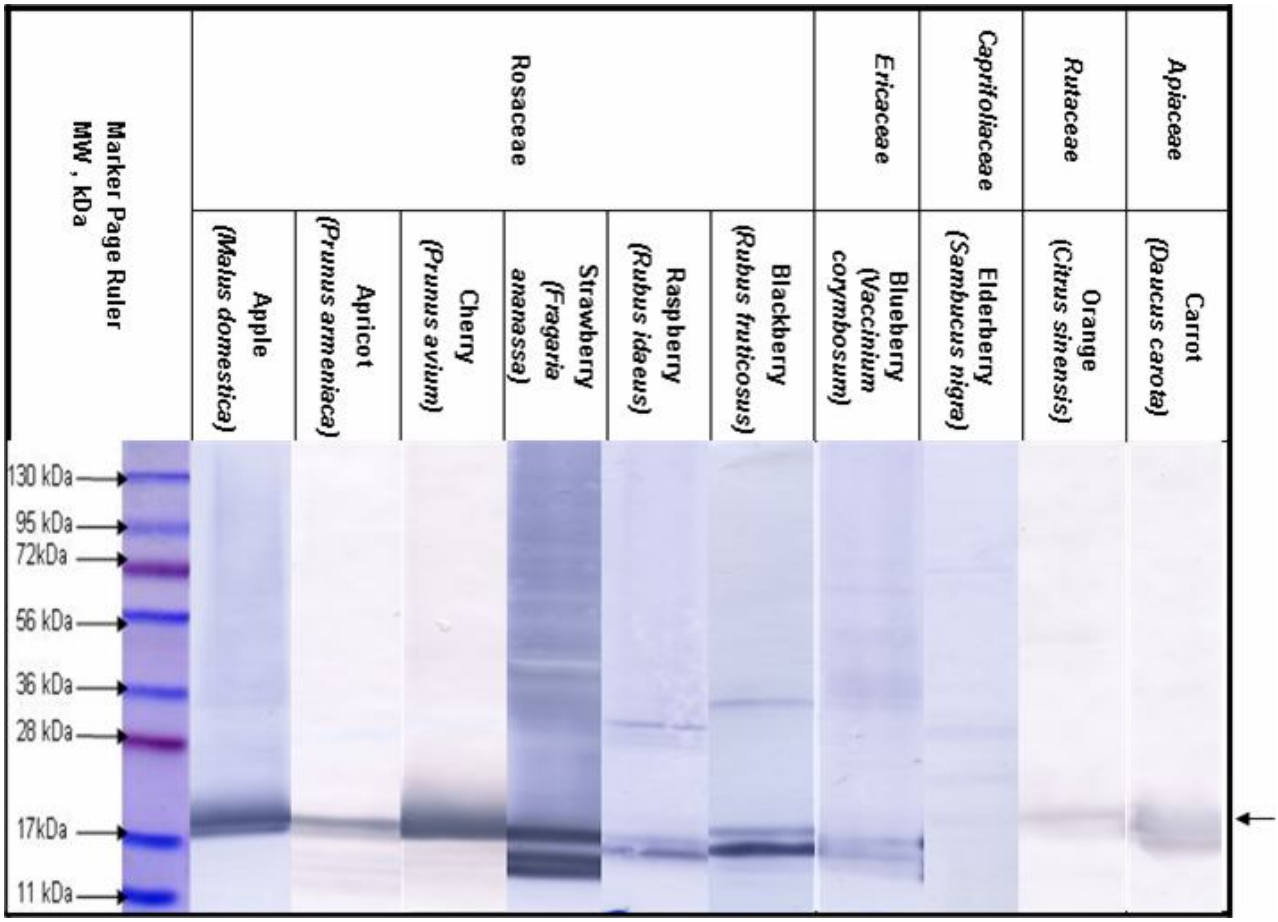


Fig. 7. SDS-PAGE followed by IgG-Western blot with polyclonal anti Mal d 1 antibody for the detection of protein extracts of fruit and vegetables. The samples were prepared under reducing conditions: 10 min. at 85˚C with β-mercaptoethanol.

The polyclonal antibodies, raised in rabbit against the 17.5 kDa protein Mal d 1, could detect proteins in all analyzed extracts with the expected molecular weight about 18 kDa, except in the elderberry extract (Fig. 7). In the Coomassie stained gel (Fig. 6) there were protein bands with molecular weight about 18 kDa. In some fruits double bands appeared which were typical for Mal d 1 (Karlsson et al. 2004). There were also some unspecific bands of higher molecular weight.

4.1.2.2. Detection of immunoreactive proteins in fruit extracts with polyclonal anti Mal d 3 antibodies

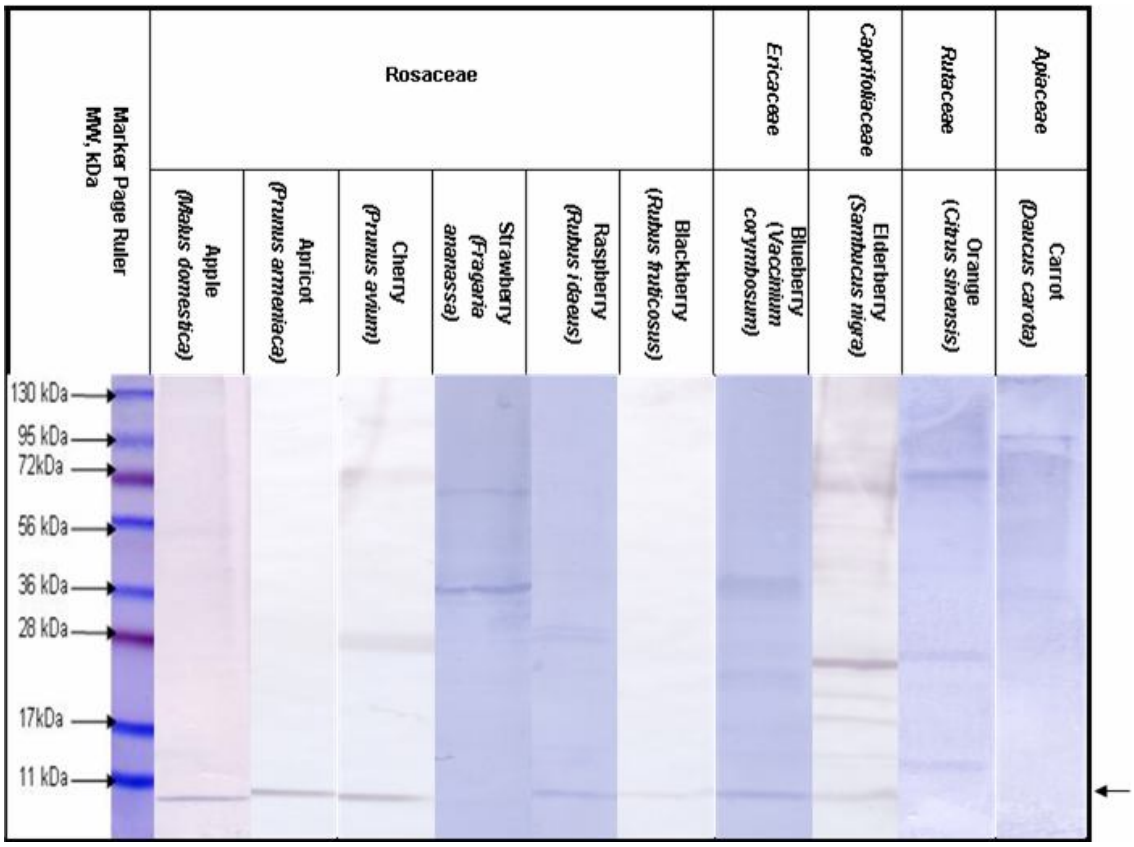


Fig. 8. SDS-PAGE followed by IgG-Western immunoblot with polyclonal anti Mal d 3 antibody for the detection of homologue proteins in fruit and vegetable protein extracts. The samples were prepared under reducing conditions: 10 min. at 85˚C with β-mercaptoethanol.

Although in the Coomassie gel (Fig. 6) no protein band was visible at 9 kDa, the anti Mal d 3 polyclonal antibody could detect proteins with the expected molecular weight of about 9 kDa in all extracts, except for the carrot extract. In orange, where a 9 kDa protein has already been determined as an LTP and named Cit s 3 (Ahrazem et al. 2005), the detected band was higher than expected. A similar situation could be observed in the strawberry extract – the detected band has a molecular weight about 36 kDa, indicating that it could be an oligomer of Fra a 3, a LTP homologue already reported by Zuidmeer (2006).

4.1.3. Detection of immuno-reactive proteins in fruit extracts with patient sera

Sera from an Austrian group of patients, who reported allergic reactions to apple and were clinically re-confirmed, were chosen to verify the findings with poly clonal antibodies and to deduce some

clinical relevance. Data are presented in order of fruits analyzed, beginning with apple as an internal reference. Furthermore, from the clinical point of view it might also represent a valid approach to analyze a patients' data to deliver a personalized diagnosis and draw dietary recommendations. The analyses visualise the entire pattern of IgE reactive proteins, indicating that there might be additional allergens to be detected (Marzban et al. 2006). The number and the sex distribution of the analyzed patients are variable, because the sera were obtained at different time (Table 1).

4.1.3.1. Apple *(Malus domestica)*

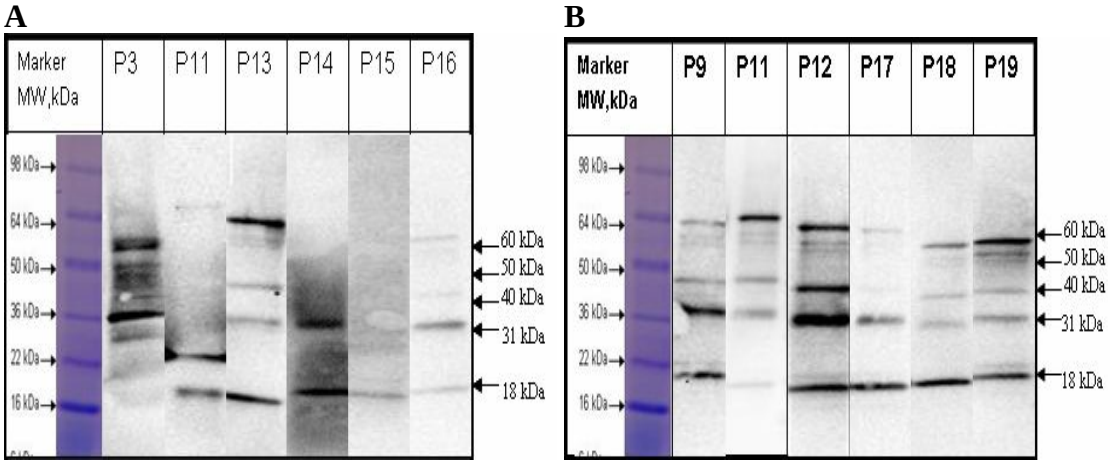


Fig 9. SDS-PAGE and IgE-binding analysis of apple fruit protein extracts. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Apple extracts were screened in reduced and not reduced form for allergens using 12 patient sera. Four of the patients were male and eight – female, all adults (see Table 1). The anamnesis for patient P13 was strawberry, and for patient P12 – peach.

The molecular weight of the detected proteins ranges from 17 kDa to 70 kDa. The protein band at about 17 kDa which corresponds to Mal d 1 (17.5 kDa), the major apple allergen (Karlsson et al. 2004) showed in five cases a double band, a phenomenon that has already been observed. The protein band at ~25 kDa (Fig. 9A, P11) corresponds to Mal d 2, a thaumatin-like protein (TLP). This allergen showed different molecular mobilities in the PAGE depending on the sample preparation: The protein band appeared at ~31 kDa under reducing conditions (Fig. 9B, P11) and ~23 kDa under non reducing conditions (Fig. 9A, P11), which was reconfirmed by further research in the same Plant Biotechnology Unit (Herndl et al. 2007, Marzban 2008a).

Other allergens in apple, e.g. Mal d 4, a profilin, with ~14 kDa MW and Mal d 3, an LTP, with MW ~9 kDa, were not recognized by these patients. However other allergic proteins with higher MW were detected, that have not been characterized so far. The proteins with the highest concentration in this extract with MW ~25, 28, 30, 35, 70 kDa, according to the Coomassie staining (Fig. 6) did not cause the strongest IgE-reaction.

4.1.3.2. Peach *(Prunus persica)*

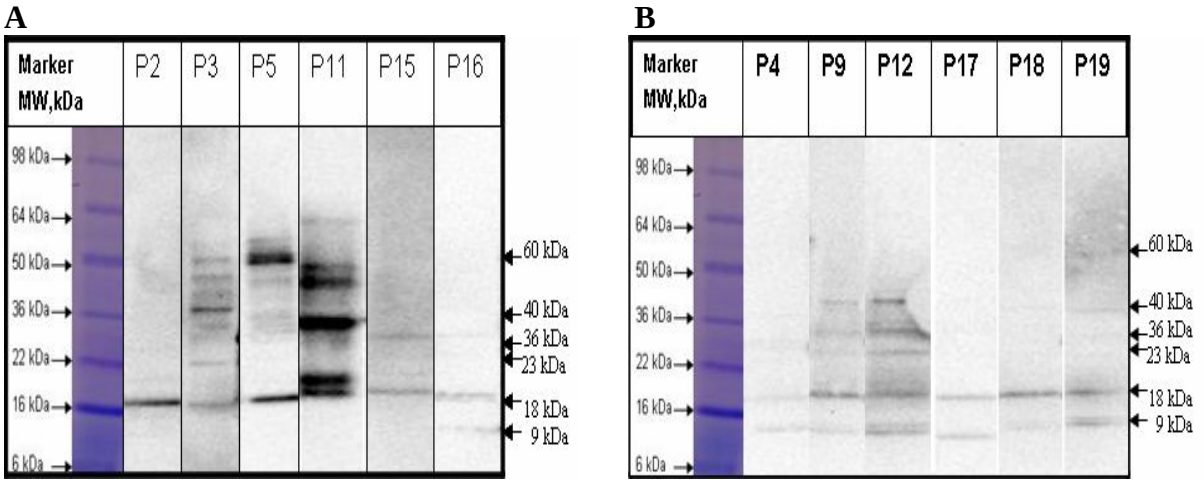


Fig 10. SDS-PAGE and IgE-binding analysis of peach fruit protein extracts. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Twelve patient sera (seven female and five – male) were used for the IgE detection of peach fuit extract. Only the anamnesis of patient P12 was peach (Fig. 10B). Although the anamnesis of P5 was originally only cherry, there is strong IgE reaction against peach protein. All other patients were reacting against apple.

The patient sera showed a strong reactivity with an 18 kDa protein band in peach fruit extract separated by SDS-PAGE, which corresponds to Pru p 1 (Ebner et al. 1995). Other proteins (4-5 bands) were detected at a molecular weight range of 9 kDa to 50 kDa. Weaker bands were observed at higher MW. The 9 kDa protein band corresponds to one of the major peach allergen Pru p 3, a lipid transfer protein (LTP). Lleonart et al. (2002) reported first that the allergenicity of the peach is confined to the skinn and corresponds to a protein dublet with an estimated MW of 8-10 kDa. There are still no reports on peach allergens with a higher molecular weight than 20 kDa yet.

Although Pru p 3 is considered as the major allergen in peach (Pastorello et al. 2003a), in the tested patient population only 6/12 sera recognized this protein in the reduced samples (Fig. 10B). This could reconfirm the raports of Scheurer et al. (2001a) that Bet v 1 homologues are the significant allergens in northern Europe and LTP homologues - in southern Europe.

4.1.3.3. Cherry *(Prunus avium)*

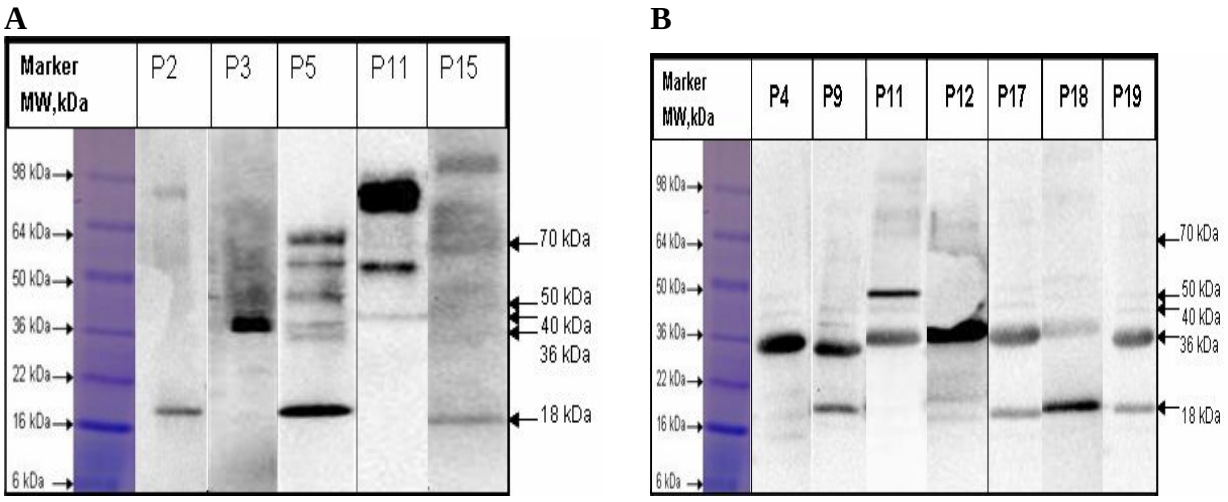


Fig 11. SDS-PAGE and IgE-binding analysis of cherry fruit protein extracts. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Twelve patients were chosen for the IgE detection of cherry fruit extact – four male and eight female. Only patient P5 had originally an anamnesis for cherry.

Allergy to cherries is widespread in Europe (Reuter et al. 2006). The IgE-reactive protein bands detected in cherry extract were in a MW-range of 18 kDa to 90 kDa. Mal d 1 (17.5 kDa) homologous proteins in cherry have been already characterized. Pru av 1, a Bet v 1 homologous protein with ribonuclease function, has a MW of 18 kDa (Neudecker et al. 2003, Wiche et al. 2005). Although a 9 kDa lipid transfer protein culd not be detected with the used sera, a Mal d 3 homologue in cherry - Pru av 3 (9 kDa pritein) has already been reported (Scheurer et al. 2004). Cherry allergy has been shown to differ between northern – Bet v 1 homologue, and southern Europe – cherry LTP homologue as ore important allergen (Scheurer et al. 2001a). The 14 kDa protein band represents cherry profilin, Pru av 4, a homologue to Bet v 2 (Scheurer et al. 2001b). The sera used did not react strongly with this protein. Another detected IgE-reactive protein band was at a MW of ca 31 kDa, which is supposed to be the Pru av 2, a thaumatin-like protein (TLP) (Dall`Antonia et al. 2005). The IgE-reactive proteins with higher molecular weight than 31 kDa have been not characterized yet. They were mostly represented in the non-reduced samples. The serum of P11 for example shows different reactions to the reduced and non-reduced extract. The band with MW~ 70 kDa in the Fig 11A might be an oligomer of the protein with MW~ 36 kDa in Fig 11.B.

4.1.3.4. Apricot *(Prunus armeniaca)*

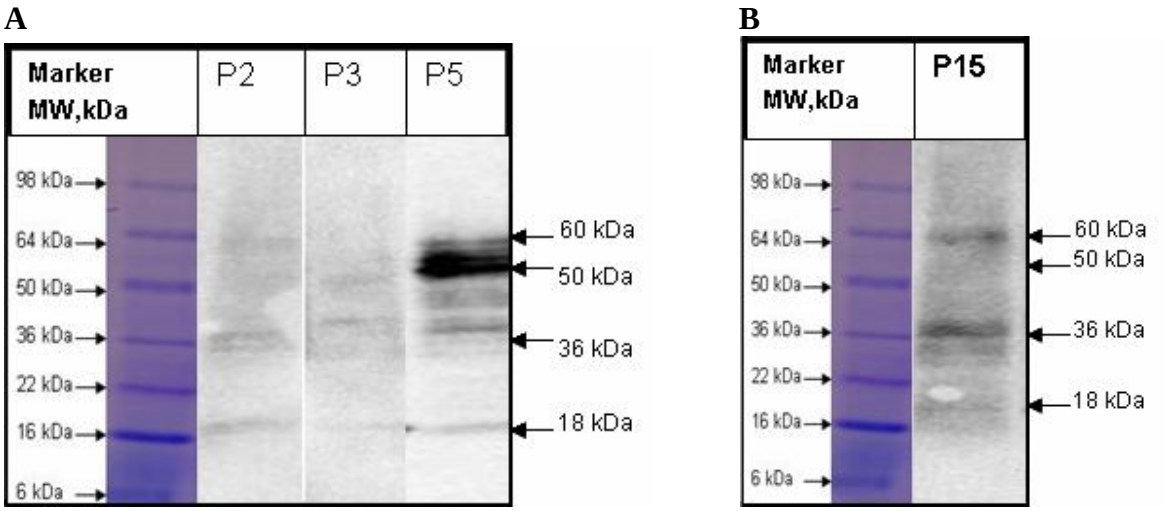


Fig 12. SDS-PAGE and IgE-binding analysis of apricot fruit protein extract. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

The extracts were tested only with four patients – one female and three male. None of them had orinally an apricot anamnesis.

The protein bands detected in apricot fruit extract were in the range from 18 kDa and 60 kDa. The allergenicity of *Prunus armeniaca* was so far not investigated in detail. The 9 kDa protein, described as an allergen with lipid transfer identity (Pastorello et al. 2000, Marzban et al. 2006) could not be detected with this extraction method and these sera. There were IgE-reactive proteins with molecular mass of about 18 kDa, 36 kDa, 50 kDa, and 60 kDa. The IgE-reactivity of apricot was not as pronounced compared to peach extract. The 18 kDa protein corresponds to Pru ar 1, a *Fagales* related protein with ribonuclease function (www.allergome.org).

4.1.3.5. Strawberry *(Fragaria ananassa)*

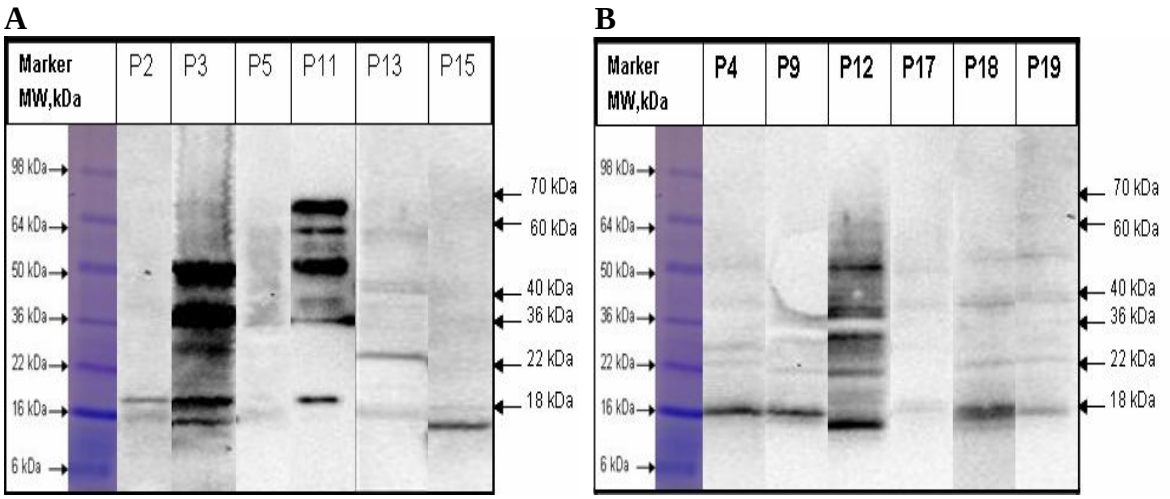
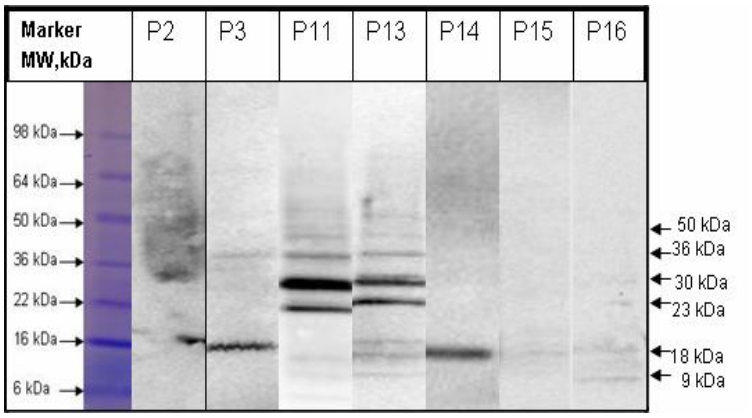


Fig 13. SDS-PAGE and IgE-binding analysis of strawberry fruit protein extract. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Sera from twelve patients were used for the analysis of he IgE binding capacity of strawberry fruit protein extract – four male and eight female. Only P13 had originally an anamnesis for strawberry. The observed IgE reactive protein bands in strawberry fruit extract were in the range from 18 kDa to 64 kDa. The strongest reaction could be observed at the ~18/20 kDa doublet – recognized by 8/12 patients; ~40 kDa - 3/12 patients and ~50 kDa also 7/12 patients. Two of the sera, tested on non-reduced extracts showed strong reactivity with proteins of 50 kDa and of 55-60 kDa, which could be also peptide oligomers. The strength of the signal on the blots was an indication for the degree of sensitization. No reaction with an LTP homologues protein band (~9 kDa) could be detected on these blots as expected. Either the patients were not sensitized to LTP, or the concentration of the LTPs in this strawberry cultivar was too low. It has been reported that the allergenicity of Fra a 3 was lower than of Prua v 3 and Mal d 3, therefore it could only be redetected by IgE-Western blotting and not by any other methodology (Zuidmeer et al. 2006). The 18/20 kDa band corresponds to the Fra a 1 (Karlsson et al. 2004).

4.1.3.6. Raspberry *(Rubus idaeus)*

A



B

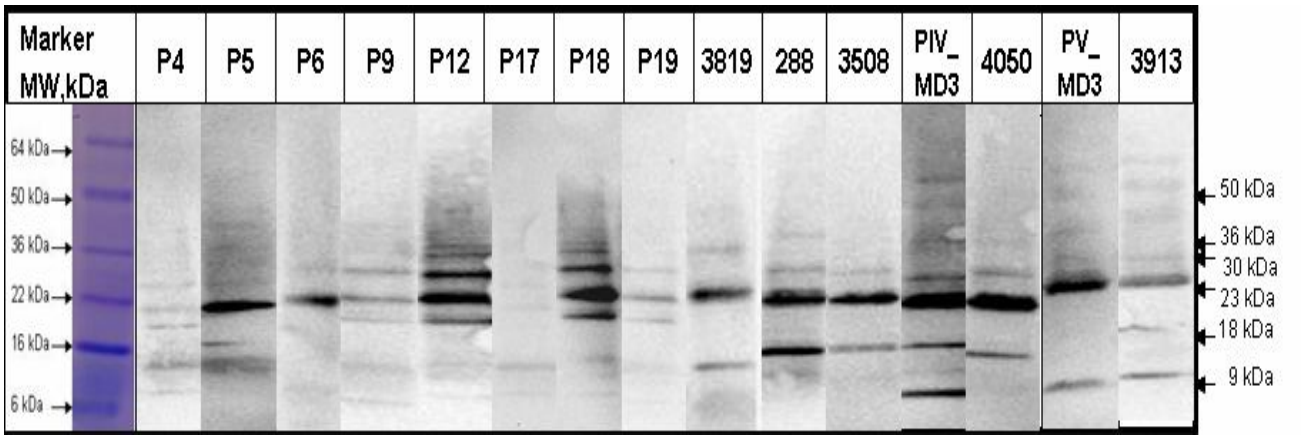


Fig 14. SDS-PAGE and IgE-binding analysis of raspberry fruit protein extract. A – samples not reduced, **B** – samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

In raspberry fruit extract a number of IgE reactive protein bands could be observed, however no differences could be detected in the intensity of reactivity to serum IgEs under reduced and non-reduced conditions. Sera from 22 apple allergic patients sensitized at different degrees to different proteins were tested – five male and seventeen female. Some patients in fact had already indicated an allergic reaction to raspberry in anamnesis (P5, PII_MALD3, see 4.2. Table 1). A pattern of seven IgE-reactive bands was recognized by eight patient sera (P11, P13 - Fig. 14A not reduced, P12, P18, PI_MALD3, 4050, PII_MALD3, 3913, Fig. 14B reduced). The strongest reactions occurred to four protein bands in the molecular weight ranges from ~22 kDa to ~50 kDa. There was an IgE-reactive protein with ~32 kDa molecular weight that seems to be the major allergen in raspberry (16/22 patients) (Marzban et al. 2008b). There were slight reactions at the ~9 kDa and ~18 kDa molecular weight level. According to Marzban et al. (2006), the IgE-reactivity at approximately 18kDa may be caused by two proteins, Rub i 1 and cyclophilin.

4.1.3.7. Blackberry *(Rubus fruticosus)*

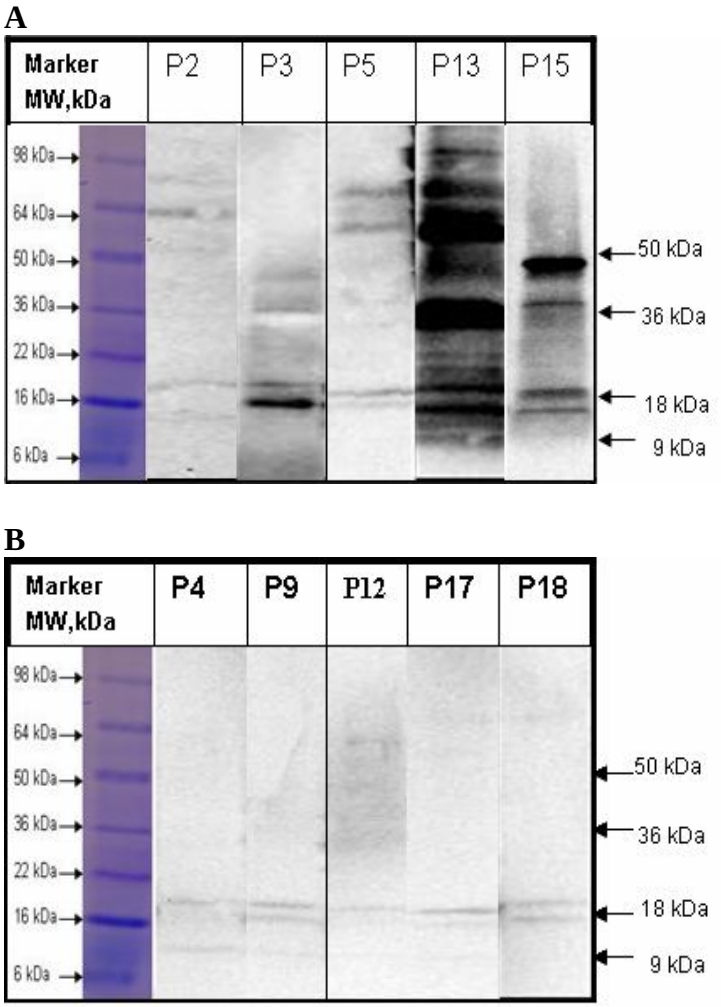


Fig 15. SDS-PAGE and IgE-binding analysis of blackberry fruit protein extracts. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Ten patients were tested for their IgE-reactivity against blackberry fruit protein extract – four male and six female. None of them had a blackberry anamnesis originally.

In blackberries the observed IgE-reactive protein bands ranges at MWs of 9 to 60 kDa. The non-reduced extracts showed allergenic proteins with higher MW and the reduced at lower MW. The IgE-reactive proteins with higher MW could be oligomers of low molecular raspberry allergens. The blackberry fruit extract did not seem to be highly reactive. There were stronger reactions only with the sera from patients P13 (with reported strawberry allergy) and P15 (with reported apple allergy). The double protein bands at 18-20 kDa MW appeared to be the most common allergen, a Bet v 1 homologous protein. 4/13 patients showed very weak reaction to protein with MW~9 kDa (P2, P13, P4, P9). The bands were weak, due to low protein concentration in the fruit extract. There are no blackberry allergens characterized so far and therefore need further reconfirmations.

4.1.3.8. Blueberry *(Vaccinium corymbosum)*

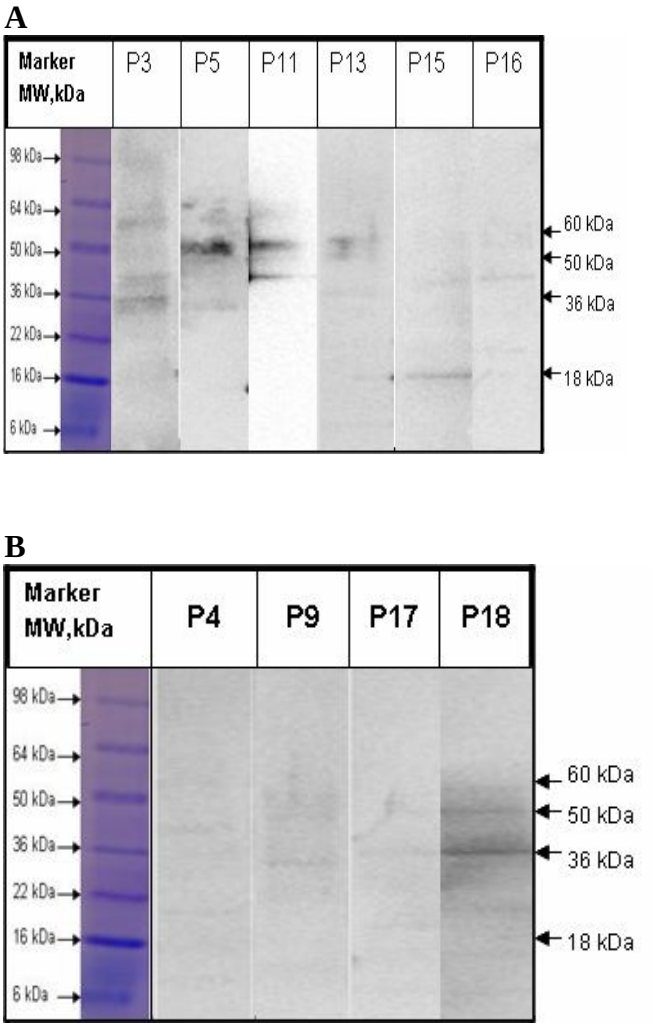


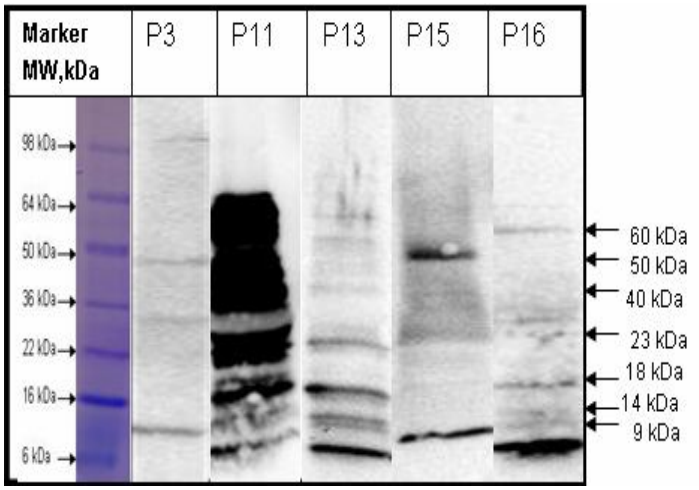
Fig 16. SDS-PAGE and IgE-binding analysis of blueberry fruit protein extract. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Blueberries are considered of value for their anti-cancer and antioxidant properties, but their allergenic capacity seems to be underestimated (Seeram et al. 2006). The analyzed sera could not detect a protein band with ~9 kDa molecular weight. Only the polyclonal anti Mal d 3 antibodies detected protein with this molecular weight (Fig. 8).

Some of the patient sera showed a reaction with blueberry fruit protein extract. There was a reaction with protein bands at ~18 kDa (3/10 patients), ~22 kDa (4/10 patients), ~36 kDa (5/10 patients), ~50 kDa (4/10 patients). The ~36 kDa band could be an oligomer of an LTP protein. None of the patients – four female and six male - clinically reported adverse reactions after blueberry consumption.

4.1.3.9. Elderberry *(Sambucus nigra)*

A



B

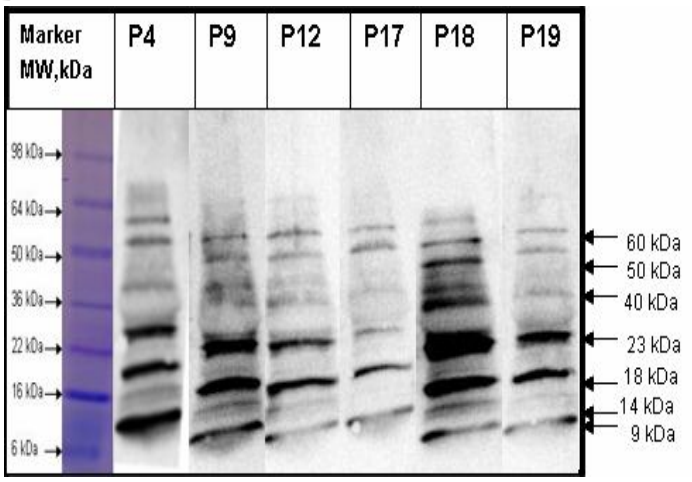


Fig 17. SDS-PAGE and IgE-binding analysis of elderberry fruit protein extract. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

Eleven patient sera were used for the IgE-binding capacity of the elderberry fruit extract – five female and six male. None of them had an elderberry anamnesis.

The protein band pattern of *Sambucus nigra* fruit extract was very complex. There were at least eight highly reactive bands detected by the IgE antibodies of allergic patients. The bands were at molecular weight ranging from ~9 kDa to ~55 kDa. All patient sera show strong reactions with very similar IgE-reactive protein pattern. One of the elderberry proteins has already been characterized as allergen – a 33,2 kDa protein that causes type I allergy (Förster-Waldl et al. 2003).

4.1.3.10. Orange *(Citrus sinensis)*

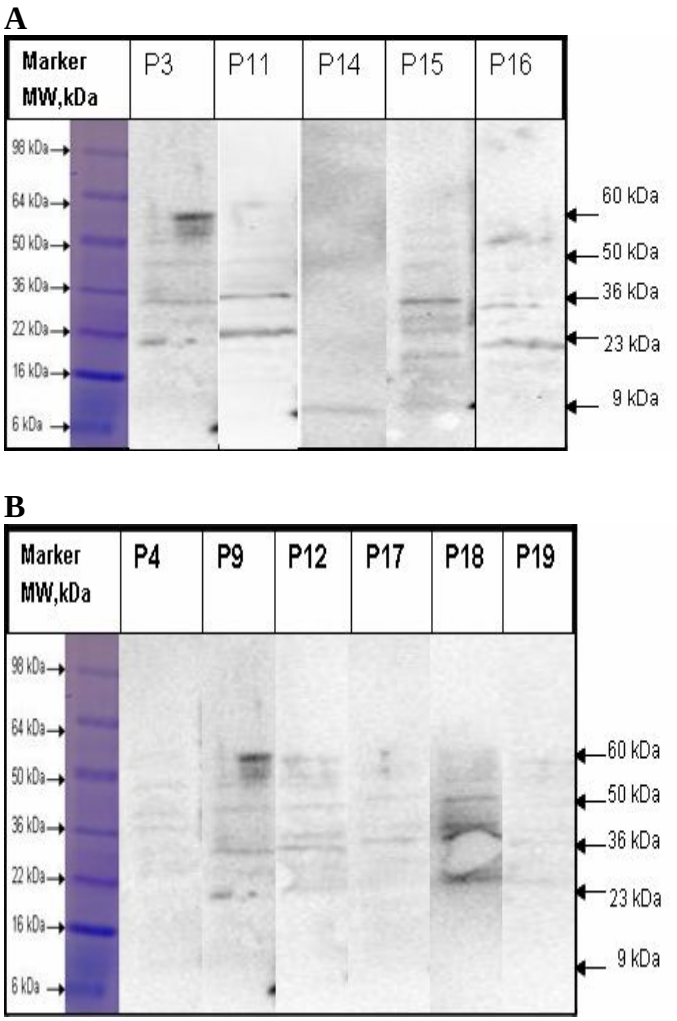


Fig 18. SDS-PAGE and IgE-binding analysis of orange fruit protein extracts. A – samples not reduced, **B –** samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

The reaction of the patient sera to Citrus sinensis fruit extract was not very strong. There was no significant difference in the reactivity between the two sample preparation procedures. Nevertheless it was possible to detect nine protein bands in the range from ~9 to ~60 kDa. The low molecular weight proteins were already characterized. The 9 kDa (P14, P15) corresponds to Cit s 3, an LTP (Ahrazem et al. 2005). The next band, ~14, corresponds to the profilin, Cit s 2, and the band ~23 kDa to the germin-like protein, Cit s 1 (Crespo et al. 2006, Ahrazem et al. 2006, Pöltl et al. 2007). An IgE-reactive 30 kDa protein, containing polypeptidic but no carbohydrate moieties, was detected in orange fruit and pollen extract (Irañeta et al. 2005). The proteins with higher molecular weight seem also to be serious target of the IgE response. None of the eleven patients analyzed – four male and seven female - have declared allergy against orange fruits. This means that they could be cross-sensitized by homologous proteins. Also orange-induced skin lesions caused by a not IgE-mediated mechanism have already been reported (Brockow et al. 2003).

4.2. Detection of immunoreactive proteins in airborne and insect-carried pollen

The IgE-binding capacity analyzes of pollen protein extracts was the second focus of this work. The current assumption is that apple sensitivity develops on the basis of primary sensitization to birch pollen. Natural exposure to apple pollen has not been considered as inducing sensitization. First insight into expression patterns of the major fruit allergens Mal d 1 and Mal d 3 was presented by Marzban et al. (2006). This study confirmed the expression of Mal d 1 and Mal d 3 homologues in pollen of *Rosaceae* family. The cross-reactivity of LTPs could have clinical significance for individuals with prolonged contact to higher concentrations of fruit pollen through their profession. This work links the attention to the presence of Mal d 1 amd Mal d 3 homologues also in pollen of other plant families. For the analysis of the pollen extracts an SDS-PAGE, Coomassie staining of the gels with following immunoblot IgG and IgE-binding capability detection were carried out.

4.2.1. Coomassie staining of SDS PAGE gels with pollen extracts

The Coomassie staining gives first insight in the protein pattern of an extract. Because apple was the reference species in this work, also a comparison 2D electrophoresis of the protein distribution in fruit and pollen extract was made.

4.2.1.1. Coomassie staining of 2D SDS PAGE gel with apple extracts

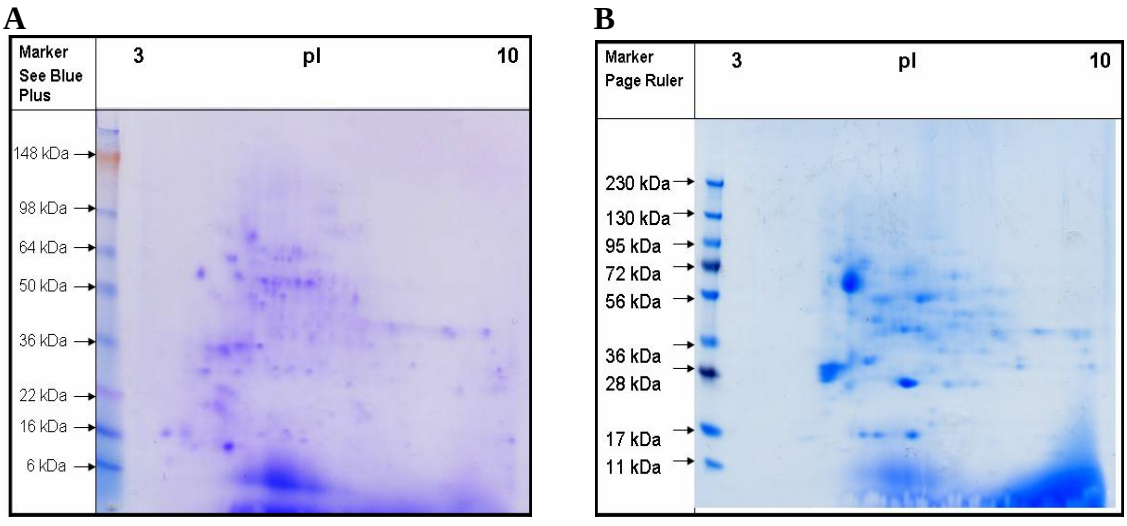


Fig 19. 2D-electrophotesis of apple pollen and fruit protein extract, Coomassie R250 staining
A – apple pollen extract, samples reduced for 10 minutes at 85˚C with β-mercaptoethanol
B –apple fruit extract, phenol precipitation, samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

The apple fruit and pollen extracts were analysed by 2-DE to determine the molecular mass and the iso-electric point of proteins. The samples were reduced for 10 minutes at 85˚C with β-mercaptoethanol to avoid the oligomerisation and cross-linkage of proteins. Most of the proteins

appeared at pI range of 4-7, however, the protein patterns of pollen and fruit showed completely different distribution and composition.

4.2.1.2. Coomassie staining of SDS PAGE gel with pollen extracts

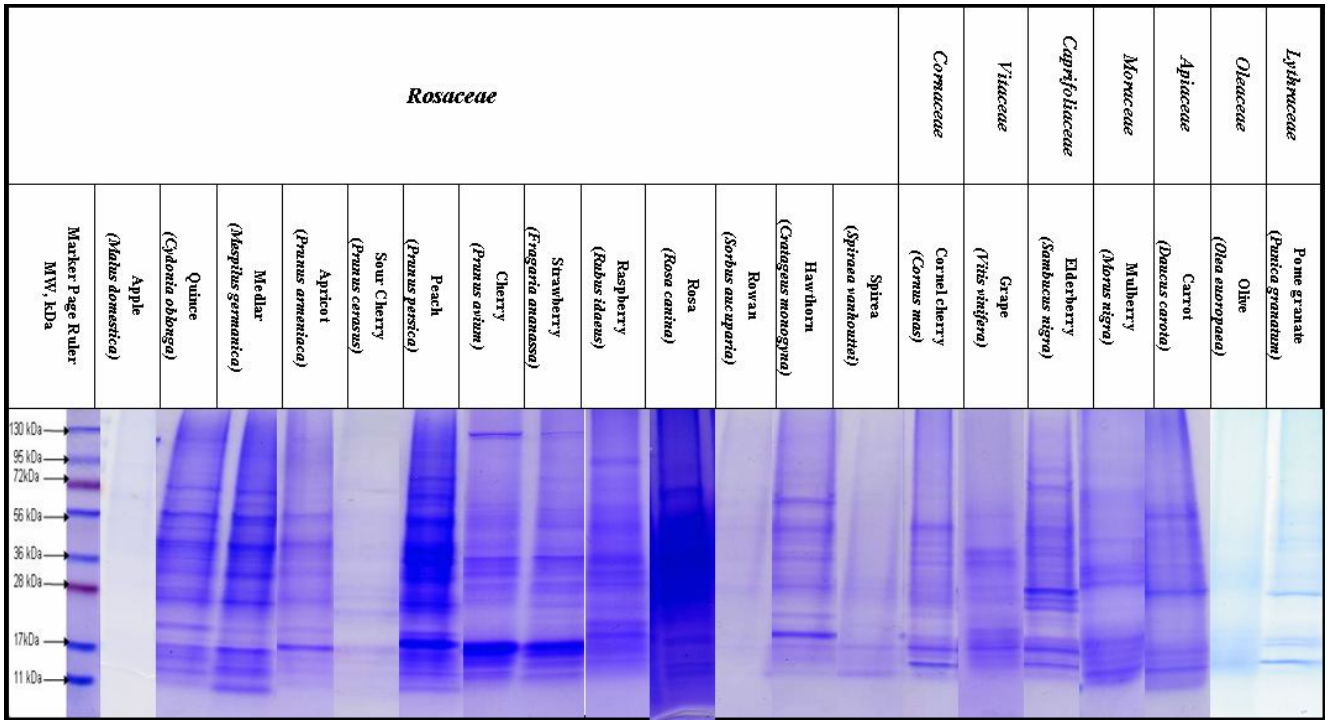


Fig 20. SDS-PAGE, Coomassie R250 stained pollen protein extracts. Samples were reduced for 10 minutes at 85˚C with β-mercaptoethanol.

SDS-PAGE separation and visualization using Coomassie staining of 20 different pollen extracts showed that a broad range of proteins can be detected. Due to a low amount of apple pollen extract, only half the volume was loaded, which yielded a band with lower intensity. Similar to the fruit extracts, there were no proteins detected at molecular masses lower than 11 kDa, except in medlar, sour cherry, peach, mulberry and carrot. This could be due either to their low concentration, or related to the staining method. Pollen extracts contain a more abundant protein pattern compared to the fruit extracts. There were many similarities in the protein pattern between the different families.

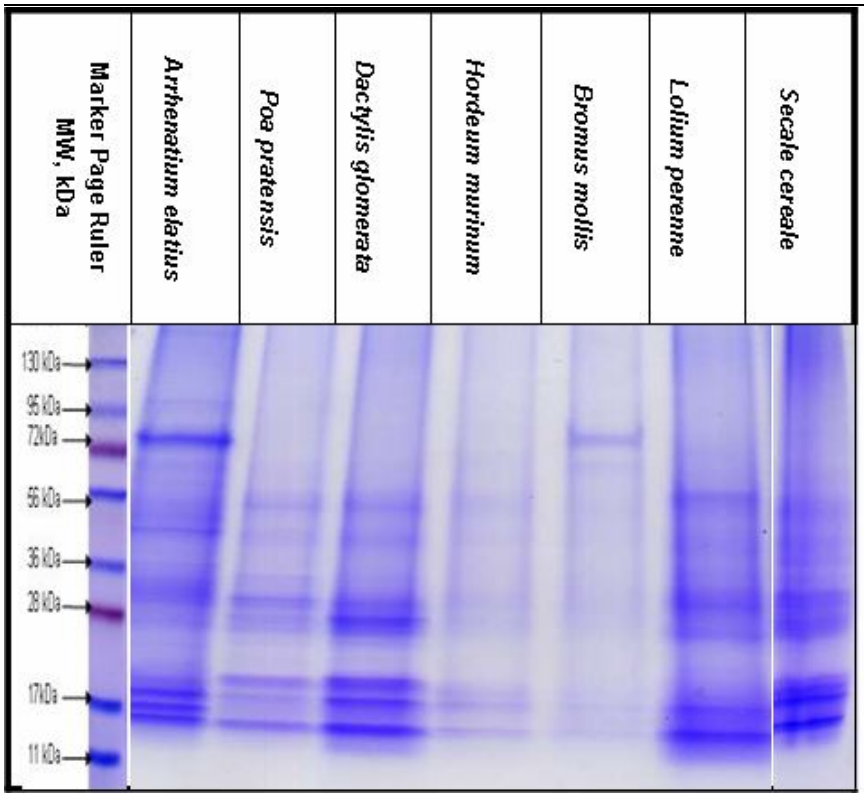


Fig 21. SDS-PAGE gel electrophoresis, Coomassie R250 stained grass pollen protein extracts. Samples were reduced for 10 minutes at 85˚C.

Coomassie staining (Fig 21) shows an abundant protein pattern with MW from 14 to 80 kDa. Again there were no stained proteins with MW below 11 kDa.

4.2.2. Detection with polyclonal antibodies

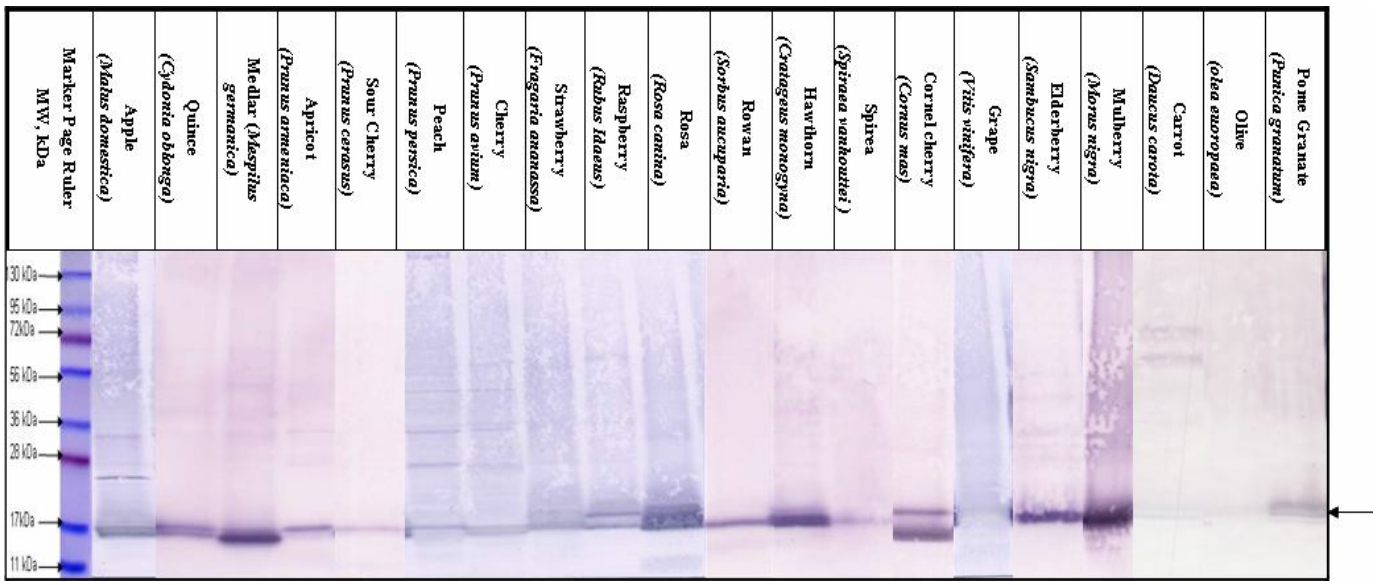


Fig 22. SDS SDS-PAGE followed by IgG-Western immunoblot with polyclonal anti Mal d 1 antibody for the detection of homologue proteins in pollen protein extracts. Samples were reduced for 10 minutes at 85˚C. Polyclonal rabbit antisera, raised against Mal d 1 (17, 5 kDa), identified the corresponding proteins in the pollen extracts.

In order to search for cross-reactive proteins, pollen extracts were incubated with a polyclonal anti Mal d 1 antibody (Fig 22). The anti-Mal d 1 antibody bound to proteins with a MW of about 18 kDa in almost every pollen extract. Mal d 1 homologous proteins belong to pathogenesis related protein and were therefore expected to be expressed in all plant species. Most of the observed plants belong to the *Rosaceae* family. Depending on the protein concentration in the extract and the incubation time with the staining solution the bands were weaker or more intensive. Such differences have been observed also in the IgE detection.

The *Olea europaea* and *Vitis vinifera* pollen extract were the only exeption. There are also many bands from unspecific binding of polyclonal antibodies.

The proteins, detected by the polyclonal anti-Mal d 1, appeared as a doublet in almost all of the observed species. This was not the case with the IgE detection, where it can be detected only one single band corresponding to Mal d 1. Probably not all of the homologues are IgE-reactive (see Fig. 7).

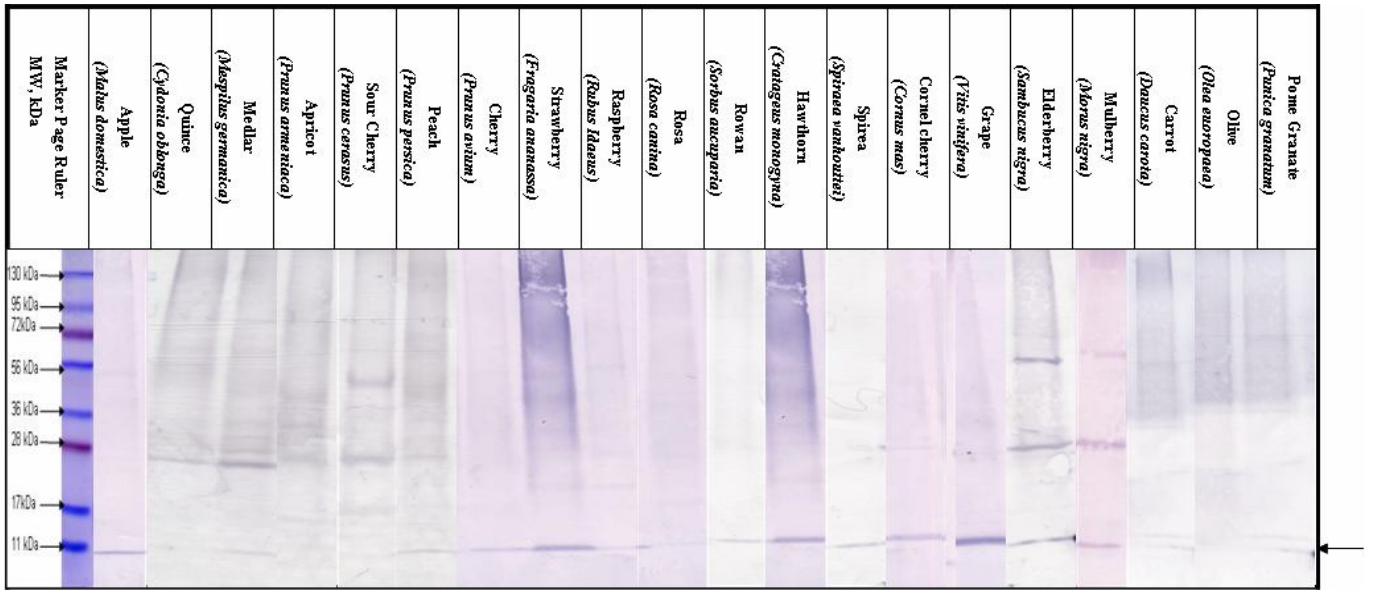


Fig 23. SDS-PAGE followed by IgG-Western immunoblot with polyclonal anti Mal d 3 antibody for the detection of homologue proteins pollen protein extracts. Samples were reduced for 10 minutes at 85˚C. Polyclonal rabbit antisera, raised against Mal d 3 (9 kDa), identified the corresponding proteins in the protein extracts.

The incubation of pollen extracts with polyclonal anti Mal d 3 antibody (Fig 23) showed, similar to Mal d 1, that all extracts contain proteins with reactive epitopes, to which the antibody could bind. The bands with higher molecular weight were most probably caused by unspecific binding or by recognition of polymers.

In the pollen of *Olea europaea* there was hardly any reaction, although, according to Tejera et al. (1999) an LTP was to be expected.

Pollen extracts from members of the *Poaceae* family were also analyzed. This family is one of the great troublemakers for pollen allergic individuals. The extracts were separated by SDS-PAGE electrophoresis, stained and detected as before.

A **B**

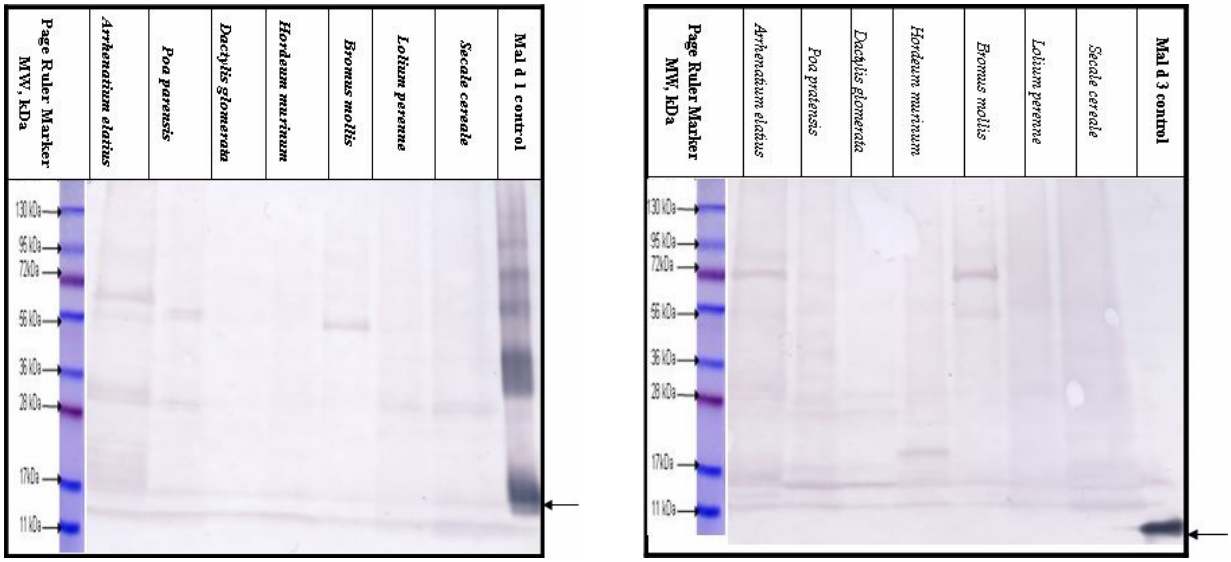


Fig 24. SDS-PAGE followed by IgG-Western immunoblot with polyclonal antibodies for the detection of homologue proteins in grass pollen protein extracts. A –polyclonal rabbit antisera, raised against Mal d 1 (17,5 kDa), identified the corresponding proteins in the pollen extracts, **B** – polyclonal rabbit antisera, raised against Mal d 3 (9 kDa), did not identify the corresponding proteins in the pollen extracts. There are only unspecific reactions.

The polyclonal anti Mal d 1 antibody (Fig 24A) showed more unspecific reactions. The result could be caused by too long incubation with the staining solution. There was a very slight line with the same molecular weight as the control, which could be Mal d 1 homologue proteins.

The detection with polyclonal anti Mal d 3 antibody (Fig 24B) did not show an explicit presence of LTP in grass pollen. There were only unspecific reactive protein bands.

4.2.3. IgE-detection with patient sera

The IgE-reactivity to pollen extracts was determined using a serum pool comprising 5 patients PIV_Mal d 3, 1105, 3913, PV_Mal d 3, 3705, Table 1). Because of the low amount of pollen protein extract, it was not possible to analyse the IgE-binding capability in separate patient sera as made by the fruit protein extracts.

In analyzed pollen extracts numerous bands of IgE-reactive proteins could be visualised, most probably due to the fact that pollen contains many different sorts of proteins. After plant growth and differentiation, part of the pollen proteins are expressed only in the leaves or in the roots e.g., depending on their function.

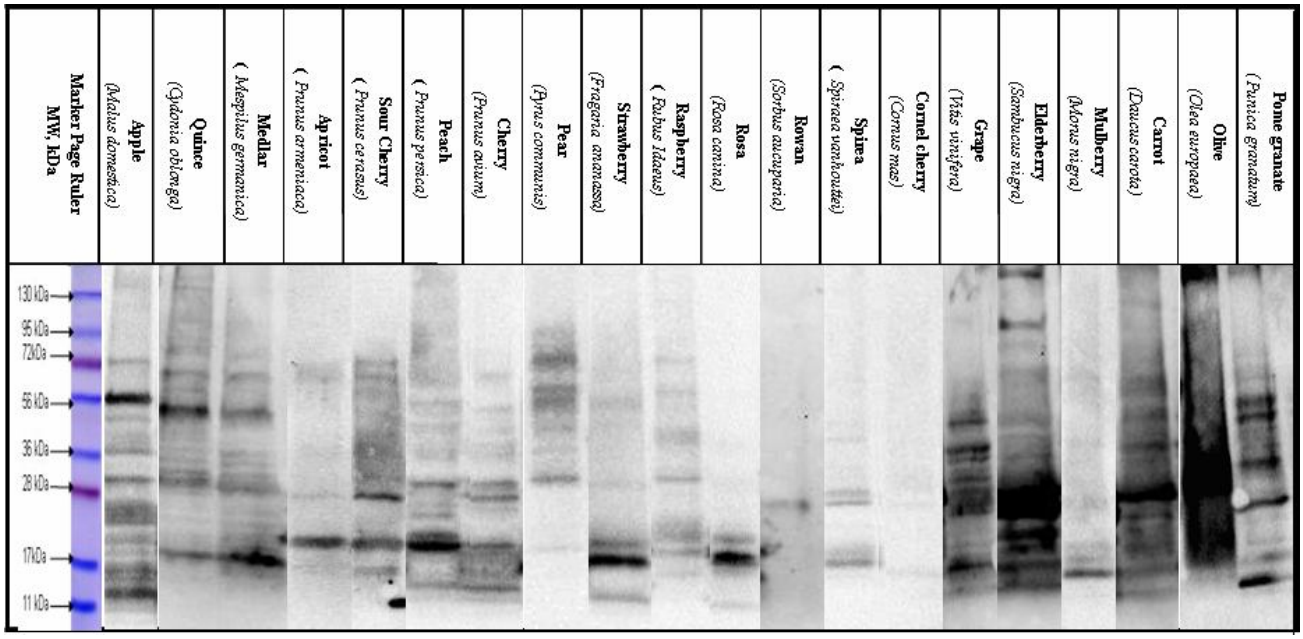


Fig 25. SDS-PAGE and IgE-binding analysis of pollen protein extracts

In the protein extract from **apple pollen** several bands with MW from ~9 to ~72 kDa could be detected. Proteins with MW similar to those of the four major allergens in apple fruit could be detected also here: Mal d 3 (LTP) 9 kDa MW, Mal d 4 (profilin) 14 kDa, Mal d 1 18 kDa double band and Mal d 2 at 23 kDa (Herndl et al. 2007).

In the **quince pollen** there were three protein bands that show strong immunoreactivity: MW ~18 kDa, ~30 kDa and ~50 kDa. There was no direct evidence about the allergenicity of quince fruits or pollen, but *Cydonia oblonga* being a member of the Rosaceous family, the presence of the main allergens, homologues to PR-proteins, was therefore expected. Quinces are mostly not consumed as raw fruits, but as jam, especially in Southern Europe, e.g. Portugal. It could be expected that only the heat stable allergens (LTP) could make troubles to sensitized individuals. A 9 kDa protein band (LTP) could not be detected in this extract with the serum pool.

The pattern of the **medlar pollen** IgE-reactive proteins was similar to those of the Rosaceous family members. There were few evidences for medlar allergy (Pajno et al. 2002, Drampain et al. 1983, García Pérez et al. 1980). But yet there was no characterized allergen in *Mespilus germanica*.

In the **apricot pollen** extract only one protein with MW of ~20 kDa showed a strong immunoreactivity. This protein could not be detected in the fruit extract (see Fig. 12).

In the **sour cherry pollen** there were allergic proteins with MW ~20 kDa and ~28 kDa. Other bands with MW ~15 kDa and ~70 kDa cause a slight IgE-reaction.

In **peach pollen** extract a protein with MW ~20 kDa seems to be the strongest IgE-reactive factor. Other allergic proteins with MW ~15 kDa and ~30 kDa could be detected.

The **cherry pollen** extract shows a broad range of IgE-reactive proteins: four bands with MW from 9 to 20 kDa and many slight reactions to proteins with MW up to 70 kDa.

Pear pollen IgE-reactive proteins have not been visualized, but a pear fruit allergen has been characterized (Karamloo et al. 2001) as member of the Bet v 1 related allergens. Here could be detected allergic protein bands with ~9 kDa, ~14 kDa and ~23 kDa MW.

In **strawberry pollen** the allergic protein detected in this extract were with MW ~9 kDa and ~18/20 kDa doublet. Slight reactions were at ~28 and ~55 kDa MW band height.

In **raspberry pollen** there were IgE-reactivity at ~15, ~20, ~25, ~30 kDa bands. In general the raeactions were weak.

The **rose pollen** showed IgE-reactive proteins with molecular weight ~9, ~14 and ~18 kDa.

In **rowan pollen** there were no direct evidences for the allergenicity of the *Sorbus* species, although the fruits of the hybrids of *Sorbus aucuparia* with *Aronia, Malus, Mespilus* and *Pyrus* species were preferred because of their antioxidant capacities and high phenolic content (Hukkanen et al. 2006). There was one band, detected with this serum pool and its molecular weight is ~23 kDa.

In **spirea pollen** the detected IgE-reactive protein bands were: ~14 kDa, ~18 kDa, ~23 kDa, ~26 kDa, ~35 kDa, which were also typical for the *Rosaceae* family.

Pollen from members of other families was also analysed.

In **cornel cherry pollen** there were very slight IgE-reactive protein bands at ~14 kDa molecular weight, ~23 kDa and ~26 kDa.

In the extract of the **grape pollen** many IgE-reactive proteins could be detected: ~9 kDa, ~14 kDa, and ~ 18 kDa, six bands between ~ 25 and ~ 40 kDa. According to Pastorello et al. (2003a), a 9 kDa allergen has been identificated as an LTP (Vit v 1), a 24 kDa one as a thaumatin like protein, and a 30 kDa one as endochitinase 4. The sensitization seems to occur through cutaneous exposure and/or minor wounding and not through the gastrointestinal tract (Kalogeromitros et al. 2004). Other minor allergens were reported: a 28 kDa expansin, a 37, 5 kDa polygalactorunase-inhibiting protein, a 39 kDa beta-1, 3-glucanase and a 60 kDa protein (Vassilopoulou et al. 2007). In none of the reports on grape and wine allergens Bet v 1 cross-reactivity was mentioned.

The **elderberry pollen** protein pattern was very similar to the fruit extract, and contains a high number of reactive bands.

In **mulberry pollen** there was only one strong IgE-reactive protein band with ~15 kDa MW, although there were many other proteins according to the Coomassie staining. Allergic reactions, caused by mulberry pollen (Muñoz et al. 1995) and cross-reactivity to fig (Caiaffa et al. 2003) have been reported. Also allergic reaction to LTP in mulberry has been recorded (Asero et al. 2007).

The bands detected in **carrot pollen** extract were with molecular weight ~9 kDa, ~15 kDa, ~18 kDa, ~20 kDa, ~25 kDa, ~ 30 kDa, ~35 kDa, ~40 kDa. It has been reported that carrot and some other foods has been tolerated by patients after oral contact and immunoblot analysis confirmed the lack of

IgE reactivity in LTP-allergic patients (Asero et al. 2007). The ~ 10 kDa band was probably a LTP, due to reaction of carrot extract to polyclonal anti Mal d 3 antibody (Fig.23). A ~12 kDa protein was reported as profilin (Fernández-Rivas et al. 2004). The ~18 kDa protein was characterized as Dau c 1, a homologue to Bet v 1 (Moreno-Ancillo et al. 2005, Moreno-Ancillo et al. 2006, Reese et al. 2007). The next band, the ~20 kDa protein, has been reported as cyclophilin, with no cross-reactivity to Bet v 7, the birch cyclophilin (Fujita et al. 2001). The strongest IgE reaction occurred to a protein with ~30 kDa MW that could correspond to a phenylcoumaran benzylic ether reductase (Moreno-Ancillo et al. 2005). The ~35 kDa protein is a Bet v 6-related allergen (Fernández-Rivas et al. 2004). Pathogenesis related proteins with molecular weight ~16, 40 and 41 kDa were isolated and detected from roots of carrots (Yamamoto et al. 1997).

The IgE reaction to **olive pollen** was so strong that the protein bands were not easily be distinguishable, nevertheless, there could be detected four bands: ~15 kDa, ~25 kDa, ~30 kDa and ~100kDa. Up to now 10 allergens from olive pollen have been identified and characterized (Rodriguez et al. 2001, Huecas et al. 2001). The major allergens were proteins with molecular weight ~18-19 kDa, ~20 kDa and ~40 kDa (Baldo et al. 1992). The best described olive allergens are Ole e 1, localized extra cellular in the vicinity of the pollen tube cell wall with trypsin inhibition function, ~20 kDa (De Dios Alché et al. 2004), Ole e 2, a profilin, ~16 kDa, Ole e 3 with a 9,2 kDa molecular weight (Batanero et al. 1996) and Ole e 8 (~18,8 kDa) with a Ca^{2+} binding function (polcalcin) (Rodriguez et al. 2001, Ledesma et al. 1998). Ole e 4 has a molecular weight ~32 kDa (Boluda et al. 1998). Ole e 5 is a Cu/Zn superoxide dismutase with ~16 kDa molecular weight (Butteroni et al. 2005, Boluda et al. 1998). Ole e 6 is a ~5, 8 kDa protein (Batanero et al. 1997). Ole e 7 with ~9,875 is a lipid transfer protein (Tejera et al. 1999). Ole e 9 is a 1,3-beta-glucanase (~46,4 kDa) (Huecas et al. 2001) and Ole e 10 with ~10,8kDa, that showes homology with the C-terminal of Ole e 9 (Barral et al. 2004).

In the **pomegranate pollen** extract protein bands with a molecular weight about ~9 kDa, an LTP (Enrique et al. 2006); ~14 kDa, ~18 kDa, ~24 kDa, ~30 kDa, ~40 kDa and ~45 kDa could be observed. There have been reported allergic reactions to pomegranate fruits (Valsecchi et al. 1998, Gaig et al. 1999, Zoccatelli et al. 2007).

Protein analysis alone could not confirm a correlation between the allergenicity of the fruit and pollen proteins, e.g. the apple pollen protein extracts contain more IgE-reactive proteins than the fruits, however, all of the allergens in apple fruit were present in the pollen. The same situation can be confirmed in the peach, cherry, raspberry, and elder (compare figures in 4.1.2. with Fig. 26). In the strawberry and apricot fruit extracts there were less reactive proteins than in the pollen (see Fig. 12, 13 and 25).

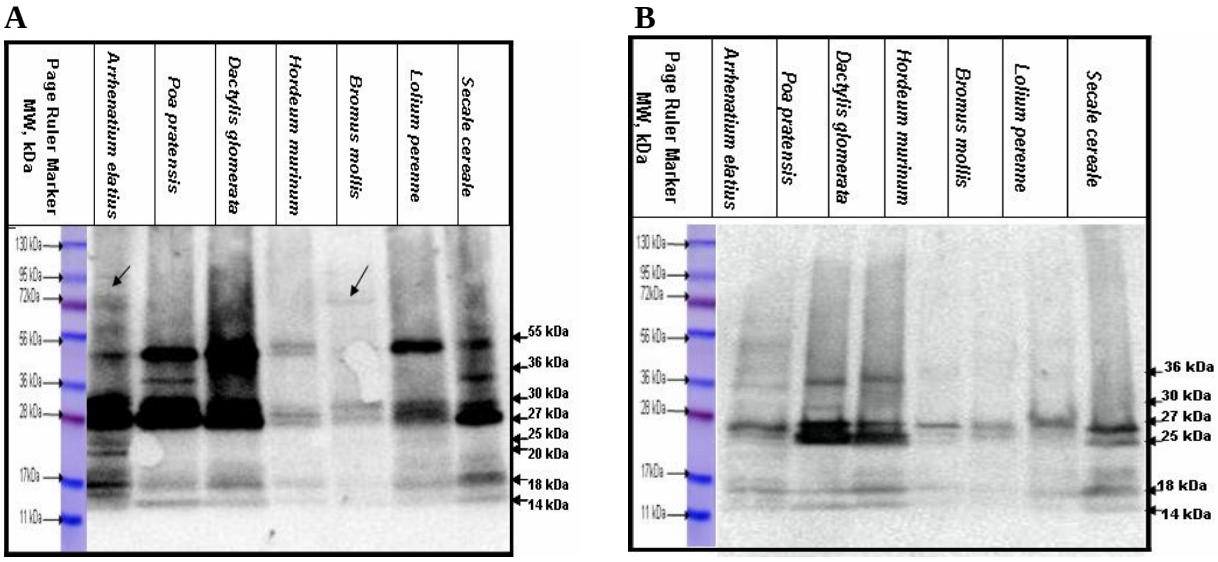


Fig 26. SDS-PAGE and IgE-binding analysis of grass pollen protein extracts. A – samples not reduced, **B** – samples reduced for 10 minutes at 85˚C with β-mercaptoethanol

The reaction of the IgE antibodies against the grass pollen proteins was weaker after reducing the extract samples (Fig. 26B). Eight protein bands with molecular weight from ~18 to ~55 kDa could be detected. Six of the bands appear as doublets: ~18/20 kDa, ~30/34 kDa, ~40/42 kDa. All grass pollen allergens display a common pattern. In *Arrhenatium elatius* extract (Fig. 26B) appeared also a doublet with ~27/29 kDa molecular weight. In the extracts from *Arrhenatium elatius* and *Bromus mollis* there was a protein band with ~60 kDa molecular weight (Fig. 26A).

Up to now, eleven different groups of grass pollen allergens (I – XI) have been identified and characterized from one or more species (Suphioglu et al. 2000).

It was not possible to detect protein bands with a low molecular weight, characterized as major allergens in grass pollen (Andersson et al. 2003): 14 kDa for group XII (profilins), described in *Lolium perenne* (rye grass); 12 kDa for group X (cytochrome c), described in *Lolium perenne* and *Poa pratensis* (Kentucky bluegrass); 13 kDa for group VI, described in *Poa pratensis*; 10-12 kDa for group II (acidic proteins) and III (basic proteins), described in *Dactylis glomerata* (orchard/cocksfoot grass) and *Lolium perenne*. The reason could be that the IgE antibodies in this serum pool could not recognize and bind these proteins.

The group I allergens (MW 31-35 kDa) were present in all extracts. Their function was reported as cell wall-loosening agents (Cosgrove et al. 1997). Poa p 1 was found to consist of a 35,8 kDa (acidic) component and a 33 kDa component (basic) (Lin et al. 1988).

Group IV allergens with MW 50-67 kDa have been described in *Lolium perenne* and *Dactylis glomerata* (Andersson et al. 2003), but they were not detected definitely.

Proteins of group V allergens (MW 27-33 kDa) were detected in the reduced extract from *Arrhenatium elatius*. Similarities in amino acid compositions and NH_2-terminal sequences of the group V allergens were reported between *Lolium perenne, Poa pratensis,* and *Dactylis glomerata* (Klysner et al. 1992).

The group XI allergens with MW 18 kDa were found in all extracts. They were detected in *Lolium perenne* (Andersson et al. 2003). The cross-reactivity among the grasses *Lolium perenne Poa pratensis,* and *Secale cereale* has been reported (González et al. 1987).

Group VII (calcium binding proteins with MW 8, 7-8, 8 kDa) has not been described in the grass species analysed above.

Group XIII allergens with MW 55-60 kDa were detected in the extracts from *Arrhenatium elatius* and *Bromus mollis* (Martin et al. 1985).

4.3. Inhibition ELISA

In general scientific praxis it is routine to confirm the obtained results using an alternative methodology. In immunological research, inhibition ELISA is commonly used parallel to Western blotting. Also in this work the affinity of paient sera to allergic epitopes of Mal d 1 and Mald 3 was determined by inhibition ELISA.

4.3.1. Inhibition of serum with apple pollen extract

The inhibition with apple pollen extract was made for optimizing the potocol. The inhibition assay for Mal d 1 and Mal d 3 was carried out using sera of two apple allergic patients: PI_Mal d 1 (Fig. 27A) and PII_Mal d 1 (Fig. 27B).

In the inhibition test for **serum PI_Mal d 1** (Table 1), the negative control (red) was the serum containing anti Mal d 1 IgEs, that was not incubated with the inhibitor. The negative serum (green) was from a non-allergic individual to Mal d 1 (no reported apple allergy and no reaction detected with polyclonal anti Mal d 1-Western blot), (Fig. 27A).

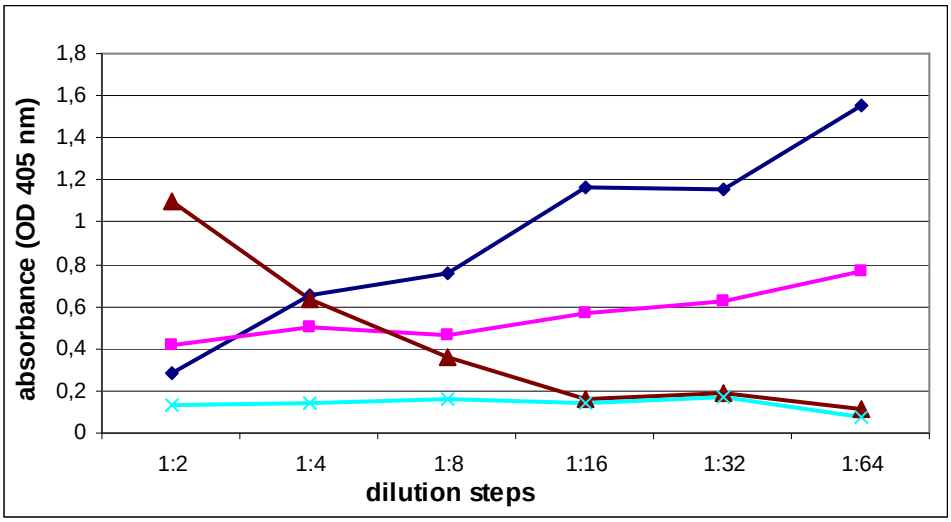


Fig 27 A. Inhibition of serum PI_Mal d 1 with apple pollen detected with the purified Mal d 1 allergen. The plate was precoated with 5 µg/mL purified Mal d 1. The serum was inhibited with 0,059 mg/mL apple pollen protein extract (blue) and autoinhibited (pink) with the 5µg/mL purified Mal d 1 allergen. The negative control (red) was the non-inhibited serum and the negative serum (green) was from a non-allergic individual to Mal d 1.

The inhibited serum IgEs (blue) bound more allergen proteins with decreasing of the inhibitor concentration (absorbance was rising). The autoinhibition (pink) showed low absorbance as expected. The autoinhibition is not 100%. The inhibitor concentration was not high enough, so that the signal was also not as low as the signal from the negative serum.

The negative serum (green), which does not react with Mal d 1, had the lowest absorbance as expected. The non-inhibited serum (red) shows decreasing absorbance with decreasing the concentration of the anti Mal d 1 IgEs.

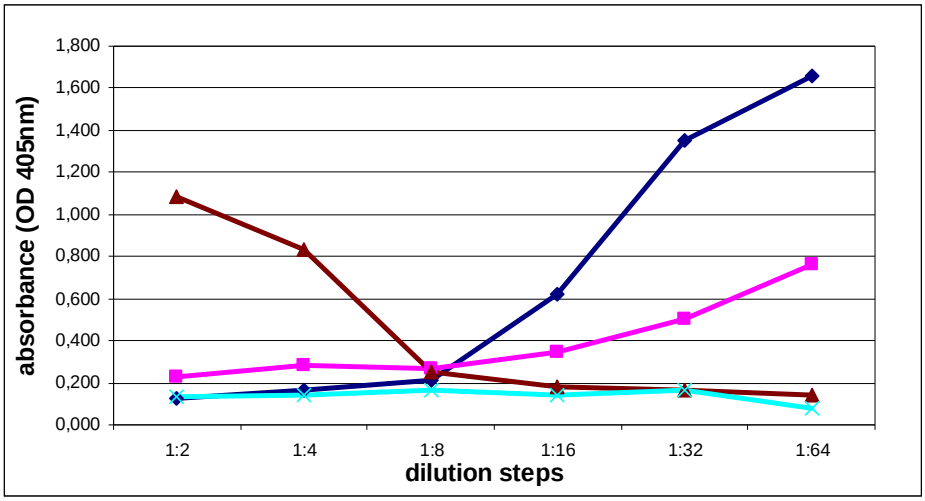


Fig 27 B. Inhibition of serum PII_Mal d 1 with apple pollen against the purified Mal d 1 allergen. The plate was precoated with 5 µg/mL purified Mal d 1. The serum (blue) is inhibited with 0,059 mg/mL apple pollen protein extract and autoinhibited (pink) with the 5µg/mL purified Mal d 1 allergen. The negative control (red) is the non inhibited serum and the negative serum (green) is from a non-allergic individual to Mal d 1.

Also the **serum PII_Mal d 1** (Table 1) yielded similar results as patient PI_Mal d 1 (Fig. 27B). The inhibited serum (blue) IgEs bound more allergen proteins with decreasing of the inhibitor concentration (absorbance is rising). The auto-inhibition (pink) showed increasing absorbance with decreasing inhibitor concentration, as expected. The negative serum (green), which does not react with Mal d 1, has the lowest absorbance as expected.

The non inhibited serum (red) shows decreasing absorbance with decreasing the concentration of the anti Mal d 1 IgEs.

The obtained results confirmed that the protocol was optimal for further analysis.

4.3.2. Inhibition of serum with peach pollen extract

The next step was to perform an inhibition ELISA to detect the presence of homologous proteins in peach pollen extract that cross-react with Mal d 1 specific IgEs. Inhibition of four different patient sera with peach pollen extract was made. Peach pollen was chosen for their high content of Pru p 1 (Ebner et al. 1995), and Pru p 3 (Gamboa et al. 2007). Four patient sera were selected for their known allergenic reactions to peach fruits, as determined by the Floridsdorf Allergiezentrum (FAZ, Vienna, Austria), see Table 1.

4.3.2.1. Cross-reactivity with Mal d 1:

The serum **PII_Mal d 1** that was successfully inhibited with apple pollen extract was used first for the inhibition woth peach pollen extract (Fig. 28A). The inhibited serum (blue) showed the lowest absorbance. The peach pollen could inhibit the anti Mal d 1 IgEs. The autoinhibition (pink) showed high absorbance. The inhibitor concentration was too low. The not inhibited serum (red) absorbed as expected, the less the IgEs (dilution), the less the absorbance.

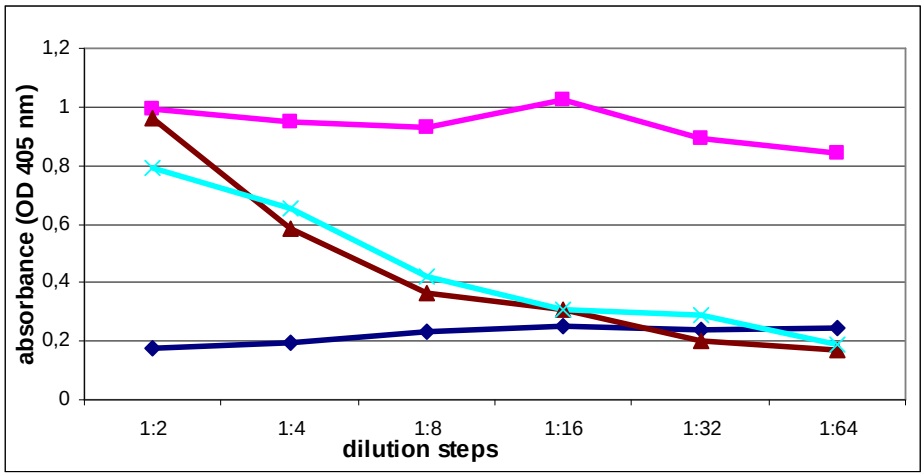


Fig 28 A. Inhibition of serum PII_Mal d 1 with peach pollen detected with purified Mal d 1 allergen. The plate was precoated with 5 µg/mL purified Mal d 1. The serum was inhibited with peach pollen protein extract (blue) and autoinhibited (pink) with the 5 µg/mL purified Mal d 1 allergen. The negative control (red) was a non-inhibited serum and the negative serum (green) was from a non-allergic individual to Mal d 1.

The negative control (green) showed also lower absorbance with the lower peach extract concentration. Possibly IgEs of this serum also recognize Mal d 1 proteins.

The situation with serum **PIII_Mal d 1** (Fig. 28 B) was identical to the one with serum PII_Mal d 1. The peach pollen could inhibit the anti Mal d 1 IgEs. The inhibited serum (blue) showed the lowest absorbance from the first dilution step onwards. The auto-inhibition (pink) showed a high absorbance. Here the inhibitor concentration was too low. The not-inhibited serum (red) absorbs as expected, the less the IgEs (dilution), the less the absorbance.

The Mal d 1 negative serum (green) contained per anamnesis no anti Mal d 1 IgEs, and therefore not reacted with the inhibitor. The observed reduction in absorbance indicated, that also this serum might recognize some Mal d 1 epitopes (Fig 28 B).

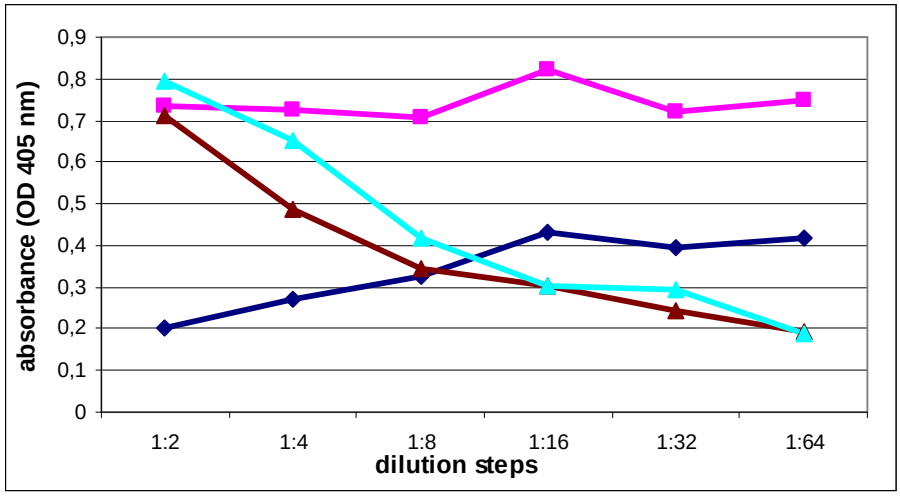


Fig 28 B. Inhibition of serum PIII_Mal d 1 with peach pollen against the purified Mal d 1 allergen. The plate was precoated with 5 µg/mL purified Mal d 1. The serum was inhibited with peach pollen protein extract (blue) and autoinhibited (pink) with the 5 µg/mL purified Mal d 1 allergen. The negative control (red) was the non inhibited serum and the negative serum (green) was from a non-allergic individual to Mal d 1.

The inhibition (%) was calculated as follows:

% inhibition= $100-[(OD_{inhib.serum}/OD_{not\ inhib.serum})*100]$

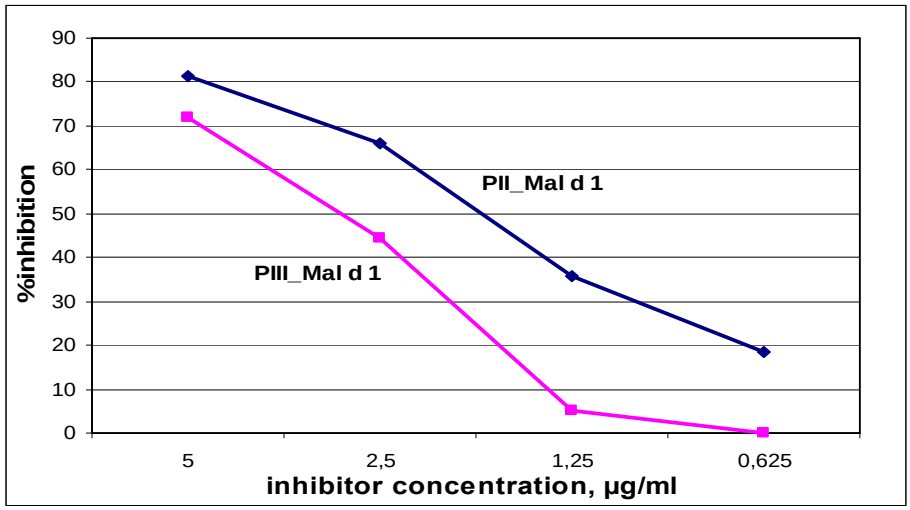


Fig 29. Inhibition (%) of sera from two apple allergic patients with peach pollen against the purified Mal d 1 allergen. The percentage of the inhibition decreases with the decreasing of the inhibitor concentration.

Due to the complex kinetic reactions and competitions between the IgE antibodies, an inhibition of 100% can not be achieved.

4.3.2.2. Cross-reactivity with Mal d 3:

Since i twas anticipated that antibodies recognizing LTPs would show cross-reactivity, purified Mal d 3 was used in the next step.

The inhibited serum **serum PIV_Mal d 3** (Table 1) (blue) showed rising absorbance, as expected (Fig. 30 A). The absorbance of the auto inhibited serum (pink) should be the lowest one. The

inhibitor concentration was obviously too low, so that not all IgEs epitopes could be blocked during the preincubation. The not-inhibited serum (red) showed decreasing absorbance, as expected. The negative Mal d 3 serum (green) showed a weak reaction to Mal d 3 (Fig 30 A).

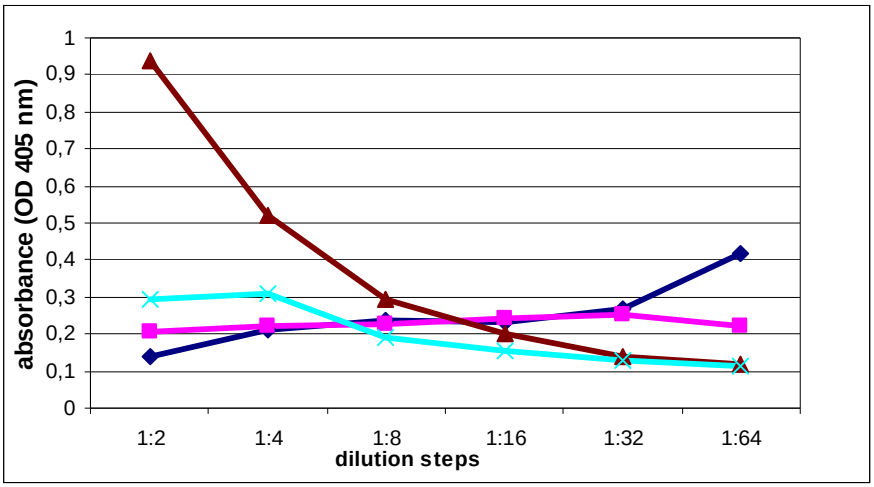


Fig 30 A. Inhibition of serum PIV_Mal d 3 with peach pollen against the purified Mal d 3 allergen. The plate was precoated with 5 µg/mL purified Mal d 3. The serum was inhibited with peach pollen protein extract (blue) and autoinhibited (pink) with 5 µg/mL purified Mal d 1 allergen. The negative control (red) was a non inhibited serum and the negative serum (green) was from a non-allergic individual to Mal d 3.

Also **serum PV_Mal d 3** (Table 1) (Fig. 30 B) showed results identical as serum PIV_Mal d 3 (Fig. 28A). The inhibited serum (blue) showed rising absorbance. The absorbance of the auto-inhibited (pink) serum should be the lowest one. The inhibitor concentration was too low. The not-inhibited serum (red) shows decreasing absorbance, as expected. The negative Mal d 3 serum (green) showed weak reaction to Mal d 3 (Fig 30 B).

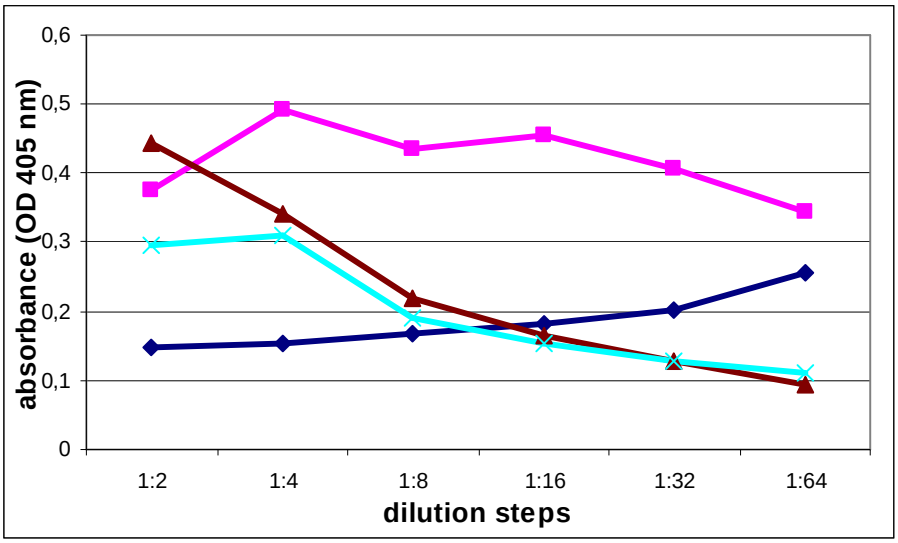


Fig 30 B. Inhibition of serum PV_Mal d 3 with peach pollen against purified Mal d 3 allergen. The plate was precoated with 5 µg/mL purified Mal d 3. The serum was inhibited with peach pollen protein extract (blue) and auto-inhibited with the 5 µg/mL purified Mal d 3 allergen (pink). The negative control was a non inhibited serum (red) and the negative serum (green) was from a non-allergic individual to Mal d 3.

The inhibition (%) was calculated as follows:

% inhibition= 100-[($OD_{inhib.serum}$ /$OD_{not\ inhib.serum}$)*100]

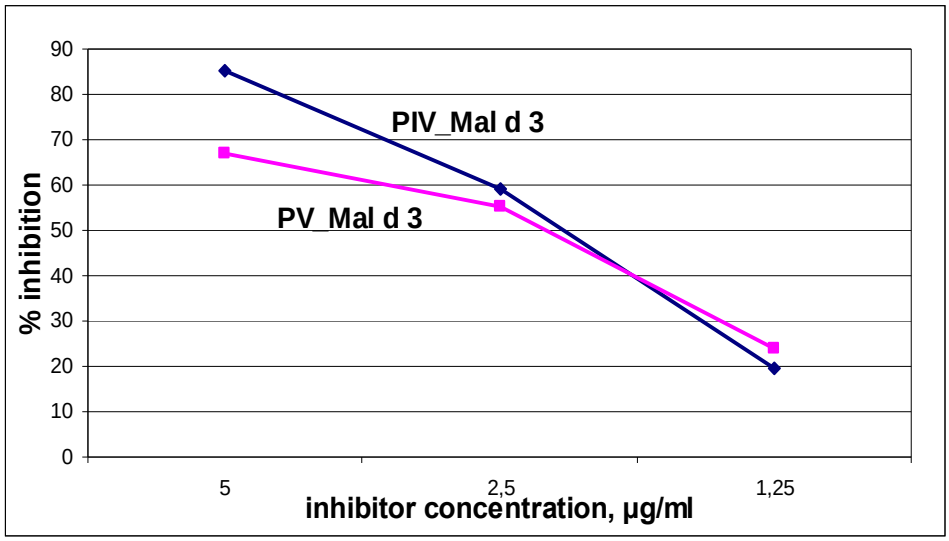


Fig 31. Inhibition (%) of sera from two apple allergic patients with peach pollen against the purified Mal d 3 allergen. The percentage of the inhibition decreases with the decreasing of the inhibitor concentration.

The high % inhibition of the serum IgEs against peach proteins with purified Mal d 1 und Mal d 3 protein indicates the strong cross-reactivity with allergens originated from Rosaceous fruit pollen. The four sera of patients with peach anmnesis showed in the inhibition tests that the allergic reaction against peach is due to Pru p 1 and Pru p 3 allergens. These two allergens are present also in the pollen, which can represent a possible way of sensitization.

5. Discussion

Plant foods are consumed as a source of vitamins, antioxidants and dietary fiber. Unfortunately, some of the proteins they contain are also the cause of adverse allergic reactions. The successful analyses of these proteins require optimization of the extraction methods according to the plant tissue and the presence of interfering compounds.

The current investigations comprised: a) the elaboration of adapted protein extraction procedures for proteins from fruits of apple, apricot, cherry, peach, strawberry, raspberry, blackberry, blueberry, elder and orange, roots of carrot, and also pollen of apple, quince, medlar, pear, apricot, sour cherry, peach, cherry, strawberry, raspberry, rosa, rowan, hawthorn, spirea, grape, cornel cherry, elder, mulberry, olive, pomegranate, carrot, and the grasses: false oat, Kentucky blue grass, cocksfoot, mouse barley, soft chess, ryegrass and cereal rye; b) the detection of homologous allergens using polyclonal rabbit and human sera; c) the study of cross-reactivity among different allergenic proteins and d) the determination of the influence of sample preparation procedures on the electrophoretic separation of proteins, both under reducing and non reducing conditions.

In the present study, major attention was devoted to two of the four major allergens in apple, Mal d 1 and Mal d 3, and their homologues in members of the *Rosaceae, Caprifoliaceae, Ericaceae, Rutaceae, Vitaceae, Cornaceae, Moraceae* and *Poaceae* families. The polyclonal IgG antibodies raized against apple Mal d 1 and Mal d 3 could successfully detect homologous proteins in most extracts analyzed.

Apple fruit extract was considered as the reference. The sera could detect all apple allergens known so far. The different molecular mobility of Mal d 2 could be shown in the apple extract (Fig. 9). This phenomenon was confirmed to be dependent on the sample preparation - ~25 kDa not reduced and ~31 kDa under reducing conditions (Herndl et al. 2007).

The major allergen in **peach** – Pru p 3 – was recognized only by the half of the sera (6 of 12 individuals), all in the reduced samples (Fig. 10B). This could be related to the fact, that in Central Europe, allergy to peach and other Rosaceous fruits was initiated by the birch pollen and more precisely by the two main cross-reactive allergens Bet v 1 and Bet v 2 (birch profilin) (Ebner et al. 1995, Reuter et al. 2006). In Southern Europe, where other sources of sensitization prevail, although they are not completely elucidated so far (Gamboa et al. 2007), peach allergy is mostly mediated by the nsLTP Pru p 3. Allergy to peach is a major risk factor for sensitised individuals, since it may lead to anaphylaxis and even death (Garcia et al. 2004).

The patient sera analyzed also detected the already characterized **cherry** allergens Pru av 1 ~18 kDa (Neudecker et al.2003), Pru av 3 ~9 kDa (Scheurer et al. 2004), Pru av 4 ~14 kDa (Scheurer et al. 2001a), Pru av 2 ~31 kDa (Dall`Antonia et al. 2005) (Fig. 11), although only one of the patients had

a recorded cherry anamnesis (P5, Table 1). The protein bands with a molecular weight above 40 kDa were detected in the non reduced samples, so they could be possibly oligomers. In the pollen extract there are many IgE reactive bands under 30 kDa – all of the characterized cherry allergens yet, in comparison to the cherry fruit extract, where the detected bands are with higher molecular weight. This is an indication that all of the potentially allergic proteins are represented in the pollen and few in the fruits, which could be due to an organ specific expression pattern, a hypothesis that is confirmed below.

The **apricot** fruit extract (Fig. 12) showed very weak bands. Since also the Coomassie staining of the fruit extract (Fig. 6) indicated a low protein concentration, the extraction protocol was optimized and provided data on the LTP expression in *Prunus sp.* (Marzban et al. 2006). The apricot pollen extract contained much higher protein concentrations (Fig. 20), possibly also due to the phenol precipitation method used for protein extraction. Nevertheless also the pollen extract showed only a weak IgE binding capability. It should be noted, however, that none of the sera used was from patient with a known apricot anamnesis.

Fragaria ananassa, member of the *Rosoideae* subfamily, is an interesting model species for small fruits, since it is used to a large extent in the fruit industry as fresh fruits and food additive. Although there are many reports for adverse reactions to strawberry, since many of the skin prick tests were negative, it was questioned if they belong to IgE-mediated allergies (Eriksson et al. 2003). The patient sera analyzed could detect many proteins (Fig. 13), also with higher molecular weight, maybe including also IgE-reactive protein glyco-structures (Altmann 2007). Karlsson et al. (2004) identified a strawberry protein of ~18 kDa as a Bet v 1 homologue for the first time, setting an end to these discussions. Although a LTP homologue, Fra a 3, was reported in strawberry by Zuidmeer et al. (2006), in the strawberry extract of this study the reactivity to sera was very weak. This might be due to the not optimized extraction method or to the missing sensitivity of the analyzed patients. The presence of profilins and Bet v 6 homologous proteins in strawberry was also reported in the meantime (Zuidmeer et al. 2006, Vieths et al. 2002, Marzban et al. 2006). The pollen protein extract showed a similar result as the cherry pollen extract – high protein concentration according to the Coomassie staining (Fig. 20), but low IgE reactivity.

A protein pattern similar to the one of apple was detected in **raspberry** (Fig. 14). A protein with molecular weight ~30 kDa turned out as the major allergen (class III chitinase) (Fig. 14). This and cross-reactivity of Rub i 1 and Rub i 3 to apple allergens was confirmed by Marzban et al. (2008b). Proteins from fruits with high molecular weight could not be extracted (Fig. 6). In the pollen extract there are many proteins higher molecular weight present in high concentrations (Fig. 20), which did not cause any IgE reaction (Fig. 25).

The **blackberry** extract (Fig. 15) also indicated that proteins in this species could cause allergic reactions in already sensitized individuals; so far none of the analyzed sera was from patient with blackberry anamnesis. The protein concentration in the fruit extract is very low (Fig. 6). Also the sera showed much weaker IgE reactivity to the reduced samples (Fig. 15B) as to the not reduced samples (Fig. 15A), although a direct comparison of individual sera is still missing.

Since the Coomassie staining of the **blueberry** extract (Fig. 6) showed 5 protein bands, the extraction protocol appeared to be acceptable. However, the IgE-reactions were very weak (Fig. 16). The ~36 kDa (P18) band was present also in the Coomassie stained gel and the IgE-reaction is induced by protein in a reduced sample, therefore it is possible that it is not an oligomer. So far, there are no reports on *Vaccinium corymbosum* allergens yet. There is an allergen Vac m 3 (LTP) in *Vaccinium myrtillus* annotated in allergome.org.

In this work it was possible to discover allergens even in species, where no homologues have been reported yet, e.g. Mal d 3 in **carrot** pollen extract (Fig. 22) (allergome.org). On the other side, in the carrot root extract Mal d 3 could not be detected as expected (Fig. 8), which could be due to an organ specific expression pattern or to the fact, that the extraction procedure requires additional optimization. The IgE antibodies could detect protein band with ~9 kDa (Fig. 25).

A comparable situation occurred with **elderberry**: polyclonal antibodies detected Mal d 1 in the pollen extract (Fig. 21), but not in the fruit extract (Fig. 7). Generally, the elderberry extract (Fig. 17) shows a very interesting pattern of IgE-reactive proteins. Since there is only one characterized allergen in this species, its allergenicity seems to be underestimated (Förster-Waldl et al. 2003). The examples on carrot and elderberry can be again mentioned as an indication for organ specific expression pattern.

Pollen extracts of ***Vitis vinifera*** and ***Olea europaea*** did not bind polyclonal anti Mal d 1 antibodies (Fig. 21). Hoewever, a 18,8 kDa protein in olive has been reported to have a Ca^{2+} binding function (polcalcin) (Rodriguez et al. 2001, Ledesma et al. 1998).

Although a 9 kDa protein, named Cit s 3, has been reported in **orange** as an LTP (Ahrazem et al. 2005), the detected band in the peel extract analyzed was of higher molecular weight than expected (Fig. 8). There are also many unspecific bands, which could be unspecific reactions due to an extended incubation period or to the formation of oligomers, although the samples were prepared under reducing conditions. The IgE reactivity of the sera to orange proteins was generally weak. This may be due to low protein concentration or to a low IgE antibody concentration in the sera.

The Coomassie stained gels provided a valuable overview of the protein composition and content in the various fruit species and grasses. The analyzed fruit extracts from apple, strawberry, raspberry, blueberry, elderberry and orange were rich in proteins, indicating that the method for protein extraction was appropriate for these fruits. The extracts of apricot, cherry, blackberry and carrot

showed a lower protein concentration, which might be due either to a high water content of the tissue or to the fact, that the extraction procedure required additional optimization. Protein bands with molecular weight below 11 kDa could not be stained, although they could be detected with polyclonal antibodies and patient sera. This might be due to the fact that the protein concentration was too low or the staining method was not sensitive enough. Other staining methods, e.g. silver staining could be used for proteins present in lower concentrations in the extract.

Due to the different characteristics of pollen as source material alternative protein extraction methods were used. Either water extraction or phenol precipitation yielded reliable results. The phenol precipitation resulted in higher protein concentrations. The pollen extracts showed a more variable protein pattern than the fruit extracts. This probably is due to the fact, that plants express proteins in a tissue specific manner, once differentiation occurred in individual organs, while in pollen most proteins are present, since it represents a more complex developmental stage.

The IgE-Western blotting provided highly variable IgE-reactivity patterns. The patient sera were from individuals living in Austria (Central Europe). It should be mentioned that most of the patients were female; an observation that has been confirmed recently by researchers from the Viennese General Hospital (Jenssen-Jarolim et al. 2008). The IgE content of each serum is to be considered as an individual characteristic, variable also according to the season. Therefore, the reaction strength does not depend only on the protein concentration, but also on the concentration of IgE antibodies present in the serum. In all of the gels the detected bands with higher molecular weight might represent di- or oligomers of the allergenic proteins or proteins with IgE-reactive glyco-structures (Altmann 2007, Jin et al. 2008).

The pollen protein extracts (Fig. 25) displayed very complex IgE reaction patterns. Most of the proteins, stained with Coomassie blue were detected also by the serum pool used for the pollen analysis. Many slight bands could be considered as unspecific reactions or again – due to protein glyco-structures. There strongest reactions occurred to pollen of species that are usually not very common for Central Europe, where the patients are originating from or their concentration in the air should be very low, i.e. carrot, olive and pome granate.

The allergens in the grass pollen could be also successfully detected with IgE antibodies, also differences in the protein pattern, according to the sample preparation (Fig. 26). The grass pollen proteins seem to loose their IgE binding capacity, respectively allergenicity, under reducing conditions (Fig. 26B).

The changes in the protein structure under the reducing or non-reducing conditions during the sample preparation played also a role for the strength of the IgE-reaction, e.g. grass pollen, where not reduced samples caused stronger reactions than the reduced ones. Probably the allergens not being thermo-stable, their structures were altered by heating/reduction or even the allergenic

epitopes were destroyed. The sample preparation influenced the IgE reactivity in the peach, strawberry, blackberry and orange fruit extract. The reduced samples showed weaker reactions, or the IgE binding proteins were of lower molecular weight. The IgE binding proteins were not thermo-stable, or the oligomers were turned to monomers after reducing of the samples, for comparison P11 in cherry extract (Fig. 11).

Inhibition ELISA reconfirmed the cross-reactivity between the proteins detected with the polyclonal anti-Mal d 1 and anti-Mal d 3 antibodies in fruit pollen. In this work peach pollen was chosen as representative model and the data obtained clearly delivered the ultimate proof for an alternative way of sensitization via fruit pollen. The negative serum (green) was initially not reported to have peach allergy – the reason why it was chosen as negative controll, but apple and peach pollen proteins could inhibit anti Mal d 1and Mal d 3-IgE antibodies in this serum (Fig. 27 and Fig. 28) and this fact indicates that the inhibition ELISA can be another alternative method to determine sensitization that has not provoked a severe allergic reaction yet, but is a possible threat for the patient's health.

The studies on pollen need further investigations that can be based on the methods established in this work.

Abbreviations

AP (**a**lkaline **p**hosphatase)

BCIP (5-**B**romo-4-**C**hloro-3-**I**ndolyl-**P**hosphate)

BSA (**b**ovine **s**erum **a**lbumin)

CBPs (**c**alcium-**b**inding **p**roteins)

EDTA (**e**thylene**d**iamine **t**etraacetic **a**cid)

DIECA (sodium **die**thyldithio**ca**rbamate trihydrate)

DTT (**d**i**t**hio**t**hreitol)

EIA (**e**nzyme **i**mmuno**a**ssay)

ELISA (**e**nzyme-**l**inked **i**mmunosorbent **a**ssay)

HRP (**h**orse**r**adish **p**eroxidase)

IgE (**i**mmuno**g**lobulin **E**)

IgG (**i**mmuno**g**lobulin **G**)

IEF (**i**so**e**lectrical **f**ocussing)

IEX (**i**on **ex**change chromatography)

kDa (**k**ilo**Da**lton)

MW (**m**olecular **w**eight)

NBT (**n**itro**t**etrazolium **b**lue chloride)

ns LTP (**n**on **s**pecific **l**ipid **t**ransfer **p**rotein)

OAS (**o**ral **a**llergy **s**yndrome)

OD (**o**ptical **d**ensity)

ON (**o**ver **n**ight)

PBS (**p**hosphate **b**uffer **s**olution)

PNPP (**p**-**n**itro**p**henyl**p**hosphate)

PR-protein (**p**athogenesis **r**elated protein)

PVDF (**p**oly**v**inily**d**en**f**luorid)

PVPP (**p**oly**v**inyl**p**oly**p**yrrolidone)

RIA (**r**adio **i**mmuno**a**ssay)

rpm (**r**otations **p**er **m**inute)

RT (**r**oom **t**emperature)

SDS-PAGE (**s**odium **d**odecyl **s**ulphate-**p**oly**a**crylamide **g**el **e**lectrophoresis)

2 DE (**t**wo **d**imensional **e**lectrophoresis)

SPT (**s**kin **p**rick **t**est)

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Cross-reactive allergens in pollen of Rosaceous trees

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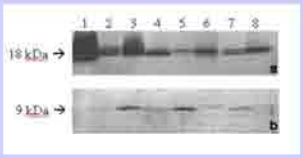
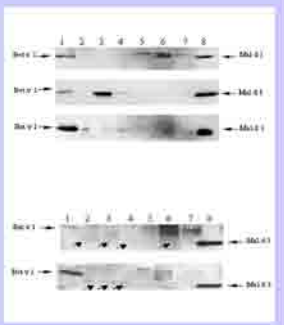
The allergen content of fruit pollen has been underestimated for a long time. Since fruit pollen are mostly classified as insect-carried, and considered to be available in the atmosphere in a lower number, with higher weight and larger size than airborne tree pollen, they were not considered as a potential trigger of allergies. Most fruit allergens have been characterised as pathogenesis-related proteins, which are expressed constitutively and mainly up-regulated upon pathogen attack. Recently, a report on orange allergy has shown a possible co-sensitisation to orange fruits by digestion as well as inhalation of fruit pollen [1], suggesting an alternative route for allergy development. The allergy to Rosaceous fruits like apple, pear, peach and cherry has been described generally as a consequence of cross-reactivity to the major birch pollen allergen Bet v 1 [2]. The possibility that exposure to Rosaceous fruit pollen might induce a similar sensitisation pattern in allergic individuals has not ever been taken into consideration.

Detection of Mal d 1 (a) and Mal d 3 (b) in fruit pollen extracts by Western blot using polyclonal antibodies: 1: *Betula sp.* (birch), 2: *Malus domestica* (cultivar Rheinischer Winterrambour), 3: *Malus domestica* (cultivar Lavantaler), 4: *Pyrus communis* (pear), 5: *Prunus armeniaca* (apricot), 6: *P. persica* (peach), 7: *P. cerasifera* (sour cherry), 8: *P. avium* (cherry).

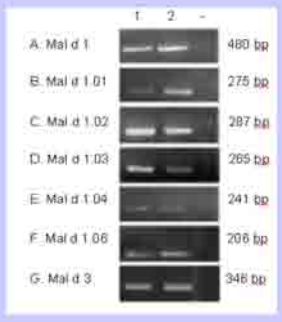


Rosaceae-pollen index (annual total pollen counts divided by number of days with incidence): none: 0-3; very low: 3, low: 13; medium: 29; high: 51; very high: 80. Data collected over large areas of Europe over a period of up to 29 years indicate considerable differences in the exposure to the relevant pollen according to the geographic distribution of *Rosaceae* species. The high incidence in Northern Europe might be due to *Sorbus sp.*, while in Central and Southern Europe mainly *Malus* and *Prunus sp.* are relevant.

Detection of Mal d 1 (A) and Mal d 3 homologues (B) in *Rosaceae* pollen was performed using IgE-immunoblot with sera of five birch/apple allergic patients. As positive controls purified native Mal d 1 and Mal d 3 (17.5 kDa, and 9 kDa) were applied to the gels. (1: *Betula sp.* (birch), 2: *Malus domestica* (Rheinischer Winterrambour), 3: *M. pumila* (wild apple), 4: *Pyrus communis* (pear), 5: *Prunus armeniaca* (apricot), 6: *P. persica* (peach), 7: *P. cerasifera* (sour herry), 8: *Positive controls*. IgE-reactivity pattern to Mal d 1 homologues of three patients indicate individual differences. However, the IgE reactivity of two LTP-sensitized patients clearly appears in apple and pear pollen (the positive LTP bands are highlighted by arrows →).



Detection of allergens transcripts. Mal d 1 (A-F) and Mal d 3 (G) in apple pollen.
Amplified transcripts are representative for all described subfamilies of Mal d 1 expressed in apple pollen: Mal d 1.01 and Mal d 1.02 – subfamily I, Mal d 1.03 – subfamily IV, Mal d 1.04 – subfamily II, Mal d 1.06 – subfamily III.
PCR products amplified from cDNA were separated on a 2% agarose gel. The length of the PCR products is defined in base pairs (bp).
Panels: (A) total Mal d 1 transcripts; (B) Mal d 1.01, (C) Mal d 1.02, (D) Mal d 1.03, (E) Mal d 1.04, (F) Mal d 1.06, (G) Mal d 3.
Lane 1: pollen cDNA from cultivar Lavantaler
Lane 2: pollen cDNA from cultivar Rheinischer Winterrambour
Lane - : negative control (Aqua dest.)



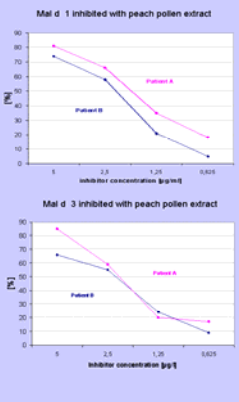
Conclusions

The expression of the major apple allergens Mal d 1 and Mal d 3 in various Rosaceous fruit tree pollen was confirmed using independent methods, including patient sera. Measurements of pollen concentration in the Vienna region demonstrate a continuous Rosaceous pollen burden without eminent peaks, which can be considered as a permanent pollen allergen exposure to Mal d 1 homologues for sensitive individuals [3].

The potential allergenic relevance of fruit pollen allergens has been confirmed by inhibition ELISA using native purified Mal d 1 and Mal d 3. Each allergen two patient sera with clinical pre-investigation are used. As inhibitor peach pollen extract have been used, since it contains both Mal d 1 and Mal d 3 homologous proteins.

The protein content of extracts after buffer exchang has been determined using BCA Protein Kit from Pierce. The plates have been coated with 1 µg/ml of each allergen and the sera inhibited with increasing amounts of pollen extract.

Overall similar inhibition values have been obtained for both allergens (70% for Mal d 1, and 65 % for Mal d 3), which indicate strong cross-reactivity with allergens originated from *Rosaceous* fruit pollen.



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Screening of major allergens in strawberry and blueberry

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Introduction

Consumption of strawberry and blueberry has been shown to comprise several health benefits like improved short time memory, reduced cancer, reversion of normal decline of motor skills in animal models, if still not in human trials. Recently small fruits like strawberry and blueberry have found their place in the nutritional daily intake recommendations for both children and adults, since these fruits provide rich source of vitamin C and contain high amounts of antioxidants. An increased production volume and consumption rate are expected consequences on European market. Although both fruits contain beneficial substances, they may represent a health risk for allergic individuals sensitized by homologous proteins in pollen or food allergens.

According to previous assumptions by clinicians, the skin reactions, that many patients exhibited upon strawberry consumption, were not to be considered as a type I allergy. Karlsson et al. (2004) reported the presence of Bet v 1 homologues in strawberries. This prompted us to investigate the expression of heterologous allergens, which we previously found in the related species *Malus*, e.g. Mal d 1, Mal d 2, Mal d 3 and Mal d 4 (Marzban et al. 2005). These proteins are constitutively expressed and can be up-regulated by pathogen-stress factors. Reported allergies to these proteins include symptoms beginning from swollen lips and irritated mouth tissue, urticaria, and ending with anaphylaxis. Even in slight forms of fruit allergies, the triggered symptoms upon fruit intake have an influence on the nutritive behavior of the allergic patients, responding with an avoidance diet. It is therefore important to address the allergenicity of different strawberry and blueberry varieties containing different amount of allergens. The development of novel selection markers will allow to choose breeding lines with low allergenic contents for future breeding strategies.

Known apple and birch allergens

Fig. 1 Schematic presentation of allergens detected in apple fruit and birch pollen extracts

Specific antibodies recognize the allergen- homologues in Rosaceous fruits

Polyclonal antibodies raised in rabbits against different allergenic proteins in apple recognize homologous proteins in most rosaceous fruits. Missing signals in some extracts might be due to very low concentration of proteins or heterologous binding sites in different species.

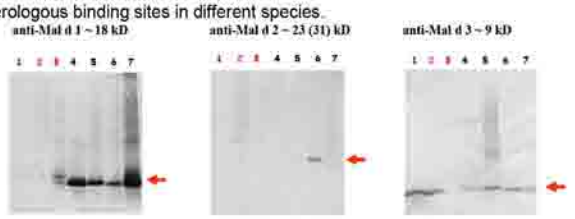

Fig. 2 Western blots with rabbit pAb of fruits extracts 1. blueberry 2. raspberry, 3. strawberry, 4. cherry, 5. peach, 6. apricot, 7. apple

Patients sera cross-react with rosaceous fruit proteins

Two patients, reported as birch pollen and apple allergic, exhibit different cross-reactivity patterns with rosaceous fruits proteins. The molecular bases of such differences in the epitope recognition are still not known.

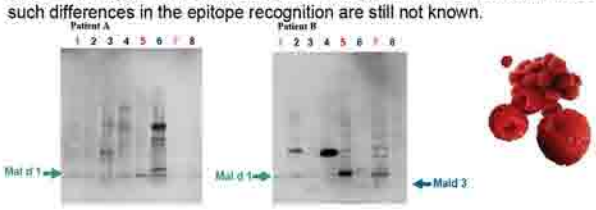

Fig. 3 Western blots with birch pollen and apple allergic patient IgE on fruits extracts; 1. native Mal d 1, 2. apple, 3. peach, 4. cherry, 5. strawberry, 6. blackberry, 7. raspberry, 8. blueberry

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Detection of IgE-reactive proteins in strawberry and blueberry extracts

For screening the patient sensitization pattern to cross-reactive allergens in strawberry and blueberry extract, sera of 15 birch and apple allergic patients were analysed.

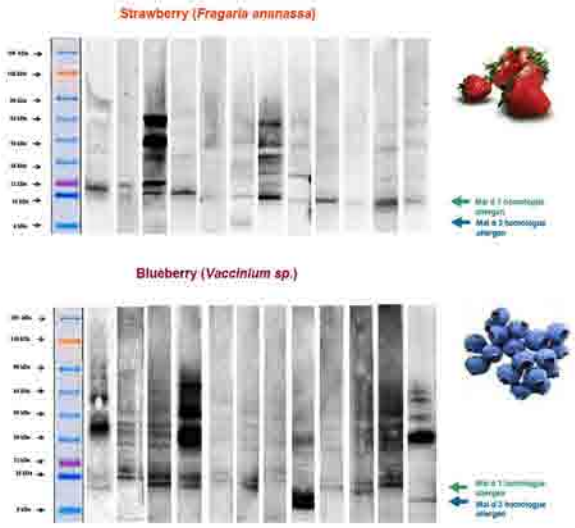

Fig. 4 Screening of allergenic proteins in strawberry and blueberry extracts using apple/birch allergic patient sera

Discussion and Conclusions

Fruits like blueberry, strawberry and raspberry may be nutritionally underestimated, but scientific research shows that they have a huge impact on human health due to their high content of antioxidants and unknown biofactors, which may play a role in ageing, cancer and infection prevention. Reports on allergenicity of small fruits such as strawberry, raspberry, blackberry and blueberry are still rare. Whether this is related to a general low allergenicity, the small amounts consumed or the restricted time frame of exposure still remains to be answered.

Since many identified allergens belong to plant pathogen-related proteins, their presence is expected in almost all type of plant-derived food. According to our results at least two major fruit allergens, Mal d 1 and Mal d 3 homologous proteins, are expressed in strawberries and blueberries, whereas, the reaction of patients to berry extracts implicates a more complex pattern for allergenic responses.

A reduction of fruit-pollen-syndromes demands a change of lifestyle, or even profession, adhering to an elimination diet or avoidance, long-term symptomatic treatment and immunotherapy. Therefore apart from an effective therapy, research on mechanisms of allergies and methods for avoiding exposure to allergens is a prerequisite to improve the life quality of affected individuals.

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Mini Review

Fruit cross-reactive allergens: A theme of uprising interest for consumers' health

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Abstract. Regular consumption of fruits has a positive influence on human health by disease prevention. However parallel to dietetic benefits, IgE-mediated fruit allergies have been shown to be an increasing health risk for children and adults in the Northern hemisphere. The spectrum of food allergies ranges from chronical symptoms to more acute problems and even anaphylaxis. Fruit proteins with high primary sequence similarity display also homologous tertiary structures, resulting in similar epitopes to IgEs and consequently in cross-reactivity. In this review we present the major allergens of stone and pome fruits and discuss the presence of homologous proteins in small fruits. Interestingly these proteins, which might pose an allergenic potential for pre-sensitised individuals are expressed also in strawberry, raspberry and blueberry, otherwise rich in beneficial biofactors.

Keywords: Fruit allergy, small fruits, cross reactivity, pathogenesis related proteins (PRPs), cross-reactive carbohydrate determinants (CCDs)

1. Introduction

The importance of regular consumption of fruits and vegetables in the prevention of cancer and cardiovascular diseases has been widely acknowledged [4,17,24,49]. The World Health Organisation recommends the daily intake of some 400 g of fresh fruits and vegetables per person [21]. However, consumption of particular fruits and vegetables may elicit adverse effects in allergic individuals [36]. Epidemiological data estimate the prevalence of food allergy to be 1.4 to 2.4% [22,51], with children, adolescents and young adults being particularly affected [22,33,43]. Recent studies indicate higher numbers of food allergic patients in the European population, particularly when also secondary food allergies resulting from cross-reactivity with common inhalant allergens, such as pollen, house dust mite or rubber latex, were taken into account. According to investigations in Germany, Sweden and UK, 12–16% of adult people report food allergy and exhibit at least one positive reaction in skin prick test to food allergens [43]. While clinical symptoms elicited by secondary food allergens are often mild, although not always, this type of food allergy is much more common among adults than "true" primary food

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Table 1
Designated cross-reactivity for main apple allergens with proteins from other fruits. Allergenic proteins were identified in fruits of *Rosaceae* with high sequence and conformational homology. These similarities have been repeatedly confirmed to be responsible for cross-reactivity in the patients response

Family	Apple allergen	Other fruit allergens
PRP-10	Mal d 1	peach (Pru p 1), apricot (Pru ar 1), cherry (Pru av 1), pear (Pyr c 1)
PRP-5	Mal d 2	cherry (Pru av 2), kiwi (Act c 2), grape (Vit v TLP)
PRP-14	Mal d 3	peach (Pru p 3), apricot (Pru ar 3) plum (Pru d 3), cherry (Pru av 3), grape (Vit v 1)
–	Mal d 4	cherry (Pru av 4), pear (Pyr c 4), apple (Mal d 4), peanut (Ara h 5), hazelnut (Cor a 2)

allergies. It is obvious from these studies that concomitant respiratory and food allergy is particularly found in individuals primarily sensitised to birch pollen [43]. The observation, that patients allergic to birch pollen develop more frequently hypersensitivity to fresh fruits and vegetables than patients allergic to pollen of mugwort, grasses, ragweed or plane, can be explained by the presence of common epitopes in birch pollen and fruits allergens, as suggested by RAST inhibition studies (Table 1 [5,10,12]). The cross-reactivity between proteins from birch (*Betulaceae*) and apple (*Rosaceae*) indicates that structural homology of the allergens can occur in distantly related taxa.

Most plant allergens belong to only four protein families, indicating that conserved structures and biological activities may play a central role in determining or promoting allergenic properties [23]. The conservation of both surface residues and main chain conformations in the Bet v 1 family plays an important role in conservation of IgE-binding epitopes, which explains cross-reactive allergy syndromes [34]. In contrast to a common opinion, sensitisation to rosaceous fruit allergens can occur without related pollinosis and may have severe consequences [16].

Fruit allergens can be classified depending on their stability to digestion and modification in the gastrointestinal tract and denaturation e.g. during thermal processing of food. Bet v 1-related allergens like Mal d 1 or Pru a 1 are unstable. Therefore, allergic reactions to raw apples and cherries in birch pollen-allergic subjects remain localised to the oral mucosa, while cooked apples or cherries do not elicit allergic reactions at all in the majority of patients. The allergenicity of profilin-related allergens and of allergenic cross-reactive carbohydrate determinants (CCDs) in celery and hazelnut might be derived from their relative stability against digestion or cooking.

So far four major allergens have been reported in apple and included in the official allergen list of the WHO allergen nomenclature subcommittee (see Table 1) (http://www.allergen.org). They include Mal d 1 (PRP-10), homologous to Bet v 1 with a molecular mass of 17.5 kD, Mal d 2 (PRP-5), a thaumatin-like protein (TLP), with a molecular mass of 23 kD, Mal d 3 (PRP-14) a lipid transfer protein (LTP) with a molecular mass of 9 kD and Mal d 4 (profilin), homologous to Bet v 2 with a molecular mass of 14 kD.

Mal d 1 is the major allergen in pollen-associated fruit allergies, belonging to the pathogenesis-induced proteins PRP-10. Hybridisation studies uncovered Bet v 1-homologue genes in apples, pea, parsley, bean and potato [11] with sequence homologies between 50–70%. Recovering the three-dimensional structure of Bet v 1 [14,19,45] and Pru a 1, the major cherry allergen, confirmed significant structural homologies (see Fig. 1) [35]. Unlike other PRP-10, whose expression is strongly induced under stress conditions or pathogen attack, the fruit allergens appear to be constitutively expressed. Particularly interesting is the double band of strawberry and blackberry extract reacting with a pAb raised against Mal d 1 (see Fig. 2A and 2C).

Mal d 2 belongs to the thaumatin-like proteins (TLPs), reported as a novel family of fruit allergens. TLPs are generally resistant to pH- or heat induced denaturation due to the presence of 8 disulfide bridges [6]. Up to now several TLPs from fruits have been reported with ability to bind IgE antibodies of allergic patients e.g. apple, kiwi, grape and cherry with about 50% sequence identity [31]. TLPs with

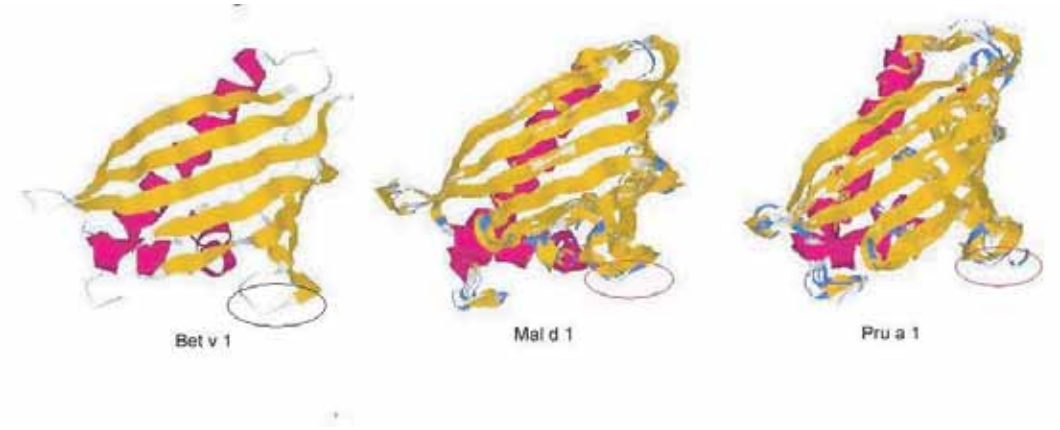

Fig. 1. The 3D structure models are based on the crystal structure of Bet v 1. The models of fruit allergens using cDNA sequences of: Mal d 1: AF020542.1; Pru av 1: AY540508 were generated using Swiss model server (http://swissmodel.expasy.org//SWISS-MODEL.html) and visualized in the Protein Explorer program (http://molvis.sdsc.edu/protexpl/frntdoor.htm). Ovals indicate a conserved glycine-rich loop crucial for Bet v 1 epitope-binding [32].

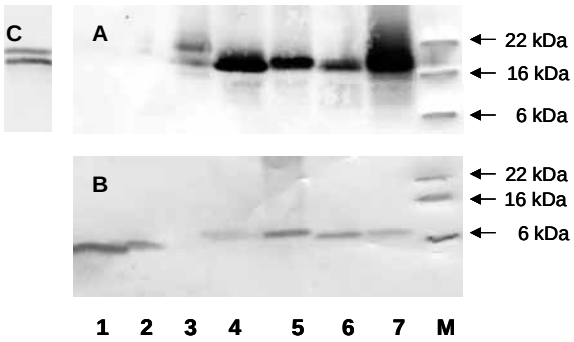

Fig. 2. Mal d 1 (A) and Mal d 3 (B) homologous proteins detected in different fruits of *Rosaceae* and *Ericaceae* using polyclonal antibodies raised in rabbit against purified Pru p 3 of peach (kindly provided by R. van Ree) and apple Mal d 1. The lanes contained: 1) blueberry 2) raspberry 3) strawberry 4) cherry 5) peach 6) apricot 7) apple M) molecular weight marker. In almost all fruit extracts at least one of the two main allergen homologues was found. Strawberry (lane 3A) and blackberry (C) showed a comparable double band reacting with the polyclonal anti-Mal d 1 antiserum. The absence of Mal d 1 homologous protein bands in raspberry and blueberry or Mal d 3 in strawberry might be due to the low sensitivity of alkaline phosphatase western blotting or reduced cross-reactivity.

pollen-origin have been also described in pollen of Cupressaceae and reported as an important allergen source in Spain, United States and Northern Mexico [26].

Mal d 3, a non-specific lipid-transfer protein (LTP), has been identified as the major allergen in Rosaceae fruit as peach and apple, particularly in the birch-free Mediterranean area [41]. This protein and its homologous in other fruits and vegetables have been claimed as elicitors of the true food allergy [3]. LTPs can resist denaturation by heat processing and degradation by digestion long enough to induce both sensitisation and systemic symptoms through the gut mucosa [47]. Currently, an increasing number of LTP-homologous proteins with more than 80% similarity, have been identified in several plant foods, particularly in stone fruits, e.g. Pru p 3 (peach), Pru a 3 (apricot), Pru av 3 (cherry) (see Fig. 2B). However, blueberry and raspberry also contain an LTP homologous protein. Besides, LTPs could be identified in grape [39], chestnut [8], hazelnut [38], maize [37], barley [20], asparagus [9], carrot [2] and

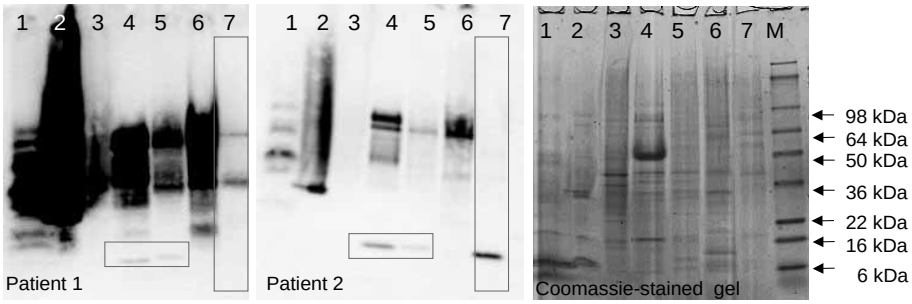

Fig. 3. Cross reactivity to different fruits was characterized by IgE Western blot for 1) blueberry 2) raspberry 3) strawberry 4) cherry 5) peach 6) apricot 7) apple M) molecular weight marker using patient sera. Two patients with birch-apple-syndrome and a well-studied allergy history demonstrated a very different sensitisation pattern to fruit extracts. While patient 2 shows a clear reaction against apple Mal d 1 and homologous proteins (17.5 kDa) in peach and cherry, patient 1 is reactive to proteins with a molecular weight of $>$36 kDa in apple but not with Mal d 1. However patient 1 serum recognizes the Mal d 1 homologous proteins in cherry and peach. Very strong reactivity could be detected for high molecular weight proteins in blueberry, raspberry, cherry, apricot, and peach, which reflects indeed their clinical case history.

lettuce [42].

Mal d 4 belongs to the profilins, a group of plant allergens representing minor allergens in pollen allergy and playing a role in pollen-associated food allergy [48]. Profilins, present in all eukaryonts, regulate the polymerization of cytoskeletal actin and mediate signal transduction in the cell [44]. Bet v 2 (birch pollen profilin) sensitizes approximately 20% of the pollen-allergic patients. Human IgE recognizing pollen profilins are highly cross-reactive to profilins in cherry (Pru av 4), pear (Pyr c 4), apple (Mal d 4), celery (Api g 4), soybean (Gly m 3), peanut (Ara h 5), hazelnut (Cor a 2), tomato (Lyc e 1) and bell pepper (Cap a 1) [50].

Fruits like blueberry, raspberry and strawberry may be nutritionally underestimated, but scientific research shows that they may have huge impact on human health due to their high content of antioxidants and unknown biofactors, which play a role in ageing, cancer and infection prevention. Reports on allergenicity of small fruits such as strawberry, raspberry, blackberry and blueberry are still rare. Whether this is related to a general low allergenicity, the small amounts consumed or the restricted time frame of consumption still remains to be answered. As a matter of fact, low exposure to certain allergens might be the reason for the limited complaints recorded so far. However, with the ongoing encouragement for the consumption of small fruits, this situation might change. About 30% of patients reported hypersensitivity or adverse effects after consumption of strawberries, although many of these reactions might be related to food intolerance rather than food allergy [13]. Small fruits are not only consumed fresh but are also eaten as common ingredients in different food products as main or additional component e.g. jam, ice cream, cornflakes. Strawberries are under investigation and strong evidence for the existing Bet v 6, Bet v 1 and Mal d 3 homologous proteins have been confirmed by existence of IgE-binding assays and skin prick test [26]. Allergic reactions were also reported in contact to raspberry proteins (see Fig. 3). Inhalation of frozen raspberry powder was reported to cause occupational asthma [7], however no raspberry allergens have been identified so far.

People vary in their reactivity to food and show a different pattern of reactivity depending on their individual characteristics. Persons following specific diets tend to show a different pattern of allergic response. Clinical reactivity depends on a variety of factors including frequency of exposure to foodstuffs [25]. The interpretation of the phenomena depends strongly on the availability of patient independent detection tools and the sensitivity of the method. In many cases it is still unclear how results between *in vitro* assays, skin prick tests and oral challenge are correlated and what is the clinical

importance of *in vitro* results. Will the patient, whose serum recognises a certain protein epitope develop an allergy in the near future? We actually do not know it, but we are developing tests to answer this in the near future.

From apple data we know that we have a high variability in Mal d 1-content among different cultivar, years and production systems. Analogous studies on small fruit allergens content are still under study. In Southern blot analyses DNA fragments homologues to Mal d 1 and Mal d 3 could be detected in genomic DNA from apple [29], strawberry, raspberry, blueberry and cranberry, and the respective genes are being cloned (unpublished data). The putative allergenicity of the recombinant gene products remains to be defined.

Recent studies have demonstrated that not only epitopes on peptides, but also on carbohydrates, so called cross-reactive carbohydrate determinants (CCD), can act as cross-reacting elements [40]. It has been shown that α1–3 linked fucose and β1–2 linked xylose present in a common cross-reactive antigenic determinants [15,40]. Due to their small size, when compared to peptide epitopes, carbohydrate residues appear to stabilize the binding to IgE [27,30]. IgE antibodies specific for certain N-glycans of plant glycoproteins, which are highly cross-reactive with almost all foods of plant origin, may occur in as much as 10–20% of pollen-allergic individuals [18]. The clinical relevance of CCDs is very controversial [46]. Allergic reactions to celery and kaki fruit in some individuals have been reported to be caused by specific IgE reactivity with CCDs without additional recognition of any other proteins [1,28].

Since many identified allergens belong to plant pathogen-related proteins, their presence is expected in almost all type of plant-derived food. The question about the clinical relevance of *in vitro* assays still remains to be answered, and a challenging field of investigations. The minimization of fruit-pollen-syndrome demands a change of life style, or even profession, adhering to an elimination diet or avoidance, long-term symptomatic treatment and immunotherapy [7]. Therefore apart from an effective therapy, research on mechanisms of allergies and methods for avoiding exposure to allergens is prerequisite for improved life quality of affected individuals.

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ORIGINAL PAPER

Direct evidence for the presence of allergens in *Rosaceae* fruit tree pollen

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Abstract The allergen content of insect-carried fruit tree pollen is poorly investigated. A recent report on orange allergy indicates a possible co-sensitisation to fruits by digestion and inhalation, suggesting an alternative mechanism for allergy development. Allergy to *Rosaceae* fruits like apple, pear, peach and cherry has been described as cross-reactivity to the birch pollen allergen Bet v 1. The expression of two major apple allergens Mal d 1 and Mal d 3 and their homologues in *Rosaceae* pollen was investigated. Transcript expression of five Mal d 1 isoforms and of Mal d 3 was examined by RT-PCR. A Mal d 1 specific ELISA was used to quantify the content of Mal d 1 homologues in *Pomoideae* pollen. Polyclonal antibodies against Mal d 1 and Mal d 3 were used for allergen detection in pollen extracts in Western blots. The cross-reactivity of pollen allergens with patient sera was confirmed by IgE-Western blotting. Pollen counts were carried out by using Hirst-type volumetric samplers, revealing an intermittent *Rosaceae* fruit tree pollen load, without remarkable peaks across large parts of Europe. The expression of Mal d 1 was confirmed in pollen of all tested *Rosaceae* fruit tree species by ELISA, Western blot and RT-PCR. IgE-Western blots showed a strong cross-reactivity, particularly with Mal d 1 and Mal d 3 homologues in different *Rosaceae* pollen. Although the pollen load of *Rosaceae* is rather low as a rule, there is certified evidence for temporary peaks, indicating that allergen exposure for sensitized individuals is likely.

Keywords *Rosaceae* · ELISA · Western blot · SDS-PAGE · Fruit tree pollen · Bet v 1 · Mal d 1 · Mal d 3 · IgE reactivity · RT-PCR (Reverse transcription-polymerase chain reaction)

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1 Introduction

The observation of associative allergic reaction to pollen of birch tree and immediate type of food allergy to fruits confirms the general hypothesis of cross-sensitisation for most allergic patients (Caballero & Martin-Esteban, 1998; Dreborg, 1988). In the Northern hemisphere, up to 70% of birch pollen allergic individuals develop fruit

allergies to apple or other members of *Rosaceae* (Vieths, Scheurer, & Ballmer-Weber, 2002). Among pollen-related food allergies, the birch and grass pollen-associated ones are very important and have been studied extensively. It was claimed, that pollen-related food allergies in adults are more frequent than real food allergies (Eriksson, Formgren, & Svenonius, 1982; Etesanifar & Wütrich, 1998). Indeed, the most recent studies on the prevalence of adverse reactions to food in Germany confirmed the previous data and showed the highest rate of affected patients at the age of 40 years (Zuberbier et al., 2004).

Allergy to *Rosaceae* fruits and birch pollen is linked to cross-reactivity of IgE antibodies to Bet v 1, the major birch pollen allergen. In regions without birch vegetation, like Southern Europe and Japan, allergy to fruits is linked to grass, *Ambrosia* and *Artemisia* pollen or can be induced by pollen from other species related to birch like *Corylus*, *Alnus* and *Carpinus* (Fernandez-Rivas, van Ree, & Cuevas, 1997; Mari, Wallnert, & Ferreira, 2003; Wopfner et al., 2005; Yamagiwa et al., 2002).

The currently favoured assumption is, that apple sensitivity develops on the basis of primary sensitisation to birch pollen. Natural exposure to apple pollen has not yet been considered as inducing sensitisation. In fact, in contrast to the anemophilous birch pollen, apple pollen is insect-carried and thus unlikely to cause pollinosis (Vaugham, 1954). Since apple allergens have been detected in seeds, leaves, pulp and peels of apples (Marzban et al., 2005), the presence of cross-reacting allergens in pollen of apple trees seems plausible. First studies in fact revealed similar IgE cross-reactivity among the pollen proteins of birch and apple trees (Berrens, van Dijk, Houben, Hagemans, & Koers, 1990). Recently significant IgE-mediated allergy to papaya and orange tree pollen was demonstrated as a consequence of occupational fruit pollen exposure (Blanco et al., 1998; Iraneta et al., 2005).

In the current study we focused on the presence of different allergens in *Rosaceae* pollen, in order to get a first insight into expression patterns of major fruit allergens Mal d 1 and Mal d 3. Different methods were employed for specific detection of allergen proteins, including PCR, Mal d 1 ELISA, IgG-Western blotting and patient IgE-Western blotting. These investigations demonstrate an alternative source of allergen and suggest a potential new route of sensitisation, particularly in the light of pollen data collected and compared across Europe.

2 Material and methods

2.1 Pollen material

Flowers were harvested from the orchard of the Institute of Pomology, BOKU (apple, pear, peach, apricot and cherry) and fence trees (birch, alder and hazel) in Vienna, during flowering time from January till May of 2005, according to the species. Pollen material was collected by manual separation. Samples were stored at –80°C prior to RNA and protein extraction.

2.2 Pollen concentration monitoring

Standardised volumetric pollen monitoring (Hirst, 1952) was performed by the European pollen information service. About 600 sites reported throughout a European network at weekly intervals. Due to the high morphologic similarity of different *Rosaceae* pollen, only the family classification was used and the patient-relevant index (annual total/number of days) calculated.

2.3 Protein extraction

About 100 mg pollen material was extracted in 1.5 ml distilled water at 4°C on ice for 60 min. The vials were vortexed every 10 min during extraction. Finally solutions were centrifuged for 10 min at 4°C and by 13.000 rpm (HERMLE Z230MR, Germany). Supernatants were stored at –20°C.

2.4 Total protein determination and Mal d 1 ELISA

Concentration of total protein was determined with the BCA protein assay kit (PIERCE, USA) according to manufacturer's instructions. All extracts were buffer-exchanged to PBS, using PD-10 columns (Amersham Biotech, USA)

before protein determination due to high contents of reducing agents expected in pollen tissue. ELISA was performed as described before (Marzban et al., 2005). Purified native Mal d 1 was used as standard. All determinations were run in duplicates and Mal d 1 amounts were converted to microgram Mal d 1 per gram fresh weight of pollen.

2.5 RNA isolation, reverse transcription, and PCR detection of pollen allergens

The Mal d 1 gene family in apple consists of 18 members (Gao et al. 2005). Expression of five isoforms was confirmed in ripe fruits of Royal Gala: Mal d 1.01 (A), Mal d 1.02 (B) (the most abundant allergen), Mal d 1.03E (E), Mal d 1.06A and Mal d 1.06B (Beuning et al., 2004). Mal d 1.06 is considered a genetic marker for molecular estimation of allergenicity in apple cultivars (Gao et al., 2005). Therefore the expression of fruit-expressed and marker-related Mal d 1 transcripts was analysed in apple pollen.

Total pollen RNA was isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. About 2.5 μg of total RNA was treated with RNase free DNAse I (Roche) and subjected for reverse transcription using SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen). The obtained cDNA was diluted 1:1 and used as a template for PCR reactions.

PCR reactions were performed using the HotStarTaq DNA Polymerase (Qiagen) in a TRIO thermocycler (Biometra) according to the HotStarTaq DNA Polymerase handbook, and contained 2–4 μl diluted cDNA in a final volume of 25 μl. The sequences of primers used for amplification of Mal d 1 and Mal d 3 fragments are listed in Table 1. The degenerated primers MD1F-EcoRI and MD1R-BamHI (Pühringer, Zinöcker, Marzban, Katinger, & Laimer, 2003) recognize highly similar 5′ and 3′ regions of several Mal d 1 isoforms. Newly designed primers Mal d 1.06F/R recognize specifically the members of the gene cluster Mal d 1.06: A, B and C (Gao et al., 2005). Amplification conditions for these primers were: 95°C-15′ hot start (95°C-1′/65°C-1′/72°C-1′) 35 cycles, 72°C-5′ final extension. PCR conditions for Mal d 3 (LTP) primers were: 95°C-15′ hot start (94°C-1′/56°C-1′/72°C-1′) 35 cycles, 72°C-5′ final extension. About 7 μl of the PCR products were separated on a 2% agarose gel containing ethidium bromide and analysed.

2.6 IgG- and IgE-immunoblotting

Western blots were performed in a Novex-Gel apparatus using precasted 4–20% Tris/Glycin acrylamide gels (Invitrogen, Netherlands). Samples were denatured at 80°C for 10 min prior to analysis. After separation, proteins were transferred by electroblotting to a polyvinyldifluoride

Table 1 Primers sequences and annealing temperatures (*T*) used for amplification of pollen allergens transcripts

Transcript	*T* (°C)	Primer sequence
Mal d 1[a]	54	MD1F-EcoRI: NNNGAATTCATGGGTGTSTWCACATWYGAA MD1R-BamHI: NNNGGATCCTTAGTTGTAGGGRTCCKGGTG
Mal d 1.01[a]	56	Md1**A**-F: AAGCTGAAATCCTTGAAGGAA Md1**A**-R: GTGCTCTTCCTTGATTTCAATG
Ma d 1.02[a]	56	Md1**B**-F: ACACCTCTGAGATTCCACCAC Md1**B**-R:CAACTTGGTYTCGTAAGAGAC
Mal d 1.03[a]	56	Md1**E**-F: ACCTCCGTCATCCCCCCTG Md1**E**-R: TCTTCTCAATTGTCTCAGAGAT
Mal d 1.04[a]	56	Md1**D**-F: CATCGAAGGCGATGGAGGT Md1**D**-R: CCTTAGCAYGGTAGTGGCTA
Mal d 1.06[b]	65	Mal d **1.06**-F: TGTCCTCACATACGAAACCGAA Mal d **1.06**-R: TGTGCTTCACATAGCTGTATTCACTC
Mal d 3[a]	56	Mal d 3-F: ATGGCTWGCTCTGCARTGAYYAAG Mal d 3-R: TYACTTCACGGTGGCGCAGTTG

Primer names indicate the differences in the nomenclature of Mal d 1 isoforms according to Pühringer et al. (2003)[a] and Gao et al. (2005)[b]

(PVDF) membrane (Millipore, USA). First polyclonal antibodies against Mal d 1 (Marzban et al., 2005) or Mal d 3 (Herndl et al., submitted) were used, followed by anti-IgG rabbit antibodies labelled with alkaline phosphatase (Sigma, USA). Pre-stained Precision Plus Protein Standards (Biorad, USA) was applied as molecular weight marker.

IgE-immunoblotting was performed with sera of Austrian patients allergic to apple and birch using non-radioactive detection (A. Herndl et al., submitted). In brief, the separated pollen proteins were transferred to a nitrocellulose membrane (BIO-RAD, USA) and blocked in a PBS buffer (0.1% Tween/3% BSA), pH 7.4, for 2 h at 37°C. The membranes were incubated for at least 16 h at 4°C with 400 µl patient serum in 20 ml PBS buffer (0.1% Tween/1% BSA). After three times 5 min washing with PBS buffer (0.1% Tween), membranes were incubated with horse raddish peroxidase-labelled mouse anti human-IgE antibody (ZYMED, USA) (1:2000 diluted in PBS buffer 0.1% Tween/1% BSA) for 4 h at RT. After three times 5 min washing, the protein bands were detected with ECL Plus Western Blotting Detection Reagents (Amersham Bioscience, US) according to the manufacturer's protocol. Native Mal d 1 and Mal 3 were used as positive controls. Mal d 1 was purified using ion-exchange and size-exclusion chromatography. As ion-exchanger a Mono-Q column and as size-exclusion a Sephadex Column (Amersham, USA) were used. Purified Mal d 3 was obtained by a one step separation using a Mono-S column (A. Herndl et al., submitted).

3 Results

3.1 Tree pollen burden in European sites

During a period of three decades, pollen loads have been reported and analysed (Fig. 1). Analyses showed that the *Rosaceae* pollen concentrations reach noteworthy amounts and there exists a continuous level of *Rosaceae* pollen content across Europe, without drastic maximum peaks compared for example to birch pollen. Using *Rosaceae* pollen as common term, one should keep in mind the geographic distribution pattern of the different botanical species (Tutin et al., 1978). In Northern Europe *Sorbus* sp. will prevail, while in Central Europe fruit tree species like *Malus*, *Pyrus* and *Prunus* and in Southern Europe *Prunus* ssp. will prevail in the sample.

3.2 Determination of total soluble protein and Mal d 1 content of apple pollen

Due to the intensive colour and high content of reducing substances, pollen extracts were buffer exchanged prior to protein determination: the ratio of Mal d 1 content to total pollen protein was determined by quantitative ELISA in apple and pear pollen (Fig. 2), using the monoclonal anti-Mal d 1 antibody 4C3H10, which apparently recognizes a distinct epitope conserved among the *Pomoideae*. The content of Mal d 1 in extracts of fruit pollen (245–775 µg/g fresh weight) is considerably higher than of fruit pulp (from 0.8 to 33 µg/g fresh weight) (Marzban et al., 2005). This may be explained by the high protein content of fruit pollen, which reaches concentrations up to 297 mg/g fresh weight (30%), while in apple pulp protein ratios from 0.3 to 0.5% of fresh weight are observed (Fig. 2).

3.3 Expression of Mal d 1 isoforms and Mal d 3 in apple pollen

RT-PCR demonstrated the presence of the Mal d 1 and Mal d 3 transcripts in pollen material of two apple cultivars 'Lavantaler' and 'Rheinischer Winterrambour' (Fig. 3A, G). PCR data indicated transcription of at least five different Mal d 1 isoforms and of Mal d 3 in apple pollen (Fig. 3B–F) and confirmed the results obtained at the protein level.

3.4 Immuno-detection of fruit pollen allergens using IgG and IgE antibodies

Pollen extracts were analysed by SDS-PAGE and Western blot. Using polyclonal rabbit antibodies against Mal d 1, we could detect for the first time Mal d 1 and homologue proteins in all pollen extracts (Fig. 4a). This means that Bet v 1 homologue proteins are expressed also in fruit tree

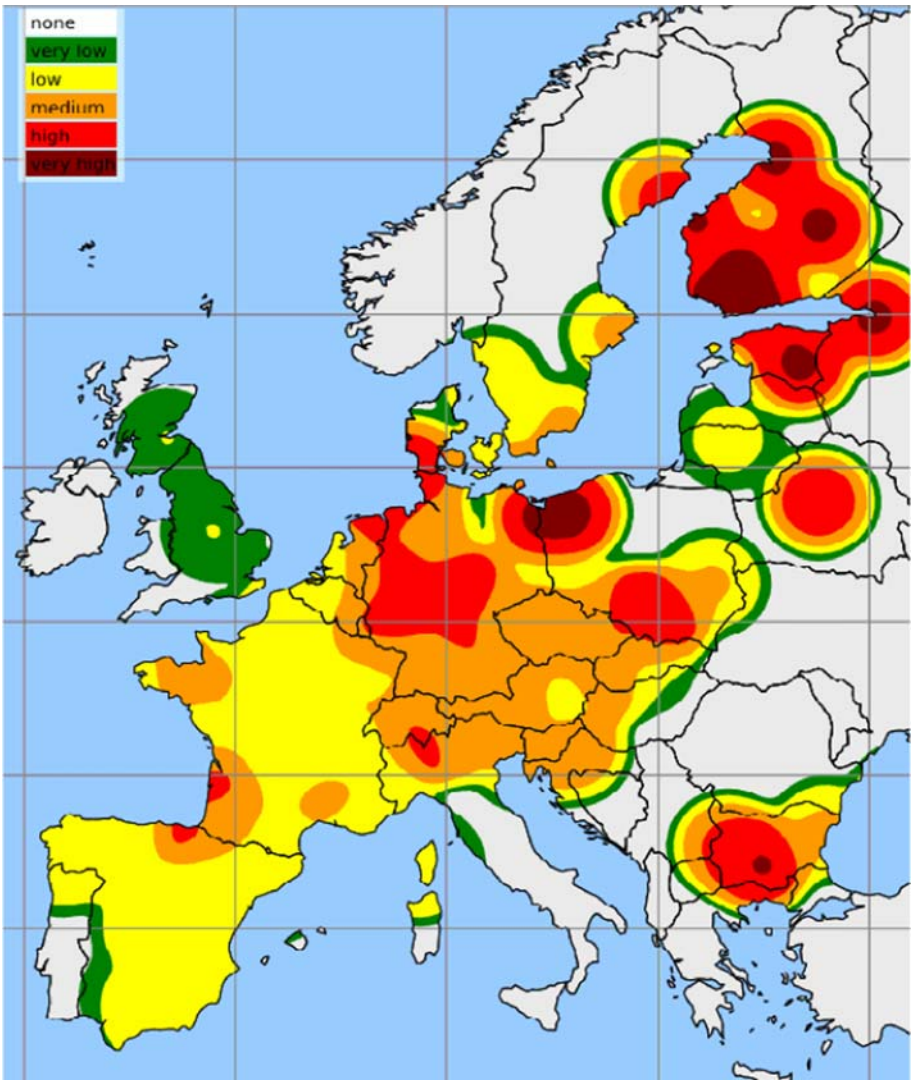

Fig. 1 *Rosaceae*-pollen index (annual total pollen counts divided by number of days with incidence)—none: 0–3; very low: 3; low: 13; medium: 29; high: 51; very high: 80. Data collected over large areas of Europe over a period of up to 29 years indicate considerable differences in the exposure to the relevant pollen according to the geographic distribution of *Rosaceae* species. The high incidence in Northern Europe might be due to *Sorbus* sp., while in Central and Southern Europe mainly *Malus* and *Prunus* sp. are relevant

pollen, and not exclusively in pollen from birch and other *Fagales*. Likewise, Mal d 3 and homologous LTPs (Fig. 4b) were found in all pollen extracts of pome and stone fruits, while no signal was detected in the birch pollen extract.

Patient sera with well-documented clinical history in relation to birch pollen and apple fruit showed a positive reaction to Bet v 1 (Fig. 5). The patient sera with a defined history of severe systemic reactions after consumption of pome and stone fruits, reacted with Mal d 1 and Mal d 3 homologues in different fruit pollen. The sera of three patients representing a typical Bet v 1 sensitisation pattern, recognized Mal d 1 homologues in pollen of apple, pear, apricot, peach, cherry and also with purified Mal d 1 as positive control. On the other hand, sera of two LTP-sensitized patients reacted with Mal d 1 homologues in apricot, peach and cherry (stone fruits), and with Mal d 3 homologues in apple, pear and peach.

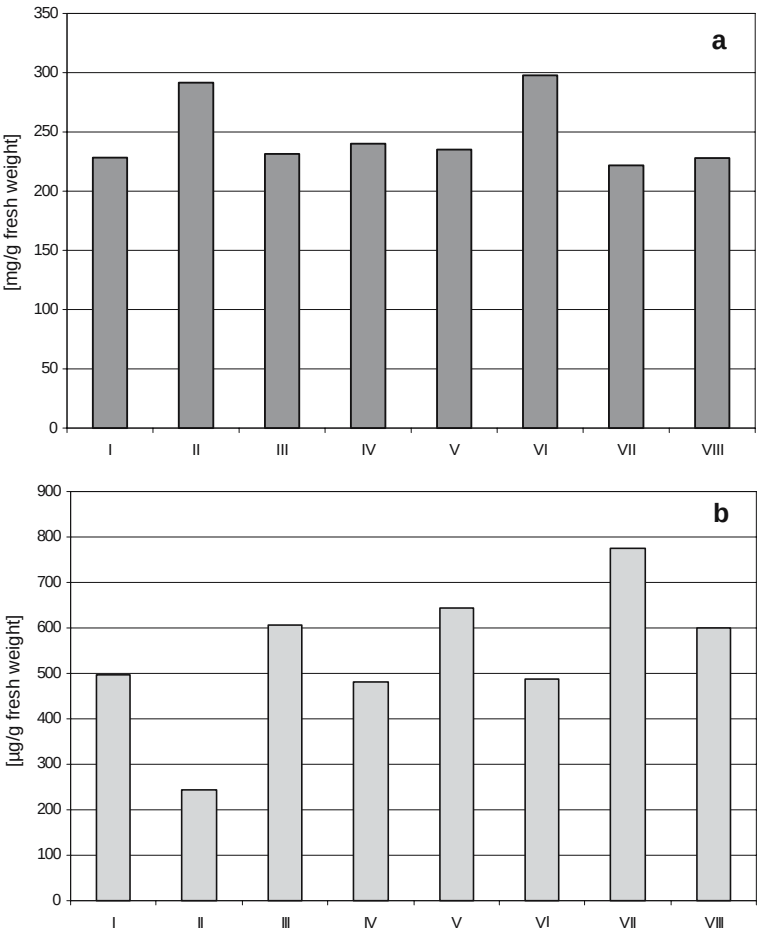

Fig. 2 The content of total soluble protein (**a**) and Mal d 1 homologue allergens (**b**) in different apple and pear pollen. I: *Malus domestica* cultivar Lavantaler; II: *M. domestica* cultivar Virginia Crab; III: *M. domestica* cultivar Golden Delicious; IV: *M. domestica* cultivar Gravensteiner; V: *M. pumila* (wild apple); VI: *M. domestica* cultivar Rheinischer Winterrambour; VII: *Pyrus communis* cultivar Delbard 51; VIII: *P. communis* cultivar Delbard 333

4 Discussion

The presence of allergens in *Rosaceae* pollen was so far not well documented, with the exception of one report on a Bet v 1 homologous protein in apple tree pollen (Berrens et al., 1990). Pollen from *Fagales* has been shown to be of high clinical impact due to airborne pollination. In contrast, it was assumed, that inhalation of insect-borne fruit tree pollen is only limited to people having a very close contact to fruit trees in the context of occupational exposure (Iraneta et al., 2005). Monitoring of *Rosaceae* pollen across Europe during the past 30 years, showed a moderate, but constant exposure. However, their concentration is low, when compared with birch pollen data.

The current study confirmed by three independent methods (ELISA, Western blot and RT-PCR), the expression of Mal d 1, Mal d 3 and their homologues in *Rosaceae* pollen. IgE-Western blots revealed a more complex pattern. Apple/birch allergic patient sera reacted with proteins in a range of 17–70 kDa. The fact, that also Mal d 3 homologues are found in *Rosaceae* pollen deserves special attention. Exposure to this protein source might be relevant for Northern and Central European countries, where allergy to fruit LTP is rare, while it might be less relevant in Southern Europe, where allergy to *Rosaceae* fruits is mainly caused by consumption of fruit LTPs. In fact the phenomenon, that allergy development of patients from different countries to a given fruit is driven by different allergens, has already been described in the case of cherry (Scheuer et al., 2001). According to previous reports, IgE-binding activities in higher molecular weight ranges (>30 kDa) are due to cross-reactive carbohydrate determinants (CCDs)

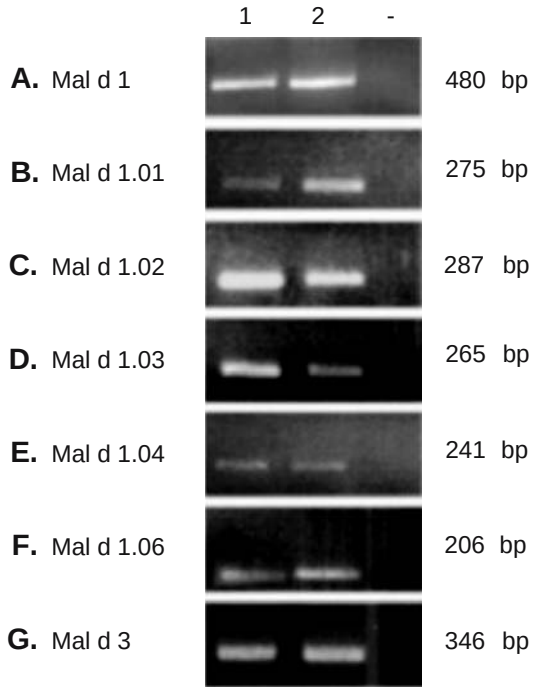

Fig. 3 Detection of allergens transcripts: Mal d 1 (**a**–**f**) and Mal d 3 (**G**) in apple pollen. Amplified transcripts are representative for all described subfamilies of Mal d 1 expressed in apple pollen: Mal d 1.01 and Mal d 1.02—subfamily I; Mal d 1.03—subfamily IV; Mal d 1.04—subfamily II; Mal d 1.06—subfamily III. PCR products amplified from cDNA were separated on a 2% agarose gel. The length of the PCR products is defined in base pairs (bp). Panels: (**a**) total Mal d 1 transcripts; (**b**) Mal d 1.01; (**c**) Mal d 1.02; (**d**) Mal d 1.03; (**e**) Mal d 1.04; (**f**) Mal d 1.06; (**g**) Mal d 3. Lane 1: pollen cDNA from cultivar Lavantaler; lane 2: pollen cDNA from cultivar Rheinischer Winterrambour; lane –: negative control (Aqua dest)

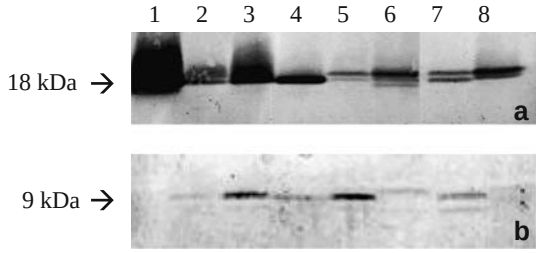

Fig. 4 Detection of the Mal d 1 (**a**) and Mal d 3 (**b**) in fruit pollen extracts by Western blot using polyclonal antibodies—1: *Betula* sp. (birch); 2: *Malus domestica* (cultivar Rheinischer Winterrambour); 3: *M. domestica* (cultivar Lavantaler); 4: *Pyrus communis* (pear); 5: *Prunus armeniaca* (apricot); 6: *P. persica* (peach); 7: *P. cerasifera* (sour cherry); 8: *P. avium* (cherry)

(Fötisch & Vieths, 2001; Petersen, Vieths, Aulepp, Schlaak, & Becker, 1996; Vieths et al., 2002), whose allergenic potential are still under study.

Data obtained indicate the presence of Mal d 1, Mal d 3 and their homologues in *Rosaceae* pollen. Mal d 1 was previously shown to be expressed in young and ripe apple fruits, old leaves and young leaves under stress conditions (Atkinson, Perry, Matsui, Ross, & MacRae, 1996; Beuning et al., 2004; Pühringer et al., 2000). Due to the limited size of samples and the low amount of total RNA extracted, we identified allergen transcripts using RT-PCR. The obtained results demonstrate that, as in the apple fruit, at least five members of Mal d 1 gene family are expressed in pollen, including the most abundant fruit-allergen Mal d 1.02 and Mal d 1.04, which was not found in the fruit. Since the expression of this protein is regulated by pathogen and stress, the presence of Mal d 3, a non-specific lipid transfer protein in apple pollen was astonishing. Further, the expression of nsLTPs could be demonstrated also in pear, peach and cherry pollen. nsLTPs have been identified as major allergens in apple and peach in patients not allergic to birch pollen (Pastorello et al., 1999; Sanchez-Monge, Lombardero, Garcia-Selles, Barber, & Salcedo, 1999) and cross-reactivity between nsLTPs in *Rosaceae* fruits, nuts, chestnut and maize has been observed (Asero et al., 2000). LTPs have also been identified in pollen of some important hayfever plants, such as mugwort (Art v 3), pellitory (Par j 2) and sycamore (Pla a 3). However, no or only limited cross-reactivity between these proteins and Mal d 3 and Pru p 3 has been found, leading to the view, that food LTPs primarily act as ''true'' food allergens (Diaz-Perales et al., 2000; Pastorello et al. 2002). The successful identification of Mal d 3 homologues in *Rosaceae* pollen in this study, along with the finding of a constant background fruit tree pollen load, might provide a new explanation for the origin of allergy to fruit LTPs.

Data presented in this study provide a new aspect of the allergen profile in *Rosaceae*, particularly for apple trees, which are the worldwide most produced and consumed fruits. A recent work on the phylogenetic relationship suggested that the

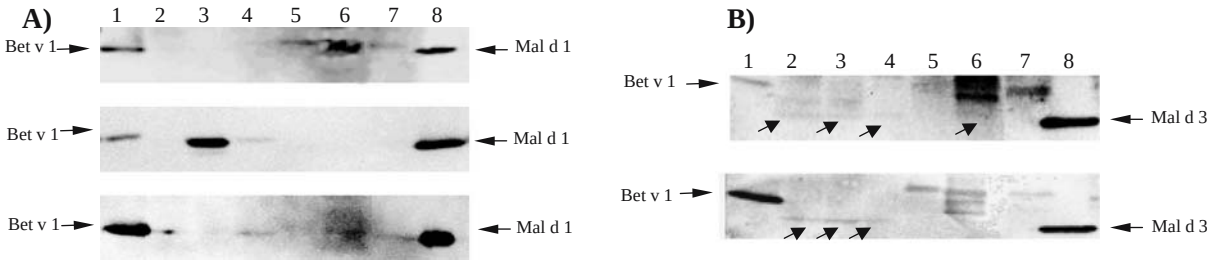

Fig. 5 Detection of Mal d 1 (**a**) and Mal d 3 homologues (**b**) in *Rosaceae* pollen was performed using IgE-immunoblot with sera of five birch/apple allergic patients. As positive controls purified native Mal d 1 and Mal d 3 (17.5 and 9 kDa) were applied to the gels. 1: *Betula* sp. (birch); 2: *Malus domestica* (Rheinischer Winterrambour); 3: *M. pumila* (wild apple); 4: *Pyrus communis* (pear); 5: *Prunus armeniaca* (apricot); 6: *P. persica* (peach); 7: *P. cerasifera* (sour herry); 8: positive controls. IgE-reactivity pattern to Mal d 1 homologues of three patients indicate individual differences. However, the IgE reactivity of two LTP-sensitized patients clearly appears in apple and pear pollen (the positive LTP bands are highlighted by arrows, →)

cross-allergenicity can reflect taxonomy in a majority of cases (Weber, 2003). Our results support, that most related fruit trees, e.g. pome fruits and stone fruits, express similar antigens with allergenic potential in their pollen. It is still to be clarified, if a baseline concentration of *Rosaceae* pollen in the atmosphere can be identified as a source for cross-sensitisation. Future studies are needed to clarify the cross-reactivity of pollen LTPs and their clinical significance for individuals with prolonged contact to higher concentrations of fruit pollen through their profession (farmers, orchard growers, greenhouse workers).

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Research Article

Mapping of *Malus domestica* allergens by 2-D electrophoresis and IgE-reactivity

The importance of apple allergens has been repeatedly emphasized, and their presence has been confirmed both in pollen and in fruits. In the present study, a combination of proteomic tools have been used to build a complete allergen map of apple. The water-soluble fraction of an apple extract was precipitated using a phenol-based procedure and separated by 2-DE. Initially four previously classified allergens, Mal d 1, Mal d 2, Mal d 3 and Mal d 4, could be identified in Western blots with polyclonal rabbit antibodies directed to the four respective allergens, and subsequently matched to the bands recognized by several patient sera. Further, all four known apple allergens were localized on a 2-DE map and they were matched with spots recognized by sera of patients with different allergic patterns. Moreover, a new, putative allergen could be identified using MS. We evaluated the influence of post-translational modifications and the immunoreactivity under different analytical conditions. The comparison of different visualization methods for 2-DE gels and blots revealed that even very low concentrations of the intact epitopes are detectable by IgEs of patients, and therefore might be sufficient to trigger allergic symptoms in sensitized individuals.

Keywords: Apple proteins, Mal d 1-4 / Apple allergen map / Fruit allergens / Pathogenesis-related proteins DOI 10.1002/elps.200600342

1 Introduction

Apple is the most widely grown and consumed fruit in the Northern hemisphere. Together with other plant-derived food, apple has been shown to have a positive effect on human health by reducing the risk of chronic diseases such as heart disease, strokes and some forms of cancer [1, 2]. However, the consumption of apples may also impose a serious allergenic risk for sensitised individuals.

So far, four main apple allergens have been reported named according to the rules of the WHO/IUIS Allergen Nomenclature Subcommittee (www.allergen.org). These are (i) Mal d 1 (pathogenesis-related protein; PRP-10), homologous to Bet v 1 with a molecular mass of 17.5 kDa, (ii) Mal d 2 (PRP-5), a thaumatin-like protein (TLP) with a molecular mass of 23 kDa, (iii) Mal d 3 (PRP-14) a lipid transfer protein (LTP) with a molecular mass of 9 kDa and (iv) Mal d 4 (profilin), homologous to Bet v 2 with a molecular mass of 14 kDa.

The importance of apple allergens, in particular Mal d 1, in Northern Europe, and Mal d 3 in Southern Europe, has been repeatedly emphasized [3–5]. Mal d 1, a homologous protein to the major birch pollen allergen Bet v 1, is known as the main cause for pollen-associated apple allergy. Symptoms related to Mal d 1 are generally mild and local, representative for a chemical labile protein [3, 5]. Mal d 3, on the other hand, is a highly stable protein due to eight cysteine residues forming four disulfide bridges [6]. Mal d 3 and its homologues in other fruits and vegetables have been repeatedly mentioned as main elicitors for true food allergy [7]. LTPs resist denaturation and degradation by thermal food processing and digestive proteases, long enough to induce sensitization in the gastrointestinal tract and to provoke systemic symptoms [8].

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Abbreviations: CCD, cross-reactive carbohydrate determinant; **LTP**, lipid transfer protein; **PRP**, pathogenesis related proteins; **TLP**, thaumatin-like protein

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Little is known about the way of sensitization to Mal d 2. However, due to the presence of 16 conserved cysteine residues, forming eight disulfide bridges, TLPs are expected to be resistant to pH- or heat-induced denaturation [9]. Mal d 4 is a minor allergen and seems to be pollinosis-related. Bet v 2, the birch pollen profilin, sensitizes approximately 20% of the pollen-allergic patients [10]. Profilins have recently been described as highly cross-reactive with other fruits and vegetables of the *Rosaceae, Vitaceae* and *Solanaceae*, as well as with pollen. Although profilins were shown to be strong sensitisers and highly crossreactive, frequently they do not provoke symptoms and are believed to be of limited clinical relevance [11, 12].

Apart from their importance as food allergens, there is growing evidence for the biological functions of these proteins in plant cells. Based on sequence and structural similarities, the major apple allergens can be classified as PRP, suggesting an important role in the plant defence system [13]. Unlike other PRP-10, Mal d 1 appears to be constitutively expressed in apple tissues, but differentially during different stages of development, suggesting an involvement in defined physiological changes [14, 15]. The interaction of low allergenic birch Bet v 1 isoforms with phytosteroids suggests a similar function for the homologous proteins in apples [16]. An antifungal activity of recombinant Mal d 2 has recently been demonstrated [17]. The expression of Mal d 2 has also been shown to be developmentally regulated [18]. Experimental data point to a role of LTPs in plant defence due to antifungal and antibacterial properties [19] and participation in the formation of extracellular lipophilic surface polymers. Profilins, ubiquitous proteins expressed in all eukaryotes regulate the polymerisation of cytoskeletal actin mediating signal transduction in the cells [20].

Although the protein sequence of Mal d 2 shows two potential *N*-glycosylation sites [17], this protein was not found to carry any post-translational modifications. Thus, any involvement of Mal d 2 in carbohydrate-mediated cross-reactivity is rather unlikely (D. Kolarich, personal communication). Glyco-structures containing core α1,3-linked fucose and β1,2-linked xylose are typical features of the *N*-glycans from many plant-derived allergens and are generally termed as cross-reactive carbohydrate determinants (CCDs) [21].

Despite the clinical importance of fruit allergens, proteomic studies are rare due to the complexity of the plant-specific tissue matrix and the low protein content [22–24]. For this reason limited data on allergen expression profiles in fruits exist. In the frame of the present study, apple proteins have been chosen as a model for analysis to accomplish two main aims: (i) to separate as many apple proteins as possible and (ii) to identify the allergenic proteins among them. The combination of 2-DE with IgE immunoblotting appeared to be the appropriate tool for the creation of a complete apple allergen map. Proteins most frequently binding to patient IgEs on 2-D-blots described the sensitisation pattern. Further alterations in IgE-binding affinity to separated proteins under different analytical conditions and changed post-translative modifications could be elaborated.

The results allowed to determine a finalized profile of IgE-reactive proteins in *Malus domestica*, and furthermore showed that the currently discussed groups of apple allergens seem to be far from being complete.

2 Materials and methods

2.1 Protein preparation from apple

Water soluble proteins were extracted from apples of the cultivar 'Vienna' according to a protocol described previously [15]. In order to isolate the proteins, 30% w/v sucrose was added to the apple extract to ensure a phase inversion before mixing it with an equal volume of cold phenol (Tris-buffered, pH 8, Sigma, MO, USA), and vortexing for at least 15 min at 4°C. After centrifugation for 15 min at 5500 rpm (Beckman Coulter, Allegra R21, S4180 rotor) and 4°C, the phenol phase was collected. Proteins solved phenol solution were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at −20°C and then pelleted by centrifugation at 5500 rpm and 4°C for 30 min. The precipitate was washed twice in 0.1 M ammonium acetate in methanol (−20°C) and twice in acetone (−20°C) and subsequently air-dried at −20°C. Finally, the protein precipitate was resolved in 8 M urea and the total protein content was determined by using the BCA Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.2 Protein separation by SDS-PAGE

Samples were diluted in a sample buffer containing 0.125 M Tris-HCl, pH 6.8, 20% v/v glycerol, 2% w/v SDS, 0.005% v/v bromophenol blue in the ratio 1:2 and denaturated for 1 h at room temperature. When reducing conditions were required, 0.05% DTT was added to the mixture. Samples were loaded on 4–20% gradient gels (4–20% Tris-glycine gel; Invitrogen, Carlsbad, USA). Electrophoresis was carried out on the Novex System (Invitrogen).

2.3 2-DE and electroblotting

Samples were diluted in rehydration buffer (6 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 2% IPG buffer and traces of bromophenol blue), and 70 μg of protein of the total protein extract or 25 μg of protein of purified Mal d 3 was loaded on 7 cm IPG strips, pH 3–10 (Amersham Biosciences, Piscataway, USA), using in-gel rehydration. Focusing was carried out in a Multiphor II System (Amersham Biosciences) at 20°C for a total of 7.2 kV·h with a voltage maximum at 3500 V. Prior to the second-dimensional separation, strips were equilibrated for 12 min in equilibration solution (50 mM Tris-HCl buffer, pH 6.5 containing 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 2% DTT and subsequently for 8 min in equilibration buffer containing 2.5% iodacetamide. When nonreducing conditions were required, all steps were performed as described above without DTT and iodacetamide. Alternatively, proteins were reduced but not alkylated. The strips were then transferred onto 4–20% gradient gels (4–20% Tris-glycine ZOOM gel, Invitrogen) and SDS-PAGE was performed on the Novex System (Invitrogen) at a constant voltage of 125 V. Gels were either stained with CBB or transferred onto suitable membranes. Electroblotting was performed on the XCell II Blot Module (Invitrogen) using a buffer containing 50 mM $Na_2B_4O_7 \cdot 10H_2O$, 0.1% w/v SDS, 20% v/v methanol.

2.4 Mal d 3 purification and characterisation

Apples of the cultivar 'Topaz' were washed with hot water, surface-sterilized with Danchlor and dried with a paper towel. Apples were peeled with a peeling machine (*ca.* 0.1 mm), weighed and immediately frozen in liquid nitrogen. The frozen material was mixed with the precooled extraction buffer (ratio 1:4) in a blender for 3 min, and stirred for 2 h in a cool room. To remove apple debris, the extract was filtered through two layers of Calbiochem Miracloth (Merck, Darmstadt, Germany) and centrifuged at 10 000 rpm (Beckman Coulter, Avanti™ J-25, JLA-10500) for 30 min at 4°C. The supernatant was lyophilized prior to separation to reduce the volume and stored at −20°C. The lyophilisate was resolved in 20 mM NaH_2PO_4, pH 7.5, in a ratio of 1:8 and centrifuged at 5500 rpm (Beckman Coulter, Allegra R21, S4180 rotor) for 30 min, at 4°C. The supernatant was filtered using a 0.2 μm filter (Minisart Flow High, Sartorius, Goettingen, Germany), and buffer-exchanged into 20 mM phosphate buffer (pH 7.5) using PD-10 column (Amersham Bioscience). Chromatographic purification was performed on an AEKTA 100 explorer chromatography system (GE Healthcare, Uppsala, Sweden). A Cation-exchange Mono S column (GE Healthcare) was used for purification. Column dimensions were 0.5 cm inner diameter and 5 cm height, resulting in a bed volume of ~1 mL. The column was equilibrated with 20 mM phosphate buffer, pH 7.5 for five column volumes at a flow rate of 0.5 mL/min. Four milliliters of buffer-exchanged apple extract was loaded in a single run at a flow rate of 0.5 mL/min. After removal of unbound proteins and other compounds with five column volumes of equilibration buffer, elution was carried out with a linear gradient from 0 to 1 M NaCl in equilibration buffer over ten column volumes. Fractions were collected and subjected to further analysis.

For protein identification and characterization, the protein was digested after SDS-PAGE separation with trypsin and subjected to LC-ESI-Q-TOF-MS/MS/MS as described by Kolarich *et al.* [25]. Prediction of potential signal sequences was performed using the SignalP 3.0 software available online on http://www.cbs.dtu.dk/service/SignalP [26].

For MS on the purified, intact protein, purified Mal d 3 was diluted to a concentration of approximately 10 pmol/μL in 50% ACN containing 0.1% formic acid. This sample solution was subjected to offline ESI-Q-TOF-MS. Spectra were deconvoluted using MaxEnt 1 function of MassLynx 4.0 SP4. All experiments were done on a Q-TOF Ultima Global (Waters Micromass, UK).

2.5 Production of antibodies and IgG Western blotting

Polyclonal antibodies against Mal d 1 were raised in rabbits as described by Marzban *et al.* [15]. Purified Mal d 3 was used for raising polyclonal antibodies in rabbits by immunization with 500 μg native protein and Freund's Adjuvans. Polyclonal antibodies for the detection of Mal d 2 and Mal d 4 were kindly provided by Drs. H. Breiteneder and I. Swoboda. Proteins were blotted on PVDF membranes (Millipore) and blocked overnight with 3% skim milk powder. Membranes were incubated with the primary Ab (see above) at a ratio 1:2000 v/v and the immunoreaction was detected using mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) in a ratio 1:2000 v/v, followed by bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma) in alkaline phosphate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris).

2.6 Patient sera and IgE Western blotting

Sera of mono- and polysensitized patients were used to identify distinct allergens and to confirm the apple allergen map. For this purpose allergic individuals were clinically characterized by anamnesis and IgE assays, and in

some cases additionally by skin prick tests (SPTs) (Table 1). The allergic reactivity of patient 2 to profilin was characterised by immunoblotting and inhibition assay.

Proteins were blotted onto NC membranes (BioRad, Hercules, USA) and blocked with 3% BSA for 2 h at 37°C. Membranes were first incubated overnight at 4°C with patient sera at a ratio of 1:50 v/v, and subsequently with goat anti-human IgE conjugated with horseradish peroxidase (ICL, Newberg, USA) at a ratio of 1:1000 v/v for 4 h at room temperature. Reacting spots were visualized by ECL (Amersham Biosciences) reaction and chemiluminescence using the Lumi-Imager TM apparatus (Boehringer Mannheim/Roche, Penzberg, Germany). To study the influence of carbohydrate residues on the immunoreaction, glyco-residues were treated prior to detection according to the method of Woodward *et al.* [27].

3 Results

3.1 Localization of the apple allergens on 2-DE maps

In order to identify the allergens previously described in apple, fruit protein extracts were enriched using a phenol-based procedure. This method has allowed the successful performance of 2-DE and Western blotting. After separation under reducing and nonreducing conditions, the 2-D gels were Coomassie-stained to visualize the apple proteins (Fig. 1). Despite being similar, the protein profiles under these two different electrophoretic condi-

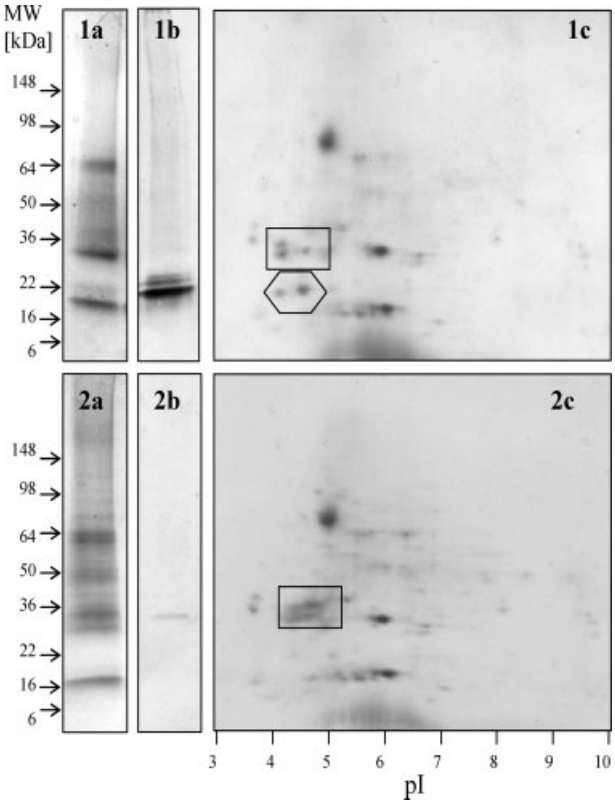

Figure 1. SDS-PAGE and 2-DE pattern of apple extracts under nonreducing (1) and reducing (2) conditions. The SDS-PAGE gel was stained with CBB (a) and blotted onto PVDF membranes and detected by pAbs to Mal d 2 (b). 2-DE gels were stained with CBB (c). The rectangle highlights the reduced form of Mal d 2 and the hexagon the nonreduced form.

Table 1. Clinical data of the allergic patients, whose sera have been used for 2-DE maps of apple allergens

	Anamnesis	IgE reactivity	SPT
P1	Apple, peach, cherry, strawberry, kiwi, peanut, almond, hazelnut, grape, carrot	Birch, apple, peach, cherry, raspberry, blueberry	n.a.
P2	Various pollen	Birch, mugwort, grasses	Birch, ragweed, grasses, mugwort, almond, peanut, hazelnut, banana, potato
P3	Peach, hazelnut, latex	Apple, peach, cherry, strawberry, raspberry	n.a.
P4	Birch pollen	Birch, apple, peach, cherry, strawberry, raspberry, blueberry	n.a.
P5	Apple	Birch, apple, peach, cherry, strawberry, raspberry	n.a.
P6	Apple and citrus fruits	n.a.	Birch, apple, citrus fruits, banana, grape, paprika
P7	Apple, peach, cherry, kiwi, almonds, peanuts, hazelnut	Birch, apple, peach, cherry, strawberry, raspberry, blueberry	n.a.

tions showed some explicit differences. The intensity of an abundant spot at 31 kDa and acidic p*I* decreased drastically under nonreducing conditions, in favor of two spots at 23 kDa missing under reducing conditions (Fig. 1c). This phenomenon was already observed in the SDS-PAGE (Fig. 1a). Both, the 23 and the 31 kDa band, under nonreducing and reducing conditions, respectively, were identified as Mal d 2 by Western blotting, using a polyclonal antiserum (Fig. 1b). Moreover, the running conditions affected not only the spot position in the gel, but also its antibody binding intensity (Fig. 1b). In contrast to the nonreduced protein, reduced Mal d 2 shows weak IgG-reactivity and cannot be visualized on reduced and alkylated 2-D blots (data not shown). Under nonreducing conditions, however, Mal d 2 can be easily detected by specific antibodies (Fig. 2b).

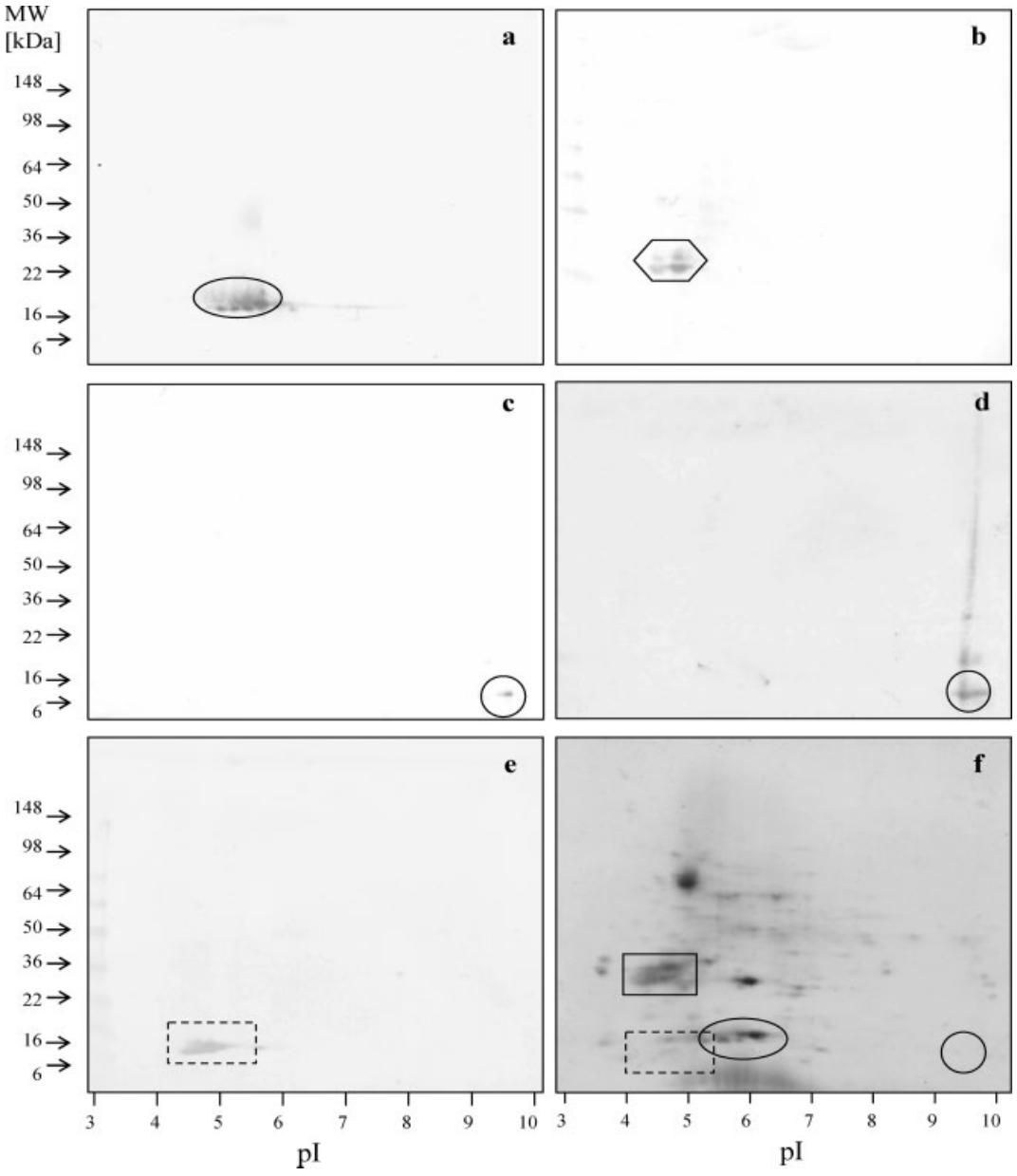

Figure 2. Localization of the major apple allergens using specific pAbs. Apple extract separated by 2-DE was blotted onto PVDF membranes. Samples were reduced and alkylated (a, d, e and f). The allergens are highlighted using an ellipse showing Mal d 1, a hexagon for nonreduced Mal d 2, a circle for Mal d 3 and a dotted rectangle for Mal d 4. The not alkylated, reduced Mal d 3 (c) and purified Mal d 3 as a positive control (d) are demonstrated in the picture above. The Coomassie-stained 2-DE gel of the apple extract is shown in (f).

The position of the known apple allergens on 2-DE maps is shown in Fig. 2. All four allergens were localized with specific antisera (Fig. 2a–d). Based on these findings, the respective spots could be attributed to the spots in the Coomassie-stained gel (Fig. 2f). In the case of Mal d 1, one reactive spot and at least three minor spots are clearly recognizable at a molecular mass of 17.5 kDa and acidic to neutral pH of 5–7. The two 23 kDa spots in the nonreduced sample (Fig. 2b) were assigned to the 31 kDa spot of the reduced sample in the Coomassie-stained gel (Fig. 2f), due to the discrepancies in the electrophoretic mobility of Mal d 2 mentioned before. Mal d 3 cannot be visualized in the Coomassie-stained 2-DE, neither under reducing (Fig. 2f) nor under non-reducing conditions (data not shown). Its position on the 2-DE map could only be determined by Western blotting (Fig. 2c). To confirm these results, purified Mal d 3 was separated by SDS-PAGE as a positive control and characterized by MS analysis. The excised band could be clearly identified as the apple allergen Mal d 3 (accession no. AY374225) (Table 2). The Mal d 3 sequence was subjected to the peptide signal cleavage prediction program SignalP 3.0. A high probability for a peptide signal cleavage was indicated between positions A24 and I25 (data not shown). These *in silico* data were confirmed by the clear identification of the peptide 25–42, which would not result from a tryptic cleavage, thus signal peptide cleavage must occur between A24 and I25. This was additionally confirmed by offline ESI-Q-TOF-MS of the purified and intact protein, whereby the protein mass of the mature protein could be determined with very high accuracy. The spectrum in Fig. 3 shows a major peak of 9076 Da and minor peaks of 9356 and 9638 Da, which possibly indicate minor amounts of differently cleaved signal peptides (Table 3). Moreover, the obtained results and the very close homology to similar proteins from other sources clearly point towards the presence of four intact disulfide bonds within the protein. Purified native Mal d 3 was used to visualize the position of this protein on the 2-D map and as a positive control as well (Fig. 2). However, Mal d 3 from apple extract reacted weakly and only in nonalkylated state with the pAb's. The purified Mal d 3 could be intensively stained even in fully reduced and alkylated state. Similar to Mal d 3, Mal d 4 was also not visualized by Coomassie staining, and its position could only be assessed by Western blotting using a specific antibody (Fig. 2e).

3.2 Serological analysis of apple allergen 2-DE maps

The characterization of the electrophoretic mobility of the four known apple allergens allowed to assign reactive proteins on the Western blots to corresponding spots on the Coomassie-stained gel. Figure 4 shows the sensitization profiles of seven fruit allergic patients. The allergens are highlighted by: elipses for Mal d 1, rectangles and dotted rectangles for Mal d 2 and Mal d 4, while circles indicate Mal d 3. Interestingly, the sera of all seven patients recognized Mal d 1, though to a different extent. It is striking that all sera except one, also reacted with Mal d 2. In contrast to the IgG antibodies, the IgEs of patient sera seemed to have a significant reactivity with the reduced Mal d 2. The nonreduced form of Mal d 2 showed no detectable reactivity with patient sera (Fig. 5).

Table 2. Tryptic peptides from Mal d 3 identified by ESI Q-TOF LC-MS/MS/MS

Tryptic fragment	Sequence	MC	CS	Detected (*m/z*)	Calculated (*m/z*)
1–24	MACSAVIKLALVVALCMAVS VAHA			Signal peptide	
25–42	ITCGQV TSSLAPCIGYVR	0	3	661.3599	661.3326
43–56[a)]	SGGAVPPACCNGIR	0	2	708.3742	708.3295
64–68	TTADR			Not detected	
57–63[b)]	TINGLAR	0	1	744.4824[b)]	744.4362
77–96	NLAGSISGVN PNNAAGLPGK	0	2	926.0446	925.9895
97–104	CGVNVPYK	0	2	468.7538	468.7340
97–115	CGVNVPYKISTSTNCATVK	1	3	700.3795	700.3468
105–115	ISTSTNCATVK	0	2	591.3082	591.2951

Sequence coverage was calculated to be 94.5% (thereof 86.8% by MS/MS). Cysteines were detected as carbamidomethyl-cysteine.
MC, missed cleavage site; CS, charge state of the most intensive detected signal.
a) Partial deamidation of N53 was detected.
b) Signal detected by MS only.

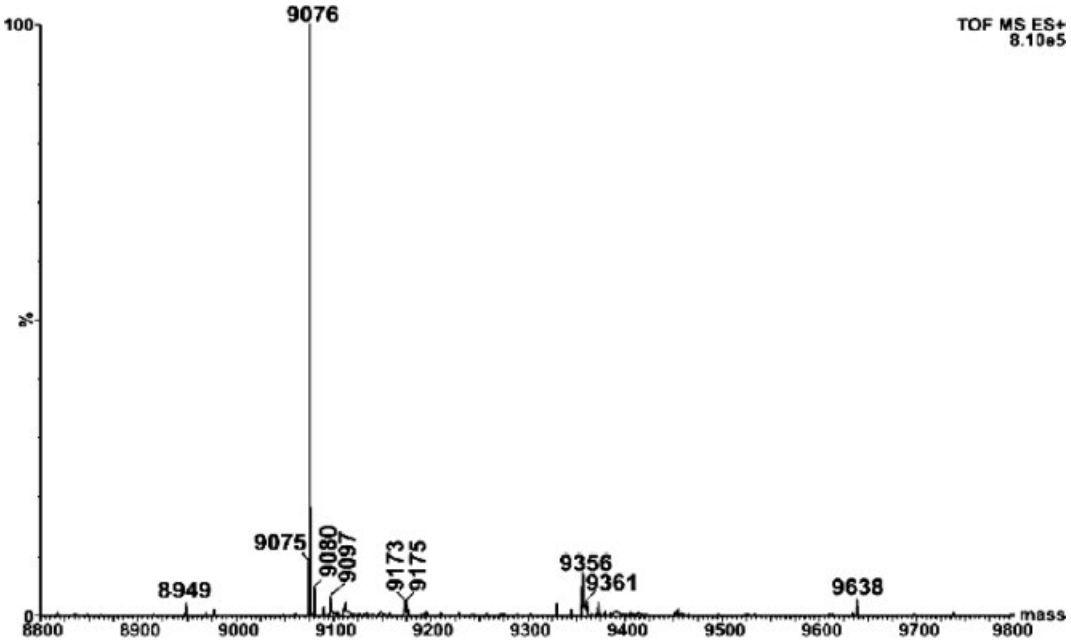

Figure 3. MaxEnt1 deconvoluted average mass spectra of Mal d 3. The determined mass is 8 Da smaller than the calculated mass of the protein, indicating the presence of four disulfide bonds within the protein.

Table 3. Theoretical and detected masses of Mal d 3 (average and monoisotopic)

Protein sequence	19–115	22–115	25–115
Calculated average mass	9641	9356	9076
Detected average mass	9638	9356	9076

As deduced from the mass only, the trace signals detected might indicate the presence of a different signal peptide cleavage.

Reactive spots corresponding to Mal d 3 are present in 2-DE blots probed with sera P2, P4 and P6, whereas only P2 reacted intensively with Mal d 4. Monosensitization was observed only to Mal d 1. Moreover, five out of seven sera (71%) showed reactivity to an unknown basic protein of approximately 45 kDa. Based on the sequence homologies identified by MS/MS sequencing, this unknown protein from *M. domestica* contains several sequence stretches showing high homology to a glyceraldehyde-3-phosphate dehydrogenase from other plants (data not shown).

3.3 Effect of periodate treatment on IgE-reactivity of apple allergens

Sera from four patients were used to evaluate the influence of carbohydrate determinants on the IgE reactivity (Fig. 6). After periodate treatment, a general decrease in the immunoreactivity of Mal d 2 was detected. The serum P1 showed some intense spots at approximately 90 kDa and one small spot at acidic pH and 18 kDa, which disappeared upon periodate treatment. The single unidentified basic spot at 45 kDa, though, was still present in all four blots.

4 Discussion

Atopic allergy is a genetically determined disorder affecting a considerable proportion of the population, whose main physiological feature is an increased ability to produce IgE antibodies in response to certain allergens by inhalation or ingestion [28]. Although IgE antibodies are produced in extremely low concentrations, they are able to cause a cascade of allergic symptoms (rhinoconjunctivitis, asthma, anaphylactic shock).

Here we demonstrated that a comparative proteomic approach using patient IgEs can be used for the mapping of apple allergens. This approach is particularly useful, since it allows a direct detection of all potential allergens present in the fruit. This has led to the identification of a novel putative allergen in apple.

Generally, proteomic studies of plant tissues face a number of obstacles, which are caused by the nature of plant material, representing a low protein content, accom-

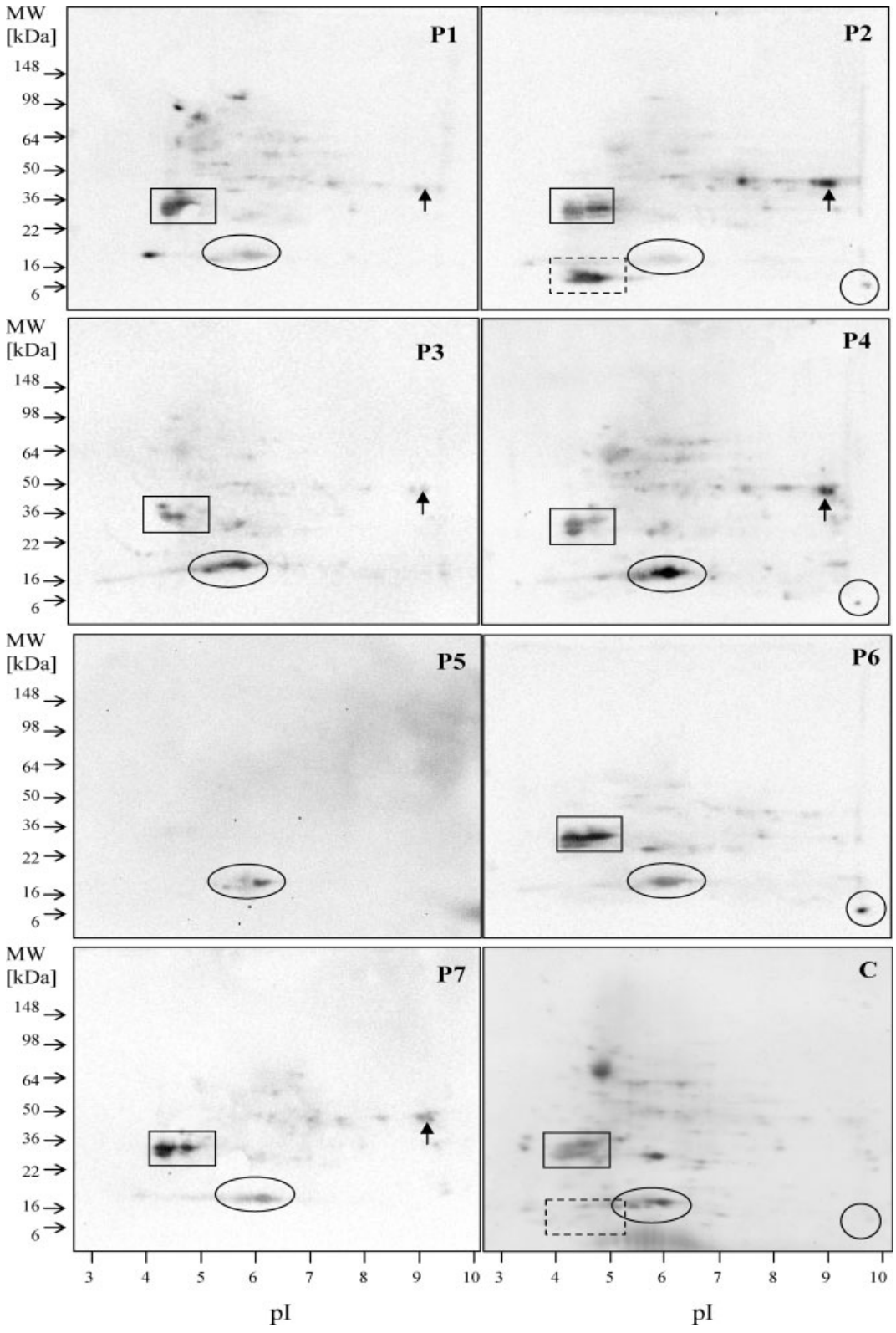

Figure 4. Identification of allergenic proteins under reducing conditions using sera of seven patients (P1–P7). Ellipses show Mal d 1, rectangles Mal d 2, circles Mal d 3 and dotted rectangles Mal d 4. An unidentified IgE reactive spot is highlighted by arrows. The Coomassie-stained 2-DE gel of the apple extract is shown in (C).

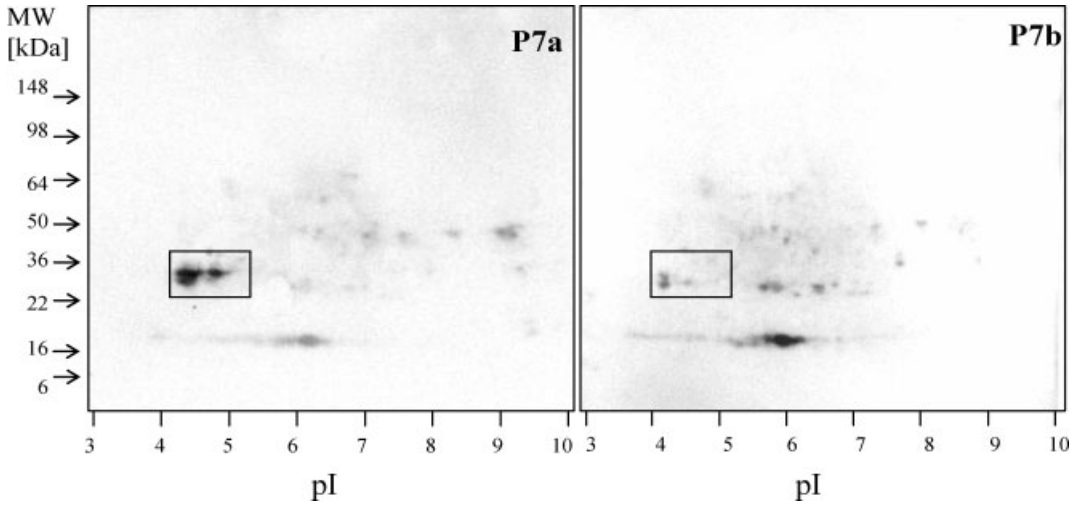

Figure 5. Influence of reducing (a) and nonreducing (b) conditions on Mal d 2 IgE-reactivity using the serum of patient 7. Rectangles highlight the serum-reactivity to reduced Mal d 2. There is no detectable binding to the unreduced form (see Fig. 1).

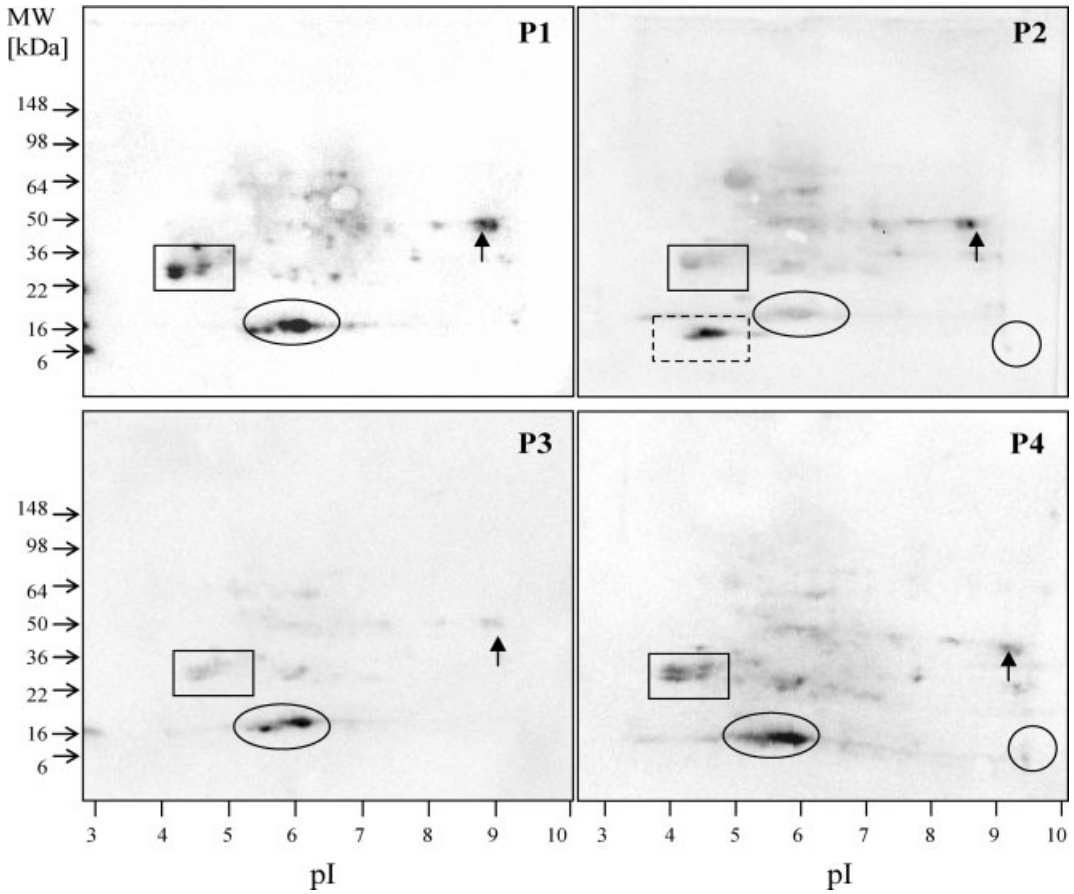

Figure 6. The influence of carbohydrate moieties on the IgE-reactivity was investigated by periodate treatment. After blotting, the membranes were subjected to a mild periodate oxidation and incubated with the sera of the patients 1–4 (P1–P4). Ellipses show Mal d 1, rectangles the Mal d 2, circles Mal d 3 and dotted rectangles show Mal d 4. The reactivity to Mal d 2 was altered after periodate treatment, whereas the other allergens remained unaffected. The unidentified spot, highlighted by arrows, demonstrated no significant alterations.

panied by a plethora of proteases and interfering compounds. These substances comprise polysaccharides, lipids, phenolic compounds and a broad range of secondary metabolites [23]. A phenol-based precipitation method as applied, overcomes these problems, and allows protein enrichment with a simultaneous removal of matrix residues. Phenol extraction has already been described as an efficient method for preparation of plant samples, combining good purification capacity with irreversible inactivation of internal proteases [29, 30].

Immunoblotting using polyclonal anti-Mal d 1 antiserum revealed four distinct spots with a similar molecular weight, which could be partly visualized by Coomassie staining. It is well documented that several isoforms of Mal d 1 are expressed in apple fruits [31, 32], and therefore it was tempting to assume that these spots represent different isoforms. However, it has been shown, both at the RNA and protein level that these isoforms are not equally expressed. Indeed only one isoform (Mal d 1.02) is abundantly present in apple fruit tissue, while the others show minor expression levels [31, 33]. Previously, it has been demonstrated that four putative Pru av 1 isoforms, separated by 2-DE, represent one single isoform [24]. The authors suggested a nonenzymatic deamination of asparagine and glutamine residues as an explanation for this phenomenon. Similar to Pru av 1, it seems more likely that the putative Mal d 1 isoforms, detected by 2-DE and Western blotting, derive from one single isoform, which is predominantly expressed in the fruit tissue.

Mal d 2 showed anomalies in its electrophoretic mobility if treated by reducing agents. Interestingly, the molecular weight of Mal d 2 seems to increase under reducing conditions compared to nonreduced protein. The altered migration of TLP proteins in SDS-PAGE has previously been reported, not only for Mal d 2 [18, 34], but also for the homologous protein in cherry [35]. The predicted M_r of 23 kDa has been confirmed by MS, although the protein migrated as a 31 kDa band in SDS-PAGE [17]. However, the influence of reducing milieu on the conformational changes was generally ignored. A similar behavior was only described for a basic TLP from kiwi, which was attributed to the high number of disulfide bonds [36].

Reduced Mal d 2 was, to some extent, also present under nonreducing conditions, probably due to partial reduction during sample preparation or reducing agents naturally present in apple tissue. Previously, Mal d 2 has been described to deliver one single distinct band of 23 kDa, if the fruit was extracted at an acidic pH [37]. At this low pH-level the activity of reducing compounds available in the fruit tissue, might be reduced, so that only nonreduced protein was detectable in SDS-PAGE.

A significant increase in IgE-binding activity under reducing condition was observed, which might be related to the refolding nature and alteration of electronegatively charged cleft at the protein surface [37]. None of the sera tested, recognised the nonreduced protein at 23 kDa (data not shown). It seems of eminent importance to investigate this phenomenon during food processing. The finding that the IgE-reactivity of native Mal d 2 is significantly lower than that of the reduced form, could be an important consideration for allergen inactivation during industrial juice production.

The attribution of Mal d 1 and Mal d 2 to the respective protein spots on the 2-DE map could be easily achieved by comparing the corresponding spots on Western blots. In the case of Mal d 3 and Mal d 4, however, no corresponding spots are visible on the 2-DE gel stained by Coomassie, although a high amount of protein was loaded. According to our findings, Mal d 3 can only be stained in SDS-PAGE, if present in high amounts, *i.e. ca.* 15 μg and without rigorous gel destaining (data not shown). This can result from the absence of basic amino acids, since there is a significant correlation between the color intensity with Coomassie staining and the number of lysine, histidine and arginine residues of a protein [38]. Additionally, the poor stainability could be due to a very low Mal d 3 content in the analyzed apple extracts. By Western blotting with pAbs Mal d 3 could be detected, though weakly and only if not alkylated. Using 25 μg of purified Mal d 3 as a positive control led to a significant improvement of the immunoreactivity, supporting our previous assumption. A reversible refolding for Mal d 3 after mild heating has been described, whereby the disulfide bonds most likely assist in this process [6]. Therefore, we expect that Mal d 3 refolds after reduction if the cysteine residues are not blocked to allow antibody binding. The Mal d 3 positive control could probably be partially alkylated, due to the very high protein concentrations and accessibility of disulphide bridges. However, Mal d 3 was easily recognised by the sera of three patients, suggesting that low amounts of this protein are sufficient for allergen recognition and subsequent allergic symptoms. This is of particular interest, since Mal d 3 is believed to be the major elicitor of severe symptoms after fruit consumption [8]. Analogous to Mal d 3, Mal d 4, could not be detected by Coomassie staining, but easily visualized by Western blotting both with IgG and IgE antibodies. Considerations made for Mal d 3, about protein concentrations and stainability, might also be valid for Mal d 4. In contrast to LTP, however, profilins are believed to be of limited clinical relevance [10].

The inactivation of carbohydrate moieties revealed a lowered reactivity for Mal d 2. Since Mal d 2 is not glycosylated, the involvement of CCDs in this phenomenon is

unlikely. It has been shown that periodate treatment of Art v 1, the major mugwort pollen allergen, resulted in a loss of IgE binding, even from sera clearly identified to be directed against peptide moieties of the protein [39]. Thus, periodate treatment appears to induce more changes on the proteins beyond deglycosylation. There is an ongoing debate on the clinical relevance of anti-CCD IgEs. The general impression is that they are of poor to no biological activity. One reason given is that proteins carrying only one sugar moiety would not be able to elicit mediator release [40, 41]. Nevertheless, anti-CCD IgEs might be responsible for the reactivity to some spots in higher M_r ranges than 35 kDa.

The sensitization profiles of the seven patients tested were characteristic. Although all sera reacted to some extent with Mal d 1, a monosensitization to Mal d 1 could only be demonstrated in one single case. Symptoms related to Mal d 1 are described as mostly mild and local, many patients tend to ignore the impact of this allergen on the dietary behavior. However, a continued consumption of apples might increase the risk of a cosensitization to more stable allergens and the risk of anaphylactic reactions [42]. The sensitization profiles of patients in the present study support this idea. Nevertheless, it needs to be mentioned that apple allergy can also occur without concomitant pollinosis, which is mainly attributed to Mal d 3 [8].

Serological screening of 2-DE maps allowed the identification of a novel allergen candidate in apple. We found this putative allergen at a basic p*I* of about 9 and an M_r of 40–45 kDa, showing reactivity to 71% of tested patient sera. The reactivity to this rather high-molecular-weight protein was probably not due to CCDs, since it did not alter upon periodate treatment. The corresponding protein was identified by MS analysis as a glyceraldehyde-3-phosphate dehydrogenase, which has already been described as an important allergen in wheat flour [43]. Further analyses to characterize this protein and its clinical relevance are in progress.

The present proteomic approach allowed to unequivocally map the four major apple allergens by IgE Western blots with patient sera recognizing the spots identified by polyclonal antibodies and delivering authentic 2-DE apple allergen maps.

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