Stability of the Bet v 1 cross-reactive allergens Api g 1 and Dau c 1

A biophysical approach

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Abstract

The allergen Bet v 1 is known as the primary sensitizer for birch pollen-related food allergy and is responsible for IgE cross-reactivity to pathogenesis-related 10 (PR-10) proteins from, in particular, fruits from the *Rosaceae* and vegetables from the *Apiaceae* families. The allergenic potential of PR-10 proteins is mainly characterized for specific recombinantly produced isoforms, which are used for research and diagnostic purposes. However, in natural food sources these allergens are often present as isoform mixtures. The first aim of this research was to purify and characterize PR-10 allergens as natural isoform mixtures to determine whether differences could be observed between natural and recombinant allergens and between plant families. The second aim was to find a relationship between the physico-chemical stability of PR-10 proteins and structural characteristics to explain differences in IgE binding potential and cross-reactivity. The PR-10 allergens Bet v 1 from birch, Api g 1 from celery, and Dau c 1 from carrot were purified under mild conditions following a standardized protocol. Different allergen isoforms were determined and circular dichorism (CD) analyses of the allergen mixtures showed a similar secondary structure composition as observed for other PR-10 proteins. The allergen mixtures and recombinant allergens were characterized by stability studies to pH, temperature and denaturant where CD was used to detect structural changes. Minor differences were observed in stability between natural isoform mixtures and between the recombinant isoforms, although recombinant Dau c 1 was likely destabilized by its attached His-tag. A general trend was observed for allergen stability, structural differences and their relationship to the IgE binding capacity in aqueous solutions. The allergenic potential decreases in the following order: Bet v 1, the primary allergen of birch pollen-related allergies, Mal d 1, Api g 1 and Dau c 1, in accordance with their amino acid sequence identity. Bet v 1 cross-reactive IgE antibodies preferably bind to the charged and polar residues of Mal d 1 for which the positive charge can be increased by the physiological pH of fruit. Api g 1 appears to be more stable than Dau c 1 as the result of a tighter hydrophobic packing. However, the thermodynamic stability of Api g 1 is similar to that of Bet v 1, but the higher proportion of hydrophobic residues and the reduced proportion of charged residues are responsible for the lower IgE binding capacity. Furthermore, the IgE binding capacity is not severely affected, as long as the protein is able to refold. The implications of these findings are discussed in the context of the development of allergic symptoms upon exposure to these PR-10 proteins.

Contents

Chapter 1

Introduction

1.1 Allergic response; Birch pollen hay fever

Birch pollen from the *Betula pendula* is a major source of allergens, which can induce hay fever as a result of flowering trees highly represented in Northern Europe. The trees flower during early springtime with the airborne allergen Bet v 1 as the major sensitizer. Bet v 1 is responsible for IgE-mediated cross-reactivity to allergens of other trees, but also fruits, vegetables and legumes. Other less abundant allergens in birch pollen besides Bet v 1 may cause allergic reactions and are numbered, Bet v 2 to 8, in order of identification. Bet v 2 (profilin) and Bet v 6 (isoflavone reductase) are two other birch pollen allergens known to cross-react with food allergens. Nevertheless, 90% of birch pollen allergic individuals are sensitized to Bet v 1 and 20% to Bet v 2, meaning that a group in the allergic population is sensitized to multiple allergens [1].

To evoke an allergic reaction, an individual first needs to be sensitized to the allergen by the T and B cell response, followed by the response that induces symptoms, the IgE-mediated degranulation of sensitized mast cells and basophils. Therefore, the allergen first needs to pass airway epithelium and submucosa either as intact protein or epitope-bearing peptides, followed by the uptake by an antigen presenting cell (dendritic cell or monocyte) present in the mucosa, which processes the allergen into peptides [2].

The subsequent selection of peptides, containing T cell epitopes, are presented by the major histocompatibility class II (MHC-II) complex, on the antigen presenting cell, to the T cell receptor of naïve CD4+ T cells. This recognition, together with co-stimulatory molecules and a polarizing micro-environment, leads to differentiation into T helper 2 cells (Th2), generating the production of cytokines (e.g. interleukin (IL)-4 and IL-13), which stimulate the allergen-specific B cells to produce IgE antibodies. IgE binds to the high affinity IgE receptor (FcεRI) on mast cells, which are then called sensitized. Upon a second exposure to the same allergen, allergens bind to IgE followed by cross-linking of two IgE molecules on the surface of the mast cell. Subsequently, the mast cell degranulates, releasing inflammatory compounds like histamine, which cause the typical allergic complaints of, e.g., hay fever and food allergy. Furthermore, the mast cell releases cytokines, including tumor necrosis factor (TNF)-α, IL-4 and IL-5, inducing the transcription of these cytokines and others as leukotrienes and prostaglandins, which contribute to the ongoing inflammatory response [2].

1.2 Allergens

Various research studies have tried different approaches to identify the characteristics that determine the allergenicity of proteins. Most proteins are non-allergenic and can be found in 9318 protein families in the Pfam (protein family) database. In this database only 184 Pfam domains (2%) are associated with allergens for which the variety of biochemical functions is limited [3]. Furthermore, in contrast to non-allergenic protein sequences, allergen sequences lack bacterial homologues [4]. Unlike plant food or pollen allergens, most animal food allergens have human homologues and it is hypothesized by Jenkins et al. [5] that the allergenicity of protein family members decreases due to their relatedness to human homologues. A set of sequences of animal foods with an identity less than 54% to human homologues are all allergens, whereas proteins with a sequence identity above 62% are rarely allergenic [5].

1.3 Structural characteristics of Bet v 1 and its homologues

The complete coding sequence of the isoform Bet v 1a was determined by screening the pollen cDNA library with specific IgE antibodies against Bet v 1 [6]. Bet v 1 has been shown to be encoded by 7 different pollen expressed genes, responsible for the expression of 14 different isoforms [7]. The sequence encodes a protein of 160 amino acids with a molecular mass of approximately 17.4 kDa. Ferreira et al. [8] divided nine of the Bet v 1 isoforms into low, intermediate and high IgE binding reactivity. Schenk [9] showed that these isoforms are abundant in birch pollen extracts and from these isoforms, 35-38% showed high, 22-24% showed intermediate and 18-19% showed low IgE binding reactivity, which leaves 19-25% of the isoforms with unidentified IgE binding reactivity.

Birch pollen related allergy is induced by cross-reactivity of birch pollen-specific IgE antibodies and binding to birch pollen-related food allergens. This is based on the structural relationship of Bet v 1 and its homologues from other plant species. These are all members of the PR-10 protein family, where PR stands for “pathogenesis related”. Birch pollen cross-reactivity is mainly observed with homologous proteins of other trees from the *Betulaceae* family (e.g. alder, hazel), fruits from the *Rosaceae* family (e.g. apple, cherry, pear), vegetables from the *Apiaceae* family (e.g. celery, carrot) and legumes from the *Fabaceae* family (e.g. soybean, mungbean, peanut). Table 1 shows a list of the main cross-reactive PR-10 allergens in order of decreasing amino acid

identity together with protein parameters (total amino acids, molecular weight, iso-electric point and % identity). With some exceptions, cross-reactive allergy seems to decrease and/or develop in the order of decreasing identity [1, 10].

Table 1. Cross-reactive PR-10 allergens and protein parameters.

PR-10	Source	Family	UniProt Acc No	PDB-entry	AA	MW	pI	Sequence identity %
Bet v 1	birch	*Betulaceae*	P15494	1bv1	160	17,571	5.39	100
Aln g 1	alder	*Betulaceae*	P38948	-	160	17,339	5.46	81.2
Cor a 1	hazel	*Betulaceae*	Q08407	-	160	17,512	5.43	72.5
Pyr c 1	pear	*Rosaceae*	O65200	-	159	17,581	5.62	56.8
Pru av 1	cherry	*Rosaceae*	O24248	2e09	160	17,660	5.87	59.3
Mal d 1	apple	*Rosaceae*	P43211	-	159	17,651	5.68	55.6
Gly m 4	soybean	*Fabaceae*	P26987	2k7h	158	16,772	4.69	46.2
Ara h 8	peanut	*Fabaceae*	Q6VT83	-	157	16,952	5.03	46.2
Vig r 1	mungbean	*Fabaceae*	Q2VU97	2flh	155	16,189	4.60	42.5
Api g 1	celery	*Apiaceae*	P49372	2bk0	154	16,321	4.63	40.0
Dau c 1	carrot	*Apiaceae*	O04298	-	154	16,049	4.63	36.8

The protein parameters shown, are total amino acids (AA), molecular weight in Da (MW), iso-electric point (pI) and % amino acid identity compared to Bet v 1. Protein sequences are taken from the UniProt database and are shown with their accession number (Acc No). The PDB-entries were taken from the RCSB protein data bank.

The primary structure (linear epitopes), secondary and tertiary structure (conformational epitopes) of allergenic proteins are important for the molecular basis of IgE-binding. However, it is believed that birch pollen cross-reactivity is based on conformational epitopes [11]. The sequence conservation of the PR-10 amino acid sequences compared to the major allergen Bet v 1 is shown in the alignment of Figure 1. The secondary structure is also indicated in this figure. The three-dimensional structures of the major birch pollen allergen and plant homologues have been resolved mainly by X-ray diffraction, but also by solution NMR, for Bet v 1a [12, 13], Bet v 1L [14], Pru av 1 [15], Api g 1 [16], mungbean [17] and lupin PR-10 [18, 19]. The 3D-structures are similar for all homologues and consist of a seven-stranded anti-parallel β-sheet wrapped around a long C-terminal α-helix (residues 130 to 154). The β-sheet consists of seven anti-parallel strands and is separated from the long helix by two

consecutive shorter α-helices (residues 15-23 and 25-34) resulting in formation of a large forked cavity. This cavity is predominantly hydrophobic, and exhibits three openings on the protein surface [13].

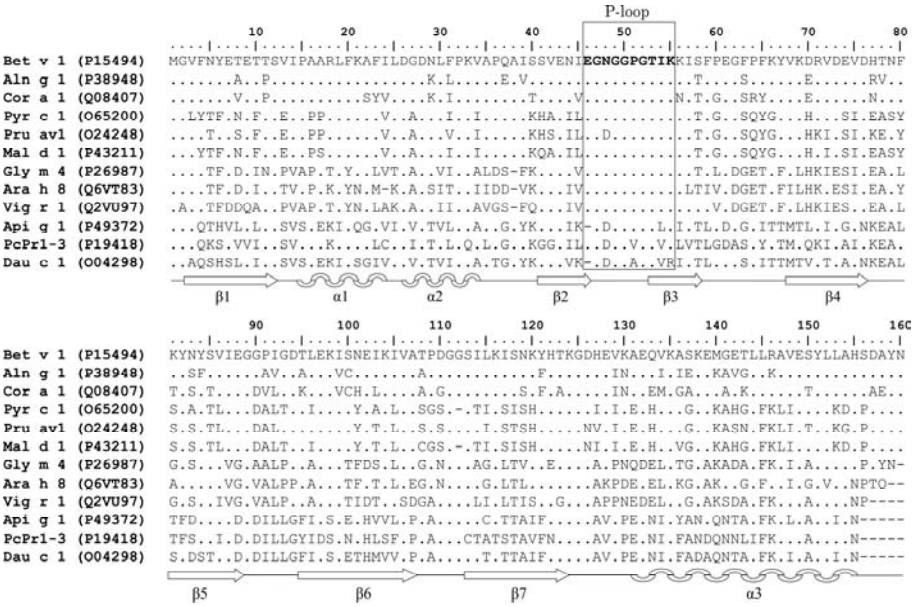

Figure 1. Sequence alignment of Bet v 1 with PR-10 homologues of different plant families. PR-10 sequences are shown, including the first methionine, with their UniProt accession numbers between brackets. The highly conserved region (emboxed), including Glu46 and the P-loop, is indicated in the alignment. Secondary structure has been indicated on the bottom of the alignment, based on the 3D-structure of Bet v 1 (PDB-entry; 1bv1).

An example of the 3D-structure of Bet v 1 is shown in Figure 2 (green) aligned with the structure of the major celery allergen Api g 1 (purple). Bet v 1 is not able to form disulfide bridges and the isoform Bet v 1a does not contain cysteines, in contrast to other isoforms such as Bet v 1L. It has been shown that Bet v 1 is not glycosylated, making it a suitable protein for recombinant expression systems. Another structural feature is a highly conserved loop region rich in glycine, GXGGXGXXK (residue 47-55), which is conserved among all Bet v 1 homologues and is referred to as the phosphate binding loop (P-loop, indicated in Figure 1) [10]. This loop is a conserved sequence motif named after Walker (Walker A motif) and is present in protein kinases and nucleotide binding proteins and has the ability to phosphorylate [20]. For Bet v 1,

however, phosphorylation has never been demonstrated and it is unknown if the P-loop contributes to the biological function of Bet v 1.

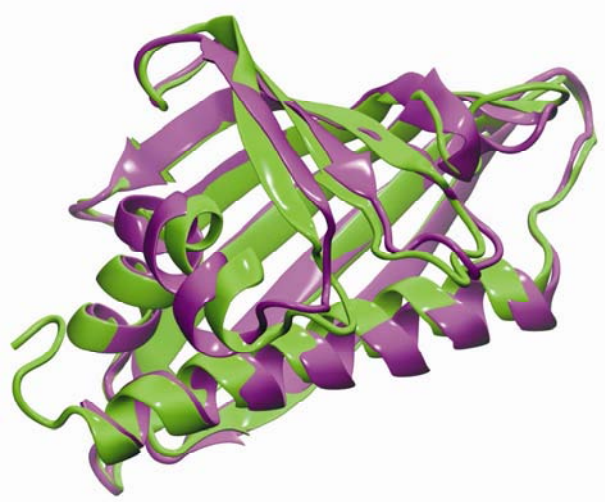

Figure 2. PR-10 homologues show a conserved tertiary fold. An overlay is shown of the 3D-structures of Bet v 1 (PDB-entry: 1bv1, green) and Api g 1 (PDB-entry: 2bk0A, purple).

1.4 Biological function of Bet v 1 and other PR-10 proteins

The biological function of the Bet v 1 homologous proteins is still unclear, but several studies have shown ligand binding properties, RNAse activity, plant pathogenesis response and lipid binding activity. Bet v 1 and other homologues bind a variety of ligands as shown by X-ray crystallography, NMR and binding studies [14, 15, 17, 21, 22]. Bet v 1a and Bet v 1L and the homologue from cherry, Pru av 1, and mungbean have been shown to bind ligands such as fatty acids, flavonoids, cytokinins and plant steroids, but it is unknown whether this property is important for the function of Bet v 1. Other studies have shown RNAse activity for some PR-10 proteins [22, 23]. Some of the PR-10 sequences are expressed as a result of external factors such as wounding, auxin treatment, copper exposure [22, 24-26].

Another structural function of Bet v 1 is the ability to bind to and permeabilize membranes by changing its conformation [27]. This feature was first observed in the structurally similar star-related lipid-transfer (START) domain superfamily. The START

domain has a similar tertiary fold to Bet v 1 and is able to bind ligands, such as cholesterol, as well as membranes [28, 29]. The ability of Bet v 1 to permeabilize membranes might facilitate the possibility to cross mucosal membranes and could provide a mechanism for Bet v 1 sensitization [27]. A recent study has shown the transport of Bet v 1 through conjunctival (eye) epithelium only for allergic patients, with the help of receptors expressed at high levels [30].

1.5 Stability of Bet v 1 cross-reactive food allergens and effects on allergenicity

In general, food allergens are known as thermostable proteins, which require high temperatures to unfold [31]. However, birch pollen related allergens are known as thermolabile and their IgE binding capacity can be easily destroyed by thermal processing in food extracts [32-35] as shown for Mal d 1 and Api g 1. IgE binding to Dau c 1 is less affected by heating even when autoclaved at 121°C [35], but Dau c 1 also has been shown to act as a primary sensitizer [36, 37]. Food matrix effects such as enzymatic and non-enzymatic reactions during heating also have a negative influence on IgE binding [38, 39]. Furthermore, the stability of cytosolic PR-10 proteins is influenced by macromolecular crowding and is different from isolated allergens in aqueous environments [40]. Most conclusions regarding changes in IgE binding are based on changes of isolated allergens in aqueous environments, which are shown to be more stable to heating and require longer heating time to destroy IgE binding [41]. In general, stability measurements of PR-10 allergens is scarce, but could be easily studied by circular dichroism.

1.6 Circular dichroism: a method to study protein characteristics

An analytical method, which is extensively used throughout this study is circular dichroism (CD), a spectroscopic technique that can be used to follow structural changes of proteins. A molecule's chirality, i.e. L-amino acids and chromophoric character allows the optically active proteins to interact differently with left- and right-handed circularly polarized light. Peptide bonds of the polypeptide backbone can be monitored at wavelengths below 260 nm, in the far UV-region, to give information on the overall secondary structure of the protein in solution. A CD spectrum of a typical β-sheet has a

maximum at 198 nm and a minimum near 215 nm and a CD spectrum of a typical α-helix has a maximum at 192 nm and two minima at 208 nm and 222 nm [42, 43]. Bet v 1, a mixed α/β-protein, has a characteristic spectrum with a maximum at approximately 196 nm and a broad minimum at 210-220 nm.

Accurate measurements of protein concentration is necessary for reliable determination of protein secondary structure. A method to determine protein concentration is the convenient and sensitive Bradford method, which gives different responses for different proteins. A second method is the bicinchoninic acid (BCA) assay, which also gives different responses for different proteins, but differences between two calibration curves for e.g. IgG and BSA are smaller than for the Bradford method [42, 43]. The protein concentration of Bet v 1 and the homologues of Api g 1 and Dau c 1 is best determined by the microBCA assay.

The concentration is used to calculate the mean residual weight ellipticity, $[\theta]_{MRW}$, of the CD spectra from the observed ellipticity signal, $[\theta]_{obs}$, in mdeg according to:

$$[\theta]_{MRW} = 100 \cdot [\theta]_{obs} / C \cdot l \cdot n \qquad (1.1)$$

Where C is the concentration in mol/L, l the path length of the cuvette in cm and n the number of amino acids. The mean residual ellipticity can be used to predict the secondary structure by deconvolution methods such as CDNN, SELCON, LINCOMB and CONTIN [44, 45]. K2D is the only algorithm that determines the protein secondary structure without the use of protein concentration, but only uses the spectral information between 200 and 240 nm. All of the various methods give a reasonable estimate of the helical content. The methods, recommended for estimating the conformation of globular proteins in solution, such as Bet v 1, are SELCON and CDNN, which both give very good correlations between predicted and observed α-helix, β-sheet and β-turn [44, 45]. SELCON and CDNN were both compared, but CDNN was used throughout this study to determine the secondary structure [46].

1.7 Protein stability studies with circular dichroism

To understand structure-function relationships of a protein, such as Bet v 1, knowledge is needed on the conformational stability. Stability is defined as a thermodynamic difference, the Gibbs free energy change, between the folded and unfolded state of a protein. The folded state of a protein can often be disrupted by an environmental change, such as temperature, pH or denaturant. The unfolded state, also referred to as

the denatured state, is usually reversible. When unfolding is irreversible, it usually involves chemical alterations of amino acid side chains taking place during or after unfolding. The reversibility of unfolding suggests a two-state mechanism, where only the folded and unfolded states are present. In this mechanism, no partially unfolded state is assumed to exist and in the midpoint of an unfolding curve (Figure 3), 50% of the protein is in the native state and 50% in the unfolded state [47]. Throughout this study, the assumption is made that proteins unfold according to the two-state mechanism.

The folded state of a protein is stabilized by a combination of hydrogen bonds, van der Waals interactions, electrostatic interactions and hydrophobic interactions. The thermodynamics of protein unfolding mainly shows the differences in strength of these interactions upon exposure to environmental changes and could therefore be used to characterize the differences between Bet v 1 isoforms and the cross-reactive allergens [47].

Thermodynamic parameters, such as the change in enthalpy ΔH_{Tm} at T_m, the temperature where 50% of the protein is unfolded, and ΔG, the Gibbs free energy change can be calculated by fitting a two-state mechanism to the data points [45]. The fitting procedure in this study is carried out by non-linear least-squares regression and is visualized in Figure 3 in steps according to the linear extrapolation approach for a thermal unfolding curve and can be followed by the description below [48]. The two-state mechanism is shown as the equilibrium between the native (N) and unfolded state (D):

$$N \rightleftharpoons D \qquad (1.2)$$

The equation for the change in Gibbs free energy (ΔG) at equilibrium is:

$$\Delta G = -RT\ln(K_{eq}) \qquad (1.3)$$

R is the gas constant ($J \cdot K^{-1} \cdot mol^{-1}$) and T the absolute temperature (Kelvin). The dimensionless equilibrium constant, K_{eq}, is defined as:

$$K_{eq} = [N]/[D] \qquad (1.4)$$

The fraction of folded protein (f_N) and the fraction of unfolded protein (f_D) changes in an unfolding curve, but the sum of these fractions ($f_N + f_D$) is equal to 1. The observed signal (Y_{obs}), ellipticity with CD, can therefore be described as:

$$Y_{obs} = Y_N \cdot f_N + Y_D \cdot f_D \qquad (1.5)$$

Y_N and Y_D are the functions of the linear fits shown in Figure 3a for the baselines of the folded (Y_N) and unfolded state (Y_D). The change in the folded fraction is similar to:

$$f_N = 1 - f_D \quad (1.6)$$

and can be written as:

$$f_D = (Y_N - Y_{obs})/(Y_N - Y_D) \quad (1.7)$$

and reformulated by using the equilibrium constant:

$$K_{eq} = (Y_N - Y_{obs})/(Y_{obs} - Y_D) \quad (1.8)$$

K_{eq} can be substituted in equation (1.3) giving:

$$\Delta G = -RT \ln((Y_N - Y_{obs})/(Y_{obs} - Y_D)) \quad (1.9)$$

This equation forms the basis for all denaturation curves shown in this thesis and will be further described for thermal denaturation curves.

The Gibbs free energy change for thermally induced unfolding is defined as:

$$\Delta G = \Delta H - T\Delta S \quad (1.10)$$

where ΔH and ΔS are the changes in enthalpy and entropy associated with unfolding. By substitution of ΔG from equation (1.3) this leads to:

$$K_{eq} = e^{-\Delta H/RT + \Delta S/R} \quad (1.11)$$

From this equation follows the van 't Hoff equation:

$$\ln(K_{eq}) = -\Delta H/RT + \Delta S/R \quad (1.12)$$

From the CD unfolding curve, the enthalpy and entropy are found by a straight line, equation (1.12), over a narrow temperature range from the transition of Figure 3A. This results in a linear plot of $\ln(K_{eq})$ versus $1/T$ (T in Kelvin), as shown in Figure 3B. The point where this line crosses the horizontal axis, defines the midpoint of thermal denaturation, T_m. The enthalpy can be calculated from the slope of this plot. By using non-linear least-squares regression, a fit will be obtained as shown in Figure 3C.

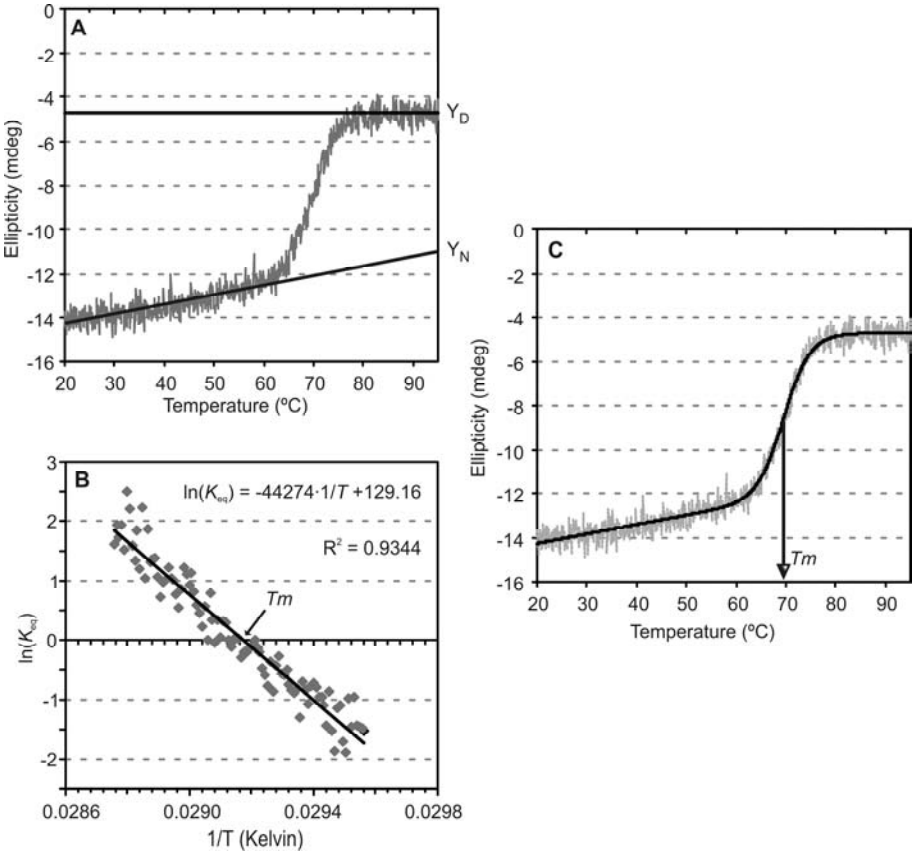

Figure 3. Fitting procedure of a thermal unfolding curve of rApi g 1 (chapter 4). measured by circular dichroism. Two baselines are drawn (**A**) that represent the native state (Y_N) and the unfolded state (Y_D). Linear extrapolation of the transition (**B**). Fit as performed by non-linear least square regression (**C**). See text for further details.

1.8 Research aim and thesis outline

Despite the considerable similarities between different PR-proteins, such as structure, relative instability towards physical processing as compared to typical food allergens, there still remain differences between members of the PR-10-family with respect to e.g. sensitization potential and towards the frequency with which their consumption leads to clinical OAS-symptoms. Furthermore, research into birch pollen allergy or birch pollen related food allergy, increasingly makes use of recombinant allergens. There is a strong desire to use recombinant allergens for e.g. diagnostic purposes, because of

relative ease of preparation, and standardizations of composition of allergen preparations. However, the natural sources of these allergens have been shown to contain a large variety of allergen isoforms for which IgE binding capacity is largely unknown and likely variable. Therefore, the first aim of this research was to purify and characterize PR-10 allergens in natural isoform mixtures to determine whether differences can be observed between natural and recombinant allergens and between plant families. The second aim was to find a relationship between the physico-chemical stability of PR-10 proteins and structural characteristics and their behavior towards cross-reactivity.

Chapter 2 describes a general purification protocol, used to purify the PR-10 allergens Bet v 1 from birch pollen, Api g 1 from celery root and Dau c 1 from carrot under mild conditions. The purified allergen mixtures are partially characterized by Q-TOF MS/MS. In **chapter 3**, the thermal stability of the natural allergen mixtures is compared to their recombinant counterparts and structural changes have been linked to the IgE binding capacity. **Chapter 4** describes the thermodynamic stability of the recombinant and natural allergens by using CD with denaturant to determine the Gibbs free energy change. The Gibbs free energy change is also determined in an alternative method by measuring the pH-dependent thermal stability of natural Api g 1 and Dau c 1. In **chapter 5**, differences in the primary, secondary and tertiary structure of PR-10 sequences are studied in a bioinformatics approach. The structural approach, on the tertiary level, is an electrostatic one and takes into consideration the physiological pH of the food of the different plant groups and the consequences for IgE binding studies. **Chapter 6** is the general discussion, which discusses the results of chapters 2 to 5 and takes into consideration new developments in allergy and the biological function and conformational flexibility of allergens.

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Chapter 2

Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery

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Abstract

Birch pollen allergy is predominantly caused by the major allergen Bet v 1 and can lead to cross-reactions with homologous proteins in food. Two major cross-reactive food allergens are Dau c 1 from carrot and Api g 1 from celery, which have never been purified from their natural source. Here we describe a non-denaturing purification method for obtaining natural Bet v 1, Dau c 1 and Api g 1, comprising of ammonium sulfate precipitation, hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC). This method resulted in 98-99% pure isoform mixtures for each allergen. Characterization of these isoform mixtures with Q-TOF MS/MS, clearly showed earlier reported isoforms of Bet v 1, Dau c 1 and Api g 1, but also new isoforms. The presence of secondary structure in the three purified allergens was demonstrated via circular dichroism and showed high similarity. The immune reactivity of the natural allergens was compared with recombinant proteins by Western blot and ELISA and showed similar reactivity.

2.1 Introduction

The major allergen from birch pollen (*Betula pendula* also *Betula verrucosa*), Bet v 1, is an important source of airborne allergen in countries of Northern and Central Europe and Northern America during early springtime [1]. Exposure can lead to sensitization characterized by allergen-specific Th2 cells and IgE antibodies in genetically predisposed individuals. These individuals may develop clinical symptoms of type I hypersensitivity, characterized by rapid and local inflammatory reactions in the upper (allergic rhinitis) and lower airways (allergic asthma) [2]. After birch pollen sensitization, many individuals also develop allergic responses to fresh fruits and vegetables of the *Rosaceae* and *Apiaceae* family, such as apple [3, 4], cherry [5], celery [6] and carrot [7, 8]. This type of food allergy mainly causes local reactions such as oral itching and swelling of the lips and oral mucosa, also known as oral allergy syndrome [9].

Birch pollen-related food allergies are the result of cross-reactivity of IgE antibodies to Bet v 1 homologous proteins, e.g. Mal d 1 from apple, Pru av 1 from cherry, Api g 1 from celery and Dau c 1 from carrot. These homologous allergens show a high similarity in primary, secondary and tertiary structure. At the amino acid level, the fruit allergens Mal d 1 and Pru av 1 show 64-66% sequence identity with Bet v 1, compared to 44% for the homologous vegetable allergens Api g 1 and Dau c 1 [10]. The secondary and tertiary protein structure also exhibit a high degree of similarity, as shown in X-ray crystallization and NMR studies of Bet v 1 and Pru av 1 [11-13]. Despite structural similarities, Bet v 1 homologues show different physico-chemical properties and immune reactivities. For example, Bet v 1L is a hypoallergenic isoform, differing in only 9 amino acids from the highly allergenic Bet v 1a [11, 14-16], despite the high degree of homology and structural similarity between these isoforms.

Current purification methods for natural allergens include steps which may induce conformational changes caused by denaturing agents, e.g. acetone used for precipitation [17] and trifluoroacetic acid and acetonitrile, used as components in RP-HPLC protocols [18]. Other methods such as affinity chromatography may be selective for specific epitopes and might thus lead to the loss of isoforms during purification. Also elution of the allergen from the affinity column with highly acidic or basic buffers could result in conformational changes [19].

Studies with purified proteins will inevitably be an approximation of *in vivo* situations, because any purification procedure or change in the physical or biochemical environment may lead to changes in functional properties. The use of allergens, isolated from natural sources, avoids possible erroneous chain folding as may occur in heterologous expression systems. In addition, when recombinant proteins are used, patients will be exposed to only one isoform and not to a proportional mixture reflecting their natural presence in food. Avoidance of organic solvents, chaotropic agents, or extreme physico-chemical conditions during isolation from natural sources is more likely to result in purified proteins that maximally resemble their natural equivalents. The aim of this study was to develop a method that not only allows purification of Bet v 1 from pollen, but also cross-reactive allergens e.g. Dau c 1 from carrot root (*Daucus carota*) and Api g 1 from celery tuber (*Apium graveolens*).

2.2 Materials and methods

2.2.1 Protein Extraction

Carrots from the cultivar *Daucus carota* var. Narbonne (de Wit & Zn., Hoogkarspel, The Netherlands) and celeriac (*Apium graveolens*), purchased from a local supermarket were used for purification of Dau c 1 and Api g 1, respectively. Small pieces of tissue were frozen in liquid nitrogen and ground to a fine powder with a Waring blender. Proteins were extracted from 300 g frozen powder in 300-400 mL 10 mM potassium phosphate extraction buffer, pH 7.0, containing 1 mM EDTA, 0.1% (w/v) ascorbate, 4 mM DTT, 1 mM PMSF, 2% (w/v) polyvinylpolypyrrolidone, and 10 mM diethyldithiocarbamate following a combination of the methods of Björksten et al. [20], Rudeschko et al. [21], and Yamamoto et al. [8]. The suspension was blended for at least 5 minutes, filtered over 4 layers of cheesecloth and centrifuged for 60 minutes at 16,000 g at 4 °C. The supernatant was used for ammonium sulfate precipitation. For extraction of Bet v 1, 5% (w/v) pollen from a single tree, *B. pendula* var. Youngii, were stirred overnight at 4 °C in the same extraction buffer. The extract was filtered over cheesecloth and centrifuged according to the same procedure.

2.2.2 Ammonium Sulfate Precipitation

Dau c 1, Api g 1 and Bet v 1 were recovered from the supernatants by adding gradually increasing concentrations of ammonium sulfate to 50, 60, 70, 80, 90 and 100% saturation followed by stirring for 30 min at 4 °C. The protein pellets were collected by centrifugation and redissolved in 10 mM potassium phosphate buffer at pH 7.0 containing 2 mM EDTA. Each fraction was analyzed with SDS-PAGE. The precipitates at 70-100 % $(NH_4)_2SO_4$ saturation for carrot and birch and at 60-80% $(NH_4)_2SO_4$ saturation for celery were collected and redissolved in 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and $(NH_4)_2SO_4$ to a final concentration of 1 M for Dau c 1 and 1.5 M for Api g 1 and Bet v 1.

2.2.3 Hydrophobic Interaction Chromatography

Protein binding was screened, on an FPLC™ System (Amersham Biosciences, Uppsala, Sweden), with the HiTrap™ hydrophobic interaction chromatography (HIC) selection kit (Amersham Biosciences), containing 5 different prepacked 1 mL columns with 3 different alkyl ligands; butyl, phenyl or octyl. The phenyl ligand was coupled to Sepharose in 3 different columns differing in mean bead size, bead size range and ligand density. Samples were centrifuged at 30,000 g at 4 °C for 60 minutes, followed by filtering on a 0.45 µm ProFill regenerated cellulose filter (Alltech Associates Inc., Deerfield, IL, USA), before loading onto the HIC columns. Buffer solutions of 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA with and without ammonium sulfate, were degassed and filtered over an OE 66 cellulose acetate 0.2 µm membrane filter (Schleicher & Schuell, Dassel, Germany) before use. The columns were first equilibrated with high salt concentration buffers of $(NH_4)_2SO_4$, 1 M for Dau c 1 and 1.5 M for Api g 1 and Bet v 1. After loading the protein and washing the column, fractions were collected by elution with buffer without $(NH_4)_2SO_4$ at 1 mL/min.

2.2.4 Size Exclusion Chromatography

The total protein fraction from HIC was concentrated in a Microsep 10K Omega centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA) to a volume of about 500 µL before SEC. The concentrated samples were loaded on a XK 16/70 column (Amersham Biosciences) packed with 120 mL Superdex® 75 prep grade (Sigma-Aldrich Inc., St. Louis, MO, USA). Proteins were collected upon elution with 10 mM potassium

phosphate buffer, pH 7.0, at 0.5 mL/min. Pooled fractions were concentrated and analyzed with SDS-PAGE, followed by quantification with the Micro BCA™ Reagent Protein Assay (Pierce, Rockford, IL, USA).

2.2.5 SDS-PAGE Gel Electrophoresis

Samples from the various purification steps were analyzed by SDS-PAGE for the presence of Bet v 1, Dau c 1 and Api g 1 by monitoring the occurrence of a band with relative masses (Mr) at 16-18 kDa. Proteins were separated on a 15% (w/v) acrylamide SDS-PAGE gel with a 5% (w/v) stacking gel, using the Mini-Protean II gel system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Gels were stained with CBB R250 and analyzed with Quantity One Bio-Rad scanner software. A Low Molecular Weight calibration Kit (Amersham Biosciences) marker was used to determine relative molecular masses.

2.2.6 Isoelectric Focusing

Homogeneity of the isolated 16-18 kDa proteins of Api g 1 and Dau c 1 fractions after SEC was analyzed by IEF on the Phastsystem™ (Amersham Biosciences). Phastgel™ IEF gels (Amersham Biosciences) with a pH range of 4-6.5 were used and calibrated with an Isoelectric Focussing Kit (Amersham Biosciences). The IEF gels were stained with CBB R350.

2.2.7 Identification of Proteins using Q-TOF MS/MS

The amino acid sequences of the purified allergens from birch, carrot and celery were determined using a Q-TOF 2 mass spectrometer (Waters, Milford, MA, USA). Samples of purified Bet v 1, Dau c 1 and Api g 1, containing 30 µg of protein, were run on a 15% (w/v) SDS-PAGE gel and subsequently stained with CBB. The Bet v 1 sample showed two protein bands at a relative molecular mass of 16-18 kDa and impurities around 29 and 35 kDa. These 4 bands were cut out of the gel and sliced into 1 mm^3 -pieces. Also the 16-18 kDa protein and minor impurities at 25 and 33 kDa for both Dau c 1 and Api g 1 were all analyzed separately.

Proteins were reduced with DTT and alkylated with iodoacetamide [22]. Gel pieces were dried under vacuum and swollen in 0.1 M $NaHCO_3$, containing 5 mM calcium chloride and sequence grade porcine trypsin (10 ng/µl, Promega Corp., Madison, WO,

USA). After overnight incubation at 37 °C, peptides were extracted from the gel with 50% (v/v) acetonitrile, 5% (v/v) formic acid and dried under vacuum. The peptides were redissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile and loaded onto a C18 Atlantis column (15 cm x 75 µm ID, Waters, Milford, MA, USA). Peptides were eluted by a linear gradient (30 min) from 0.5%(v/v) formic acid in 5% (v/v) acetonitrile to 0.5% (v/v) formic acid in 50% (v/v) acetonitrile at approximately 0.2 µL/min (resulting from a 1:20 split of 4 µL/min flow generated by the Waters CapLC pumps). The C18 column was connected to a PicoTip (New Objective, Woburn, Massachusetts) which produced an electro-spray to be analyzed by a Q-TOF-2 mass spectrometer (Waters, Milford, MA, USA). The Q-TOF mass spectrometer was programmed to determine charge states of the eluting peptides, and to switch from the MS- to the MS/MS-mode for $z \geq 2+$ at the appropriate collision energy for Argon gas-mediated CID. The resulting CID MS/MS spectra contained the sequence information for a single peptide per spectrum.

The ProteinLynx GlobalServer package V2.1 software (Waters, Milford, MA, USA) was used to process MS/MS data. Raw MS/MS spectra were deconvoluted to produce monoisotopic singly charged spectra with the proprietary MaxEnt3 algorithm. MS/MS spectra containing good quality CID products were automatically searched for sequence matches using the NCBI non-redundant protein database. Unassigned MS/MS spectra were automatically processed using the AutoMod algorithm, developed to identify amino acid substitutions, post-translational modifications and partial or non-specific cleavages. De novo sequences were generated with the MassSeq tool to search for the most likely protein homologues in the database.

2.2.8 Circular Dichroism Spectroscopy

CD spectra of Bet v 1, Dau c 1 and Api g 1 were recorded at 20 °C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) equipped with a quartz cuvette of 1 mm path length. Far-UV spectra were recorded from 185-260 nm in 10 mM potassium phosphate buffer pH 7.0 filtered through a 0.2 µm syringe filter (Schleicher & Schuell) at a protein concentration of 2.9 µM Bet v 1, 5.8 µM Dau c 1 and 6.3 µM Api g 1. To increase signal-to-noise values, 20 scans were accumulated at a scanning speed of 50 nm/min, a 0.2 nm step width and 2 nm bandwidth. The buffer spectra were

subtracted from the protein spectra and the mean residue weight ellipticity $[\theta]_{MRW}$ was calculated from the following equation:

$$[\theta]_{MRW} = \frac{100 \times [\theta]_{obs}}{C \times l \times n} \qquad (1)$$

Here, $[\theta]_{obs}$ is the observed signal in degrees, C is the concentration in mol/L, l is the path length of the cuvette in cm and n is the number of amino acids. The program CDNN was used to deconvolute the secondary structure [23].

2.2.9 ELISA

Immunodetection was performed by means of indirect ELISA. Ninetysix-well microplates (Greiner Bio-one, Frickenhausen, Germany) were coated with 2 µg/mL of natural Dau c 1, Api g 1 and Bet v 1 and the recombinant allergens rDau c 1.2, rApi g 1 and rBet v 1a (Biomay, Vienna, Austria) in coating buffer: 40 mM $NaHCO_3$, 9 mM Na_2CO_3, pH 9.6, 100 µL/well, by incubation for 1 h at 37 °C. All subsequent incubations were performed at room temperature on a microplate shaker. Coating solution was removed and 200 µL/well of blocking buffer: 2% (w/v) BSA in PBS: 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl , 130 mM NaCl, pH 7.4, was added and incubated for 1 h. Microplates were washed after each incubation step in a microplate washer (Anthos Fluido, Anthos Labtec Instruments, GmbH. Austria) with 4 times 400 µL/well of washing buffer: 0.05% (v/v) Tween 20, 0.05% (w/v) BSA in PBS. During incubation, microplates were sealed. Serum in PBS (1:5, 100 µL/well) was added as primary antibody and incubated for 3 h. Human sera of six birch pollen-allergic patients, which were previously shown to cross-react with the carrot allergen Dau c 1 and the celery allergen Api g 1, were obtained from the Laboratory for Primary Health Care (SHO, Velp, The Netherlands). Specific IgE was determined for each serum by the ImmunoCAP method (Pharmacia, Uppsala, Sweden). Sera with birch pollen-specific IgE of >100 kU/L were used. Wells were subsequently incubated for 1 h with 100 µL of 1:1000 monoclonal mouse anti-human IgE (DakoCytomation, Denmark or Sigma-Aldrich) in dilution buffer (0.1% (w/v) BSA in PBS pH 7.4) and 100 µL of 1:1000 goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) in dilution buffer, both during 1 h. Freshly prepared substrate solution, containing 1 mg/mL 4-nitrophenylphosphate disodium salt in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 1 mM $MgCl_2$, pH 9.6), by adding 200 µL/well. Color development was

measured at λ=405 nm using a microplate reader (ThermoLab Systems, Franklin, MA, USA). Sera from non-birch pollen allergic individuals with birch pollen-specific IgE of < 0.35 kU/L, were included as a negative control. Measurements were performed in triplicate.

2.2.10 Western Blot

After electrophoretic separation of protein sample containing 2-3 µg of natural or recombinant protein by 15% (w/v) SDS-PAGE, proteins were either stained with CBB or transferred to a PVDF ImmobilonTM–PSQ membrane (Millipore, USA). Semi-dry blotting was performed with a Semi-phor (Hoeffer Scientific Instruments, San Francisco, CA, USA) using a constant current of 100 mA for 75 min. Wet blotting was performed with a Mini Trans-Blot® (Bio-Rad) using a constant voltage of 100 V for 60 min. Membranes were blocked with blocking buffer, containing 2% (w/v) milk powder in PBS (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 130 mM NaCl, pH 7.4) and incubated for 1 h at room temperature. Serum (1:10) in 0.1% (w/v) milk powder in PBS 7.4 was added as primary antibody and incubated at 4-5 °C overnight. Membranes were sequentially incubated for 1 h with mouse anti-human IgE (1:1000, DakoCytomation or Sigma-Aldrich) in dilution buffer: 0.1% (w/v) milk powder in TBS: 20 mM trizma-base, 150 mM NaCl pH 8.2, followed by incubation for 1 h with goat anti-mouse IgG antibodies (1:1000) conjugated with alkaline phosphatase (Sigma-Aldrich) in dilution buffer. After each incubation step, membranes were washed for 2 times 5 minutes with washing buffer 1 (0.1% (v/v) Tween 20, 0.1% (w/v) milk powder in PBS pH 7.4) and for 3 times 5 minutes with washing buffer 2 (0.1% (w/v) milk powder in TBS pH 8.3). Freshly prepared substrate solution was added, consisting of 5% (w/v) 5-bromo-4-chloro-3-indolylphosphate and 5% (w/v) nitroblue tetrazolium in DMF added to 100 mM trizma-HCl, 5 mM $MgCl_2$, 100 mM NaCl, pH 9.6.

2.3 Results and discussion

2.3.1 Purification of the natural allergens Dau c 1, Api g 1 and Bet v 1

Birch, carrot and celery extracts were monitored for the presence of proteins, with relative molecular masses at 16-18 kDa, by SDS-PAGE during purification. Clear

protein bands were visible at 16-18 kDa in the cell free extracts of carrot, celery (Figure 1A and 1B) and birch (not shown). The majority of the 16-18 kDa protein from carrot was detected in the fractions collected at 80, 90, and 100% ammonium sulfate saturation (Figure 1A). Similar results were obtained for Bet v 1. The majority of the 16-18 kDa Api g 1 protein precipitated at 70 and 80% ammonium sulfate saturation (Figure 1B).

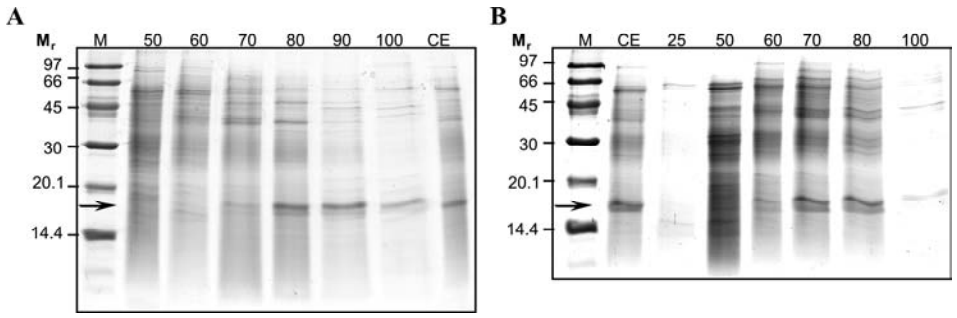

Figure 1. Ammonium sulfate precipitation of carrot and celery proteins. **A**) Precipitated protein in each carrot fraction; **B**) Precipitated protein in each celery fraction. The percentage of ammonium sulfate saturation is displayed on top of the lanes. CE: cell-free extract before ammonium sulfate precipitation. M: molecular marker. The arrow indicates the position of Dau c 1 and Api g 1.

The precipitated ammonium sulfate saturated fractions from 70-100% of Bet v 1 and Dau c 1 and from 60-80% of Api g 1 were used for concentration with HIC and further purification using SEC. From 5 different types of Sepharose HIC columns tested, the best results were obtained with a HiTrap octyl FastFlow column, with purity as selection criterion. The main aim of the HIC step was to concentrate the protein sample and to remove most of the ammonium sulfate. The unbound HIC fractions contained no protein bands at 16-18 kDa when analyzed with SDS-PAGE (not shown). This resulted in enrichment by a factor 2 of the three allergenic proteins after elution from the HIC column with low salt. Proteins were not eluted with a gradient, because the protein gradually released the column, resulting in a dilute fraction.

In HIC, proteins bind in their native state to the alkyl ligands of the column by hydrophobic interactions. This type of binding is not likely to be selective for certain isoforms, which can subsequently be recovered by non-denaturing agents. Hydrophobic characteristics of Bet v 1, Dau c 1 and Api g 1 are similar according to the ProtScale tool [24]. Therefore, we conclude that non-selective HIC is a better method to preserve the

native state of natural proteins compared to purification methods as Reversed Phase HPLC, in which highly denaturing agents are used.

SEC of HIC-concentrated Bet v 1, Dau c 1 and Api g 1 fractions showed similar results. Two peaks were clearly visible in the elution patterns as shown for the HIC purified carrot extract (Figure 2A). Peak A contained high molecular mass proteins not related to Dau c 1 (Figure 2B, lane 1). Peak B contained the 16-18 kDa Dau c 1 protein with minor impurities (Figure 2B lane 3 and 4) of approximately 25 and 35 kDa, which are clearly visible in lane 2 of Figure 2B. Fraction 4 was used for further experiments and fraction 3 was saved for a second SEC run. Analysis by SDS-PAGE indicated 98-99% purity for all purified allergens. Yields for Bet v 1, Dau c 1 and Api g 1 were estimated at 250 mg/kg birch pollen, 3 mg/kg carrot and 10 mg/kg celery, respectively.

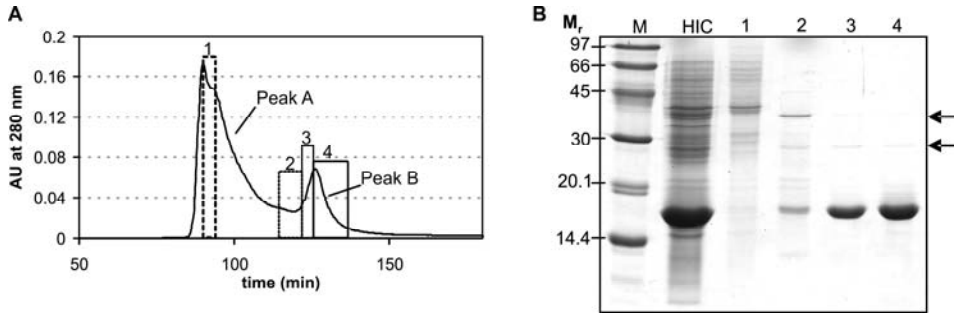

Figure 2. Size exclusion chromatography of carrot proteins, concentrated by HIC. **A**) Two major peaks were visible after SEC, peak A and peak B. Boxes 1 through 4 correspond with the numbers of the SDS-PAGE gel of Figure 2B; **B**) SDS-PAGE of the purified Dau c 1 allergen after SEC. M: molecular marker. HIC: Sample after HIC. Arrows indicate minor impurities at 25 kDa and 33 kDa.

An alternative for protein purification of natural Dau c 1 and Api g 1 might be affinity chromatography, but the available monoclonal antibodies used as stationary phases for the purification of Bet v 1 or Mal d 1, do not recognize an epitope of Dau c 1 and Api g 1 (personal communication R. van Ree, AMC, Amsterdam, The Netherlands). Affinity chromatography also results in a mixture of different isoforms, as was reported for Mal d 1 from apple [25]. Another purification method for the natural Mal d 1 allergen is described by Fahlbusch et al. [18], who used Reversed Phase HPLC and anion exchange chromatography. These methods do not exclude that isoforms are lost by selective extraction, which may lead to an altered immune reactivity as compared to

the isoform mixture in the source tissue. As Reversed Phase HPLC uses highly denaturing components e.g. TFA and acetonitrile, this may affect protein conformation and therefore immune reactivity.

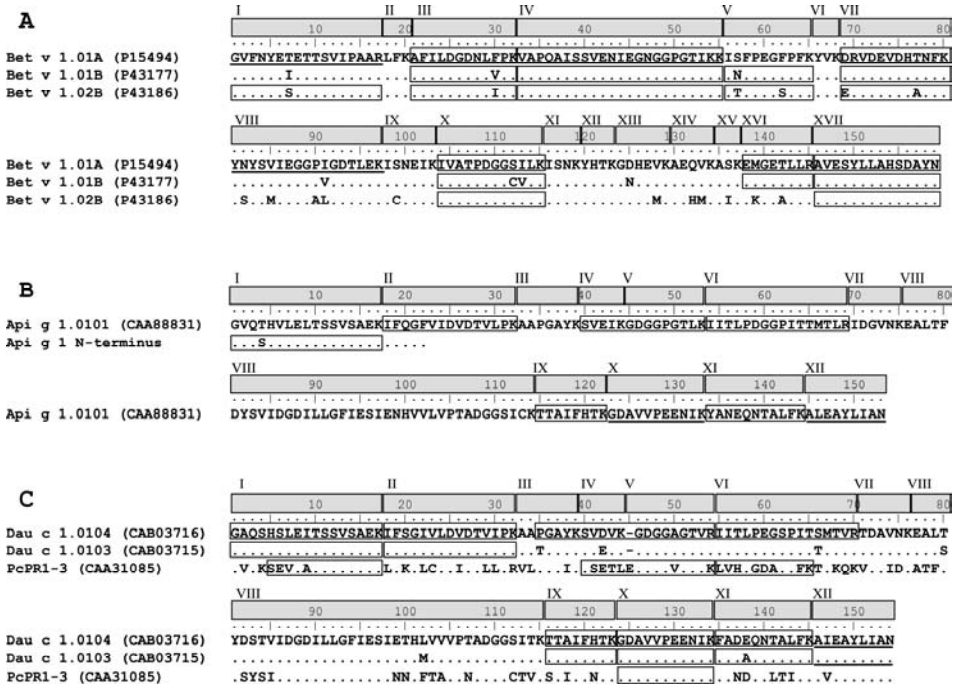

Figure 3. Alignment of Bet v 1, (**A**), Api g 1 (**B**) and Dau c 1 (**C**) with the identified peptides, as revealed by Q-TOF MS/MS analyses. The accession numbers of the different isoforms are given between brackets in the alignments. Theoretical tryptic peptides are displayed with grey-shaded boxes and Roman numbers at the amino acid positions. Peptides identified in the MS-mode and MS/MS-mode are respectively underlined and displayed in a box. Dots indicate identical amino acids with the aligned allergen and dashes indicate gaps.

2.3.2 Characterization of purified Bet v 1, Api g 1 and Dau c 1 by Q-TOF mass spectrometry

After tryptic digestion of the 16-18 kDa protein bands of purified Bet v 1, Api g 1 and Dau c 1 the resulting peptides were separated by nanoflow reversed phase LC and on-line sequenced with the Q-TOF operating in MS/MS mode. In Figure 3, tryptic peptides, from which complete amino acid sequences were obtained in the MS/MS mode, are emboxed, while peptides, only characterized in the MS-mode by their

molecular mass, are underlined. Thirteen different peptide sequences were identified in the MS/MS spectra generated for Bet v 1 (Figure 3A), representing at least three isoforms of Bet v 1 in the purified sample. These isoforms are encoded by different Bet v 1-type genes [26], namely Bet v 1.01A coding for Bet v 1a (Accession no. P15494), Bet v 1.01B coding for Bet v 1d (Accession no. P43177) and Bet v 1.02B coding for Bet v 1m (Accession no. P43186). Six of these peptides could be assigned to tryptic peptides predicted for Bet v 1.01A. Three sequences were specific for the tryptic peptides III, V and X of Bet v 1.01B, while four peptides were specific for the tryptic fragments I, III, V and VII of Bet v 1.02B. From these isoforms the following proportions of the complete protein were fully sequenced in the MS/MS mode: Bet v 1.01A 50.9%, Bet v 1.01B 57.2% and Bet v 1.02B 62.9%. In total, 13 Bet v 1-type genes are known, but only a subset is predicted to be expressed in pollen based on the detection of mRNA's in pollen tissue [16, 26].

In addition to the MS/MS mode, which provides amino acid sequence information, the sample was run in the continuous MS mode (Figure 4A). The combined MS spectrum reveals the molecular masses after deconvolution from m/z-ratios, where m= molecular peptide mass and z = peptide charge, of all peptides present in the digest. All major peaks could be assigned to tryptic peptides originating from either Bet v 1a, d or m, using the sequence information as obtained from the MS/MS run. An additional number of peptide masses in the MS spectrum of Figure 4A could be assigned to tryptic peptides originating from one or more of the three Bet v 1 isoforms based on their exact mass (Figure 3A, underlined peptides).

The protein band at an estimated relative molecular mass of 35 kDa in the SDS-PAGE gel was identified as dimeric Bet v 1 since it had similar MS and MS/MS spectra of tryptic peptides as the monomeric Bet v 1. A 29 kDa protein in the Bet v 1 sample was also sequenced from the gel, but showed no homology to Bet v 1 and could not be identified on the basis of a search in the NCBI non-redundant protein database.

Breiteneder et al. [6] first described the purification and molecular characterization of a major allergen of celery, classified as Api g 1.0101. In our sample, six of the predicted tryptic peptide sequences of Api g 1.0101 were confirmed by MS/MS analysis (Figure 3B), resulting in a total protein coverage of 41.2%. The predicted peptides IV+V were sequenced as a partial digest (no tryptic cleavage after lysine at position 44).

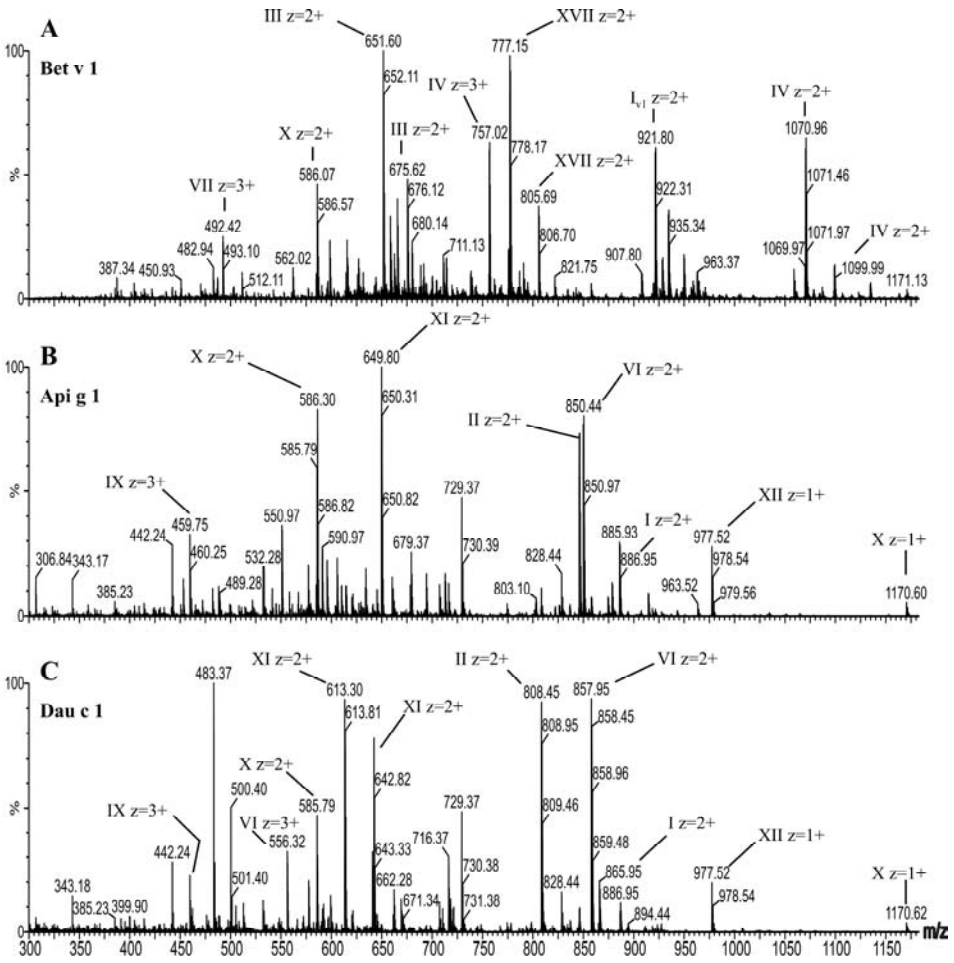

Figure 4. Q-TOF MS spectra of tryptic peptides of Bet v 1 (**A**), Api g 1 (**B**) and Dau c 1 (**C**). The x-axis represents the m/z-values of the peptides. Identified peptides are annotated with the tryptic peptide numbers, their charge state (z) and the corresponding m/z-values. Peptide numbers refer to Figure 3.

Also the homologous N-terminal part of Api g 1 was sequenced. However, we observed an amino acid substitution at position 4 (Ser4 for Thr4), which was also found by N-terminal sequencing of the Api g 1 allergen by Schöning et al. [27]. This indicates the occurrence of an Api g 1 isoform in our sample, which deviates at the N-terminus from the one present in existing sequence databases.

The sequences obtained after MS/MS analysis of the tryptic peptides present in the digested band of purified Dau c 1 are depicted in Figure 3C. Sequences of nine tryptic peptides coincided with the amino acid sequences of the predicted peptides I-VI and IX-XI of Dau c 1.0104 (Accession no. CAB03716) with a protein coverage of 62.7%. The predicted peptide III-V were found as a partial digest, in which trypsin was unable to cleave after lysine residues at positions 39 and 44. Also, an amino acid substitution was observed in peptide XI (Ala137 for Glu137), indicating the presence of one of the isoforms Dau c 1.0101, 1.0102, 1.0103 (Figure 3C, Accession no. CAB03715), or 1.0105. Four other peptides had sequences identical to the parsley PcPR1-3 Bet v 1 homologue (Accession no. CAA31085) [28]. IEF showed two major bands for Dau c 1 (not shown). This supported the findings with Q-TOF MS/MS for at least two isoforms for carrot, although we were able to identify a third one.

Two additional protein bands in the Dau c 1 sample were visible at 25 and 35 kDa, respectively (Figure 2B). These minor impurities were identified with Q-TOF MS/MS as actin and isoflavone reductase, respectively. The latter belongs to the group of the Bet v 6 homologous allergens [29]. Therefore, the 35 kDa protein band is not a dimeric form of Dau c 1.

Figure 4B-C show the combined MS spectra of the tryptic peptides present in the purified Api g 1 and Dau c 1 samples. All major peaks could be assigned to sequences as determined by MS/MS analysis of the corresponding tryptic digests of Api g 1 and Dau c 1. The two MS spectra contain several peaks with identical mass peaks. This is in agreement with the observation of identical sequences for these peptides of Api g 1 and Dau c 1 in MS/MS (see also Figure 3B-C).

2.3.3 Circular Dichroism

The CD spectra of Bet v 1, Dau c 1 and Api g 1 (Figure 5) showed that the isolated proteins were similarly folded. A broad minimum around 218 nm was observed for these allergens. Similarly, maximum ellipticity was observed at about 196 nm where the peak height for Bet v 1 was lower as compared to that for Dau c 1 and Api g 1. Other small differences were observed at the x-axis intercepts, 205 nm spectra of Bet v 1 and Api g 1 and 204 nm for Dau c 1. The CD spectra were deconvoluted using the wavelength range from 190-260 nm with the CDNN program of Böhm et al. [23]. The distribution of the estimated secondary structure is shown in Table 1. Between the

three allergens no significant differences were observed. These CD spectra were also very similar to those of natural and recombinant Bet v 1 [30] and recombinant Pru av 1 [31] reported earlier.

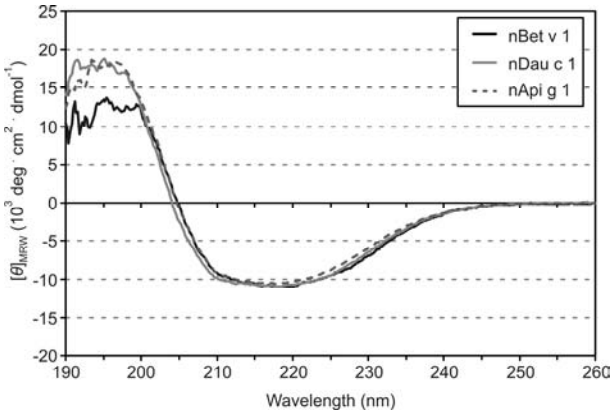

Figure 5. Circular dichroism spectra of natural Bet v 1, Dau c 1 and Api g 1.

Table 1. Secondary structure prediction of Bet v 1, Dau c 1 and Api g 1 as derived from circular dichroism analyses.

	Protein		
	Bet v 1	Dau c 1	Api g 1
α-helix	29.0%	31.2%	30.3%
β-sheet	19.7%	18.9%	20.5%
β-turn	15.8%	16.3%	16.6%
random coil	39.2%	33.6%	33.6%

CDNN was used for secondary structure prediction.

2.3.4 Immunoblotting

Natural Bet v 1 (Figure 6A), Dau c 1 and Api g 1 (Figure 6B) showed immune reactivity in Western blot experiments with Bet v 1-specific IgE patient sera cross-reacting with the corresponding recombinant birch, carrot and celery allergens. The IgE binding capacity of natural and recombinant allergens were similar for carrot and celery. A lower IgE binding capacity was observed for natural Bet v 1 compared to recombinant Bet v 1. The isoform that is used to produce the recombinant protein is Bet v 1a, which is known to be the most allergenic isoform. Natural Bet v 1 is a mixture of at least three isoforms, including the hypoallergenic isoform Bet v 1d [14]. This mixture is thus likely

to have a reduced IgE reactivity compared to the recombinant protein for the majority of patients. Both dimers from natural and recombinant Bet v 1, though not visible in SDS-PAGE, showed clear immune reactivity. Recombinant Dau c 1 showed two antibody binding proteins, while only one band was visible in SDS-PAGE. The higher molecular mass of recombinant Dau c 1, compared to natural Dau c 1, as visible in SDS-PAGE and Western blot, resulted from a His-tag, fused with the recombinant protein. The second immune reactive protein band in the Western blot of the recombinant Dau c 1 sample, at lower molecular mass, could best be explained as a result of protein degradation, revealing an important epitope for Dau c 1 with higher affinity for IgE (personal communication, M. Susani, Biomay, Austria). Possible contaminants in the allergen samples did not exhibit immune reactivity in Western blot experiments with IgE from Bet v 1 allergic patients, demonstrating that immune reactivity in the allergen isolates was caused by Bet v 1, Dau c 1 and Api g 1 only.

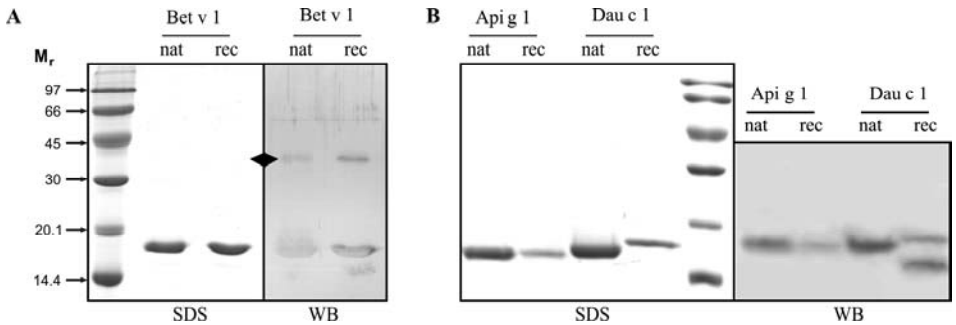

Figure 6. SDS-PAGE and Western blot with natural and recombinant Bet v 1 (**A**) and Api g 1 and Dau c 1 (**B**) with human sera from birch pollen allergic patients. The diamond–shaped marker indicates the location of the Bet v 1 dimer. WB: Western blot, M: Molecular marker.

The immune reactivity of natural Bet v 1 (Table 2), Dau c 1 and Api g 1 (Table 3) was also confirmed in two different ELISAs, where they reacted with 6 different birch-allergic patient sera. OD-values with standard deviations are expressed as relative values with the highest OD value set to 100, showed that reactivity was not exactly identical for each serum and allergen. However, the natural allergens bind to Bet v 1-recognizing IgE antibodies. Differences in ELISA reactivity between the natural and recombinant proteins could be explained by the presence of isoform mixtures of the natural proteins and possibly by serum-specificity. A lower immune reactivity for the natural Bet v 1

allergen was expected due to the presence of a hypoallergenic isoform, as shown by Q-TOF MS/MS. The natural Dau c 1 mixture was shown to be primarily composed of the Dau c 1.0104 isoform, while the recombinant protein consisted of isoform Dau c 1.0103, which may explain the difference in immune reactivity of these two samples.

Table 2. Results of ELISA with purified natural and recombinant Bet v 1 using several human patient sera A-F of Bet v 1 allergic patients.

	Bet v 1			
	nat		rec	
Serum	%	sd	%	sd
A	44.3	7.24	74.2	8.97
B	59.3	3.54	93.0	15.3
C	84.1	5.00	85.2	10.4
D	81.7	8.36	**100**	6.76
E	20.8	5.88	23.8	4.20
F	33.7	9.99	17.7	5.89

IgE binding capacity with standard deviations (sd) are given as a relative value and compared to the highest score, set to 100%, indicated in bold.

Table 3. Results of ELISA with purified natural and recombinant Dau c 1 and Api g compared to recombinant Bet v 1.

	Bet v 1		Dau c 1				Api g 1			
	rec		nat		rec		nat		rec	
Serum	%	sd	%	sd	%	sd	%	sd	%	sd
G	68.5	2.00	29.5	3.68	26.7	0.464	27.4	2.56	25.2	5.66
H	49.7	3.04	25.1	4.25	14.6	0.317	18.5	0.628	15.7	2.14
I	92.2	1.76	21.9	0.912	15.0	0.293	16.5	2.60	23.9	1.07
J	43.4	2.67	21.3	3.03	14.0	1.34	14.2	4.98	11.0	0.518
K	46.6	2.11	17.3	5.58	20.1	0.488	16.7	3.09	23.7	2.62
L	**100**	4.32	18.5	3.69	26.7	1.61	20.6	1.26	28.8	3.25

Six different human sera were used of Bet v 1 allergic patients cross-reactive to Dau c 1 and Api g 1 (G-L). IgE binding capacity with standard deviation (sd) is given as a relative value and compared to the highest score, set to 100%, indicated in bold.

2.4 Concluding remarks

Until now, no purification method has been described for Dau c 1 and Api g 1 from their natural source. Purification of these allergens was hitherto performed on proteins expressed in recombinant systems. Advantages of recombinant proteins are the high degree of homogeneity, the high yield of protein and a similar reactivity in IgE binding studies. However, recombinant proteins will not be composed of isoform mixtures and they will not include ligands [12, 15, 32], as present in the natural matrix. Moreover, it is not known whether they are folded into the correct native state [33, 34].

In conclusion, natural Bet v 1, Dau c 1 and Api g 1 allergens can be obtained at 98-99% purity using a three-step-method, using ammonium sulfate precipitation, HIC and SEC, which are non-denaturing methods. The Bet v 1 and Dau c 1 samples, contained multiple isoforms as was shown by Q-TOF MS/MS. The characterization of these proteins, on the basis of CD spectra and immune reactivity, indicated a folded state of the protein comparable to that of recombinant proteins. The advantage of these allergen samples from natural sources is that they show the immune reactivity of naturally occurring isoform mixtures.

2.5 Acknowledgements

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Chapter 3

Allergenicity of natural and recombinant PR-10 allergens in relation to thermal stability

This chapter has been submitted as:
Bollen, M.A., Jeurink P.V., Wichers, H.J., Helsper, J.P., van Boekel, M.A., and Savelkoul, H.F., *Allergenicity of natural and recombinant PR-10 allergens in relation to thermal stability.*

Abstract

The birch pollen allergen, Bet v 1 is a sensitizer for food allergy to Api g 1 and Dau c 1 from celery and carrot. Purified natural isoform mixtures and recombinant Bet v 1, Api g 1 and Dau c 1 were subjected to heat treatment to correlate structural changes, measured by circular dichroism, with immune reactivity in human PBMC cultures and ELISA. All allergens refolded after heating to 95 °C, but changes in secondary structure were observed after cooling the allergen samples, especially for the natural Bet v 1. Api g 1 and Bet v 1 showed a similar midpoint of thermal denaturation, T_m, but natural and recombinant Dau c 1 were less stable to heating. Heating for 2 hours at 100 °C completely destroyed allergenicity, unlike heating for 30 minutes at 95 °C. This allergenic potential was apparent from differential activity in ELISA assays.

3.1 Introduction

In Northern European countries, birch pollen allergy is the primary cause for cross-reactive allergies towards other trees (*Betulaceae*), fruits (*Rosaceae*), vegetables (*Apiacaea*) and legumes (*Fabaceae*). The major allergen in birch pollen is Bet v 1, which belongs to the PR-10 class of pathogenesis-related proteins [1-3]. Members of this protein family in foods show a high amino acid identity with Bet v 1 and are also similarly folded as shown with X-ray diffraction and NMR studies [4-7]. The 3D-structure of PR-10 proteins consists of a seven-stranded anti-parallel β-sheet folded around a C-terminal α-helix. The β-sheet and the C-terminal part of the α-helix are separated by two small α-helices [4]. A hydrophobic cavity in the Bet v 1 structure has the ability to bind a broad range of ligands such as fatty acids, cytokinins, flavonoids and sterols. This phenomenon may be of importance for the biological function [5, 8-10].

To evoke an allergic reaction, the allergen needs to be presented as linear peptides bound within the major histocompatibility class II (MHC-II) complex to the T cell receptor on allergen-specific naïve CD4+ T cells. This recognition leads to differentiation of T helper 2 cells (Th2). Under the influence of the cytokines produced by Th2 cells (e.g. IL-4 and IL-13) the allergen-specific B cells will induce antibody isotype switching leading to the production of IgE antibodies that can bind to type I Fcε receptors on mast cells. These events together are called the sensitization phase. A second exposure to the same allergen will lead to the cross-linking of the IgE antibodies on the surface of the mast cell, resulting in the subsequent degranulation of the mast cell releasing inflammatory compounds like histamine, which cause the typical allergic complaints of e.g. hay fever or food allergy [11].

The major symptom of IgE-mediated allergic reactions to Bet v 1 is a rapid and local inflammatory reaction in the upper and lower respiratory organs. Birch pollen-related food allergy is characterized by local reactions in and around the oral cavity (oral allergy syndrome) [12, 13]. In contrast to many other food allergens in which the IgE-reactivity is preserved after some physical-chemical treatment, Bet v 1 allergens have been characterized as structurally labile as they are unstable to heating, denaturation and proteolysis [14-19]. Upon heating, the PR-10 allergen changes conformation and shows decreased IgE binding capacity after it has refolded upon cooling down [20, 21]. After purification, PR-10 allergens appear much more stable [17].

The recombinant Bet v 1.0101 (Bet v 1a) and Bet v 1.0401 (Bet v 1d) or Bet v 1.1001 (Bet v 1L) isoforms display differences in immune reactivity. Bet v 1.0101 is the most commonly used allergen in diagnostic applications, because it strongly binds to specific IgE. The other isoforms Bet v 1.0401 or Bet v 1.1001 differ in 7 or 9 amino acids from Bet v 1.0101, respectively, and have been characterized as hypoallergenic making them useful as allergy vaccines [22, 23]. Hitherto, it remains unclear why these isoforms have a decreased IgE binding capacity and whether this is due to structural differences in protein folding of the recombinant proteins. Differences in circular dichroism (CD) spectra have been observed between the allergenic Bet v 1.0101 and hypoallergenic Bet v 1.1001, whereas X-ray diffraction showed no clear difference between the crystal structure of the different variants [9, 23]. In contrast to the recombinant PR-10 proteins, purification of the natural allergens Bet v 1, Api g 1 and Dau c 1 from birch, celery and carrot, respectively, resulted in a mixture of isoforms [24].

In the present study, the heat-stability of recombinant PR-10 allergens Bet v 1 from birch, Api g 1 from celery and Dau c 1 from carrot was compared with the heat-stability of the same allergens after purification from their natural source. The aim was to study possible molecular differences of PR-10 proteins by linking differences in thermal stability to immune reactivity. By assessing both T cell responses and allergenicity (IgE binding), we were able to show that our natural and recombinant PR-10 proteins had comparable secondary structure and IgE binding capacity, whereas rBet v 1 was not capable of inducing a T cell response. However, heat-treatment affected the natural and recombinant PR-10 proteins in different manners.

3.2 Materials and Methods

3.2.1 Allergens

From each of the natural allergens nBet v 1, nApi g 1 and nDau c 1, isoform mixtures were purified according to **chapter 2**. Briefly, ammonium sulphate precipitation was followed by hydrophobic interaction and size exclusion chromatography. The obtained isoform mixtures were analyzed by mass spectrometry (Q-TOF MS/MS). The single recombinant allergen isoforms rBet v 1a, rApi g 1 and rDau c 1.2 were purchased from Biomay (Vienna, Austria). All allergens were dissolved in 10 mM potassium phosphate -buffer, pH 7.0, buffer exchanged and concentrated on a Microsep 3K centrifugal device

(Pall Life Sciences, Ann Arbor, MI, USA). Protein concentrations were determined using the MicroBCA™ Protein Assay (Pierce, Rockford, IL, USA) with BSA as a standard.

Prior to the human PBMC cultures and ELISA, five different allergen solutions were prepared at 0.1 mg/ml in potassium phosphate buffer, pH 7.0. One of the samples was kept at room temperature and three other samples were heated in a GeneAmp PCR 9700 apparatus at 1 °C/min, which was comparable to heating in the CD spectropolarimeter. The fifth sample was heated in the GeneAmp apparatus for 2 hours at 100 °C to destroy all IgE binding capacity. The different heat-treatments are described in Table 1. Explanation of the given sample labels (Untreated, TG, MG, MQ, and H2) are given in the legend of Table 1. All samples were allowed to equilibrate at room temperature for at least 1 hour before continuing the experiment.

Table 1. Heat treatment schedule of the allergen samples prior to immune experiments

Sample	Heating rate (°C/min)	Final Temp.	Time at final Temp.	Cooling rate (°C/min)
Untreated	-	24 °C	-	-
TG[a]	1.0	T_m	30 min	1.0
MG[b]	1.0	95 °C	30 min	1.0
MQ[c]	1.0	95 °C	30 min	Quick
H2[d]	Quick	100 °C	120 min	Quick

[a]TG: Heated to T_m, Gradually cooled. T_m was determined by the CD measurements as described in section 3.2.2

[b]MG: Heated to Maximum temperature reached with CD, Gradual cooling

[c]MQ: Heated to Maximum temperature reached with CD, Quick cooling

[d]H2: Heated for 2 hours

3.2.2 Circular Dichroism (CD)

CD spectra of natural and recombinant Bet v 1, Dau c 1 and Api g 1 were recorded at 20 °C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at a protein concentration of 10 µM in 10 mM potassium phosphate buffer of pH 7.0. Prior to use, the buffer was passed through a 0.2 µm syringe filter (Schleicher & Schuell, Dassel, Germany). Far-UV spectra were recorded from 190-260 nm with a quartz cuvette of 1 mm path length, by accumulating 20 scans at a scanning speed of 50 nm/min, using a

0.2 nm step width and 2.0 nm bandwidth. Spectra were corrected for buffer background. The mean residue weight ellipticity $[\theta]_{MRW}$ (units in deg·cm^{2}·dmol^{-1}) was calculated from the following equation:

$$[\theta]_{MRW} = \frac{100 \times [\theta]_{obs}}{C \times l \times n} \quad (1)$$

Here, $[\theta]_{obs}$ is the observed signal in degrees, C is the concentration in mol/L, l is the path length of the cuvette in cm and n is the number of amino acids. The program CDNN was used to deconvolute the secondary structure [25].

Thermal denaturation spectra were recorded for all allergens by heating to 95 °C and cooling to room temperature at a rate of 1 °C/min at 222 nm with a bandwidth of 1.0 nm. After cooling, full spectra were measured from 195-260 nm under the same conditions as stated in the previous paragraph. The thermal denaturation curve data were fitted for both the heating and cooling steps, according to a non-linear least square fit method [26] using the program TableCurve (Jandel Scientific, Erkrath, Germany). With this method, six parameters are estimated corresponding to equation 2, which includes the slopes and intercepts of the baselines of the folded/native (β_N) and unfolded/denatured (β_D) states with the ellipticity values (intercepts) for the folded (α_N) and unfolded (α_D) state. The other derived parameters are the temperature at the midpoint of denaturation, T_m, and the enthalpy of unfolding at T_m, ΔH_{Tm}.

$$\theta = \frac{\alpha_N + \beta_N T + (\alpha_D + \beta_D T)\mathrm{e}^{-\left[\Delta H_{Tm}(1-\frac{T}{T_m})\right]/RT}}{1 + \mathrm{e}^{-\left[\Delta H_{Tm}(1-\frac{T}{T_m})\right]/RT}} \quad (2)$$

3.2.3 Blood donors for human allergen specific PBMC culture

Birch-pollen allergic individuals were recruited into the study directly after the birch pollen season (July, 2007). Blood withdrawal was performed at the Hospital Gelderse Vallei (Ede, The Netherlands). Donors were selected based on a positive Bet v 1 – IgE test while being negative for the other birch pollen allergens Bet v 2, Bet v 4, and Bet v 6. The three donor sera contained 66.5, 58.5 and 19.7 kU/L Bet v 1-specific IgE, whereas

the specific IgE levels were < 0.1 kU/L for Bet v 2, Bet v 4 and Bet v 6. An informed consent was obtained before sample collection and experiments were approved by the local ethical committee.

3.2.4 In vitro stimulation studies

The isolation of human peripheral blood mononuclear cells (PBMC) from the birch-pollen allergic individuals, culture conditions, cell viability, determination of the immunological phenotype, the allergen-specific proliferation capacity, and the production of several cytokines were performed according to a detailed previous technical study [27]. The samples were measured on the FACSArray, using the FCAP software (BD Biosciences). Detection limits for quantitative determinations were 1.1 pg/ml for IL-1β, 0.3 pg/ml for IL-4 and IFN-γ, 0.5 pg/ml for IL-5, 2.3 pg/ml for IL-10, 2.2 pg/ml for IL-12 , 0.6 pg/ml for IL-13 and 0.7 pg/ml for TNF-α.

3.2.5 Patient sera for ELISA

Sera were obtained from the Hospital Gelderse Vallei (Ede, The Netherlands) and the Laboratory for Primary Health Care (SHO, Velp, The Netherlands). The sera of birch pollen allergic individuals with IgE levels > 100 kU/L for birch were initially screened for their response to Bet v 1 and cross-reactivity to Api g 1 and Dau c 1. For Bet v 1 ELISA, a serum pool was prepared by combining 14 sera containing high (> 100 kU/L) Betv 1-specific IgE content. For Agi g 1, a serum pool was prepared by combining 14 sera positive for Api g 1-specific IgE while all sera are also positive for Bet v 1-specific IgE. A Dau c 1 serum pool was prepared by combining 7 sera containing Dau c 1-specific IgE. As a negative control, a healthy serum pool of three volunteers was included with birch pollen-specific IgE levels of <0.35 kU/L.

3.2.6 Indirect ELISA

In an indirect ELISA, 300 ng of the heat-treated or untreated allergens in 100 µl PBS pH 7.4 were coated on 96-well MaxiSorp microplates (Nunc, Wiesbaden, Germany) by an overnight incubation at 4 °C. Untreated rBet v 1 was included as a positive control and for the negative control, a healthy control serum pool, and blanks for non-specific binding of the secondary antibody were included. All values obtained in the indirect ELISA experiments were corrected for the blank values and were expressed as

percentage of the positive control (untreated rBet v 1). Plates were blocked for 1 h with 200 µl/well blocking buffer (4% (w/v) BSA in PBS, pH 7.4). After each incubation step, plates were washed 4 times with 400 µl/well washing buffer (0.05% (w/v) BSA and 0.05% Tween-20 in PBS). Serum dilutions (1:1) in dilution buffer (0.1% (w/v) BSA in PBS) were added in duplicate at 100 µl/well and incubated for 1.5 h. After washing, 100 µl/well of an 1 µg/ml biotin-labeled mouse anti-human IgE (BD Biosciences) was incubated for 1 h, followed by a 30 min incubation of 100 µl/well streptavidin poly-HorseRadishPeroxidase (1:10,000; Sanquin, Amsterdam, The Netherlands). Color development was started by the addition of 100 µl/well TMB substrate solution (KPL, Gaithersburg, MD, USA) and stopped by adding 100 µl/well 1 M H_3PO_4. The developed color was measured with a microplate spectrophotometer at 450 nm using 690 nm as a reference wavelength.

3.2.7 Inhibition and cross-reactive inhibition ELISA

In the inhibition ELISA, untreated natural or recombinant allergens of Bet v 1 and Api g 1 were coated on the plate by an overnight incubation of 300 ng/well allergen solution in PBS at 4 °C. Untreated and heat-treated samples of natural and recombinant Bet v 1 and Api g 1 were pre-incubated overnight at 4 °C with the appropriate serum pools in dilution buffer (1:1) at a final allergen concentration of 30, 3, 0.3, 0.06, 0.012 or 0.0024 µg/ml.

In the cross-reactive inhibition ELISA, untreated rBet v 1 was coated to the plates at 300 ng/well. The serum pools responding to Api g 1 and Dau c 1 were pre-incubated with heat-treated or untreated rApi g 1 or nApi g 1 and rDau c 1 or nDau c 1, respectively, at a concentration of 30, 3, 0.3, 0.06, 0.012 or 0.0024 µg/ml.

For the inhibition control in both types of ELISA, coating was performed with the respective untreated allergen and serum pools were added without pre-incubations. As a positive control, coating was performed with rBet v 1 with the addition of the respective positive serum pools, while as a negative control the serum pool from healthy individuals was used. For all different coatings, blanks were used to determine non-specific binding, which were subtracted from all detected values.

3.3 Results

3.3.1 Circular Dichroism (CD)

CD spectra were recorded for a detailed comparison between the purified natural allergen isoform mixtures and commercially obtained recombinant allergens. The effect of heating on the protein conformation was determined by heating to 95 °C followed by gradual cooling down to 20 °C. The effect of heat treatment on the secondary structure of all measured allergens is shown in Table 2. Here, differences in secondary structure elements are not only observed between the three allergens from different plant origin, but also as a result of heating and cooling down. The latter effect is best described as a small but consistent decrease in the proportion of α-helix, which is accompanied not only by an increase in β-sheet and β-turn but also in random coil.

Table 2. Secondary structure of recombinant and natural Bet v 1, Api g 1 and Dau c 1 before and after heating to 95 °C followed by cooling to 20 °C. The secondary structure composition was deconvoluted from the 2

	nBet v 1		rBet v 1		nApi g 1		rApi g 1		nDau c 1		rDau c 1	
Before/after heating	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
α-Helix	20.9	18.0	21.7	19.5	30.2	24.9	29.8	24.3	33.3	28.9	33.4	29.5
β-Sheet	28.5	28.4	26.6	28.5	25.1	26.4	24.3	27.9	21.8	23.6	21.8	23.1
β-Turn	15.2	17.9	14.9	16.0	12.6	14.8	13.0	14.6	12.8	14.3	13.1	14.6
Random Coil	33.7	34.4	34.6	34.2	29.8	31.8	30.2	31.3	29.5	30.9	29.2	30.4
Total Sum[a]	98.3%	98.6%	97.7%	98.3%	97.7%	97.9%	97.3%	98.1%	97.3%	97.6%	97.4%	97.6%

[a]Total sum is the sum of all secondary structure elements and shows that the prediction is near 100% and in the same range for all allergens for direct comparison.

The CD spectra clearly showed differences between the natural and recombinant Bet v 1 allergens before and after the heat treatment (Figure 1), while no such spectral differences were observed between rApi g 1 and nApi g 1 or rDau c 1 and nDau c 1 (data not shown). As is clear form Figure 1, when nBet v 1 and rBet v 1 unfold, the spectra as a whole shift to a lower wavelength, whereas a decrease in amplitude for $[\theta]_{MRW}$ is observed for the maximum at 196 nm and the minimum at 222 nm. The cooled allergens nBet v 1, nApi g 1 and nDau c 1 intersected the x-axis at a lower wavelength of an approximate difference of 2.2, 1.0 and 0.5 nm, respectively, and all three showed a

decrease in the amplitudes for $[\theta]_{MRW}$ at 196 nm and 222 nm. For the three recombinant allergens, a smaller shift of about 0.5 nm was observed.

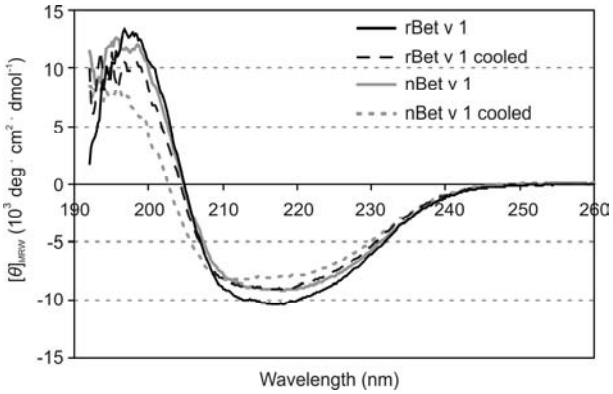

Figure 1. CD Spectra of recombinant and natural Bet v 1 before and after heating. Spectra are shown for nBet v 1 and rBet v 1 measured at 20 °C and after heating to 95 °C and cooling down to 20 °C (cooled).

Thermal denaturation curves were measured by following changes in the CD signal at 222 nm (Figure 2). From the fits of the heating data, two parameters were derived as a measure for the thermal stability; T_m, the temperature indicating the midpoint of thermal unfolding, and ΔH_{Tm}, the enthalpy of unfolding at T_m (Table 3). Dau c 1 has a lower thermal stability compared to Api g 1 and Bet v 1, and this holds true for both natural and recombinant preparations. Unfolding of rDau c 1 started at a low temperature of 30 °C (Figure 2C), whereas nDau c 1 appeared to be more stable given its start of unfolding at 40 °C. The thermal stability of rDau c 1 (T_m of 45.1 °C) differs from that of nDau c 1 (T_m of 55.7 °C). Recombinant and natural forms of Api g 1 and Bet v 1 showed higher and similar T_m-values of approximately 65 °C. Values of ΔH_{Tm} were lower for the natural allergen isoform mixtures compared to recombinant Bet v 1 and Api g 1 (Table 3). The ΔH_{Tm}-value of rDau c 1 was lower than the other recombinant allergens and was in the same range as the natural allergens.

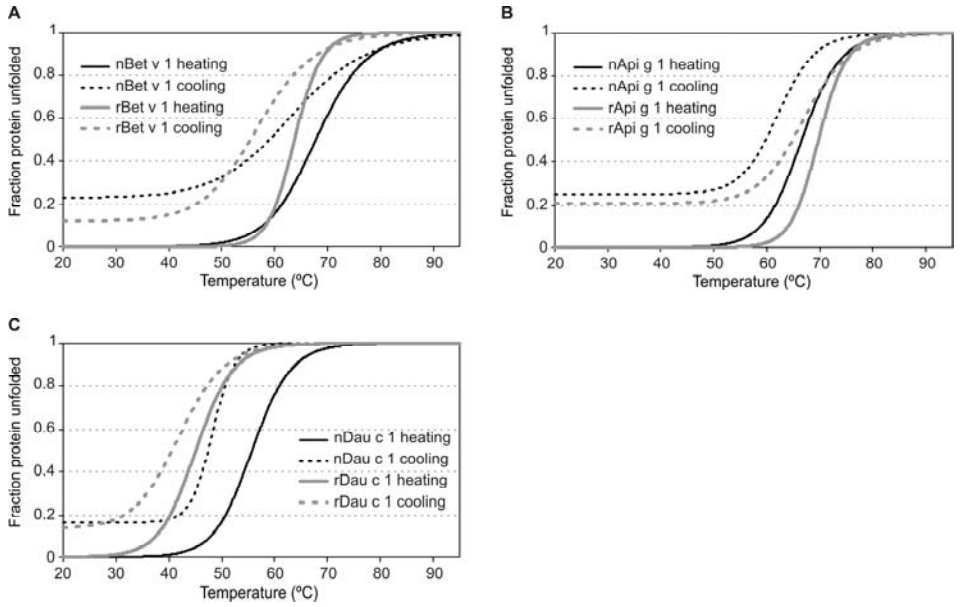

Figure 2. Thermal denaturation of natural and recombinant Bet v 1 (**A**), Api g 1 (**B**) and Dau c 1 (**C**) followed by CD at 222 nm. The curves represent the best fits of the data to a two-state unfolding transition and are shown as the fraction of unfolded protein.

Table 3. Thermodynamic parameters from CD thermal denaturation curves.

Heating		
Natural	T_m (°C)	ΔH_{Tm} (kJ/mol)
nBet v 1	67.8 ± 0.5	204.2 ± 12.0
nApi g 1	66.5 ± 0.1	265.1 ± 7.2
nDau c 1	55.7 ± 0.1	247.0 ± 5.3
Recombinant	T_m (°C)	ΔH_{Tm} (kJ/mol)
rBet v 1	63.7 ± 0.1	371.4 ± 13.0
rApi g 1	69.7 ± 0.1	368.0 ± 12.8
rDau c 1	45.1 ± 0.1	238.1 ± 6.5

T_m and ΔH_{Tm} are shown with the standard deviation from the fits of Figure 2.

3.3.2 The effect of thermal treatment of rBet v 1 on the T cell response

Heat treated rBet v 1 was exposed to the PBMC of three birch pollen allergic individuals. Viability of cells is a prerequisite for cell activation. The viability of the PBMC was assessed by using Annexin V in combination with propidium iodide. The viability of the rBet v 1 stimulated cells did not change compared to the unstimulated medium control, whereas the anti-CD3/anti-CD28 stimulated cells showed an induction of viable cells (data not shown).

As shown in Figure 3A, the amount of proliferating Ki-67$^+$ cells was low in the medium control, but strongly upregulated in the anti-CD3/anti-CD28 stimulated cells. However, this induced proliferation could not be observed in the untreated rBet v 1 stimulated control (Figure 3A) or heat-treated rBet v 1 stimulated control (data not shown). To assess which cells were mainly proliferating within the PBMC cultures, a human PBMC subset staining was performed. The assessed subsets comprised T cells (CD3$^+$), B cells (CD19$^+$), NK cells (CD16/CD56$^+$) and monocytes (CD14$^+$). As depicted in Figure 3B, mainly the T cells, B cells and NK cells were induced in the anti-CD3/anti-CD28 stimulated human PBMC culture compared to the medium control, whereas stimulation by untreated rBet v 1 (Figure 3B) or heat-treated rBet v 1 (data not shown) showed no effect on the PBMC subsets. Figure 3C shows that in the medium control both CD4+ and CD8+ cells were present, although very low amounts were activated. However, addition of anti-CD3/anti-CD28 to the culture resulted in increased numbers of CD4+ and CD8+ cells, and also induced activation of both T cell subsets. As for PBMC subsets addition of untreated (Figure 3C) or heat-treated rBet v 1 did not alter the number or the activation status of the T cell subsets.

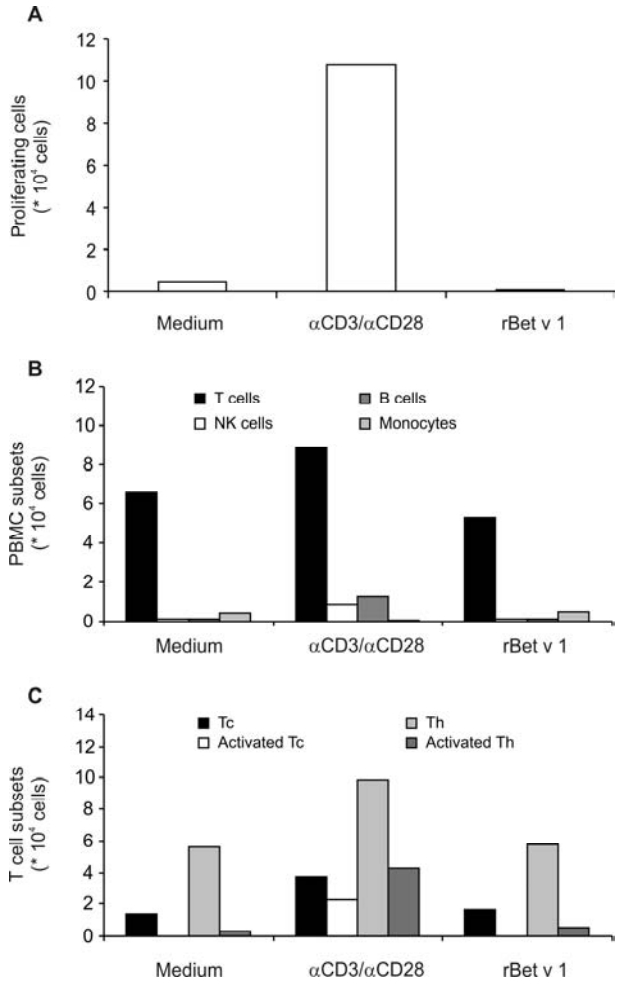

Figure 3. Human PBMC cultures stimulated with untreated rBet v 1 (rBet v 1) were cultured for 7 days. PBMC stimulated with anti-CD3 plus anti-CD28 were cultured for 3 days (αCD3/αCD28). At both time points, Ki-67 positive cells were detected and the relative number of Ki-67$^+$ cells is depicted in Figure 3**A**. Figure 3**B** shows the PBMC surface markers to analyze CD3$^+$ cells (T cells), CD16$^+$CD56$^+$ (NK cells), CD19$^+$ cells (B cells) and CD14$^+$ cells (monocytes). Figure 3**C** shows the PBMC surface markers to analyze CD3$^+$CD4$^+$ cells (Th), CD3$^+$CD4$^+$CD25$^+$ (activated Th), CD3$^+$CD8$^+$ cells (Tc) and CD3$^+$CD8$^+$CD25$^+$ cells (activated Tc).

Cytokine production of the (un-)stimulated PBMC cultures was measured by using the Flexsets (Table 4). Anti-CD3/anti-CD28 was able to induce an upregulation of the monocyte-derived IL-1β and TNF-α, whereas IL-12 was not altered compared to the

medium control. Also the Th1 cytokine IFN-γ, both Th2 cytokines IL-5 and IL-13 and the regulatory cytokine IL-10 were upregulated by anti-CD3/anti-CD28 when compared to the medium control. However, none of the measured cytokines were altered when comparing the rBet v 1 stimulated cells to the medium control, as shown in the lower part of Table 4.

Table 4. Cytokine production by human PBMC.

Stimulation	IL-1β	IL-12	TNF-α	IFN-γ	IL-5	IL-13	IL-10
Medium	12.2	2.9	2.8	6.3	1.6	4.2	3.5
αCD3/28	819	1.3	1762	59939	14.3	287	51
rBet v1	b.d.	b.d.	b.d.	47.7	1.8	2.2	b.d.
rBet v 1 MQ	b.d.	b.d.	1.5	12.4	2.7	4.0	3.8
rBet v 1 MG	b.d.	b.d.	b.d.	17.2	3.7	4.9	4.4
rBet v 1 TG	1.5	2.3	1.6	36.2	2.6	3.9	3.1

Cytokine values are presented in pg/ml.

b.d., Below detection limit

3.3.3 Effect of heating on the IgE binding capacity

Allergens were heated and labeled with a code for heat treatment as described in Table 1. In an indirect ELISA, heat treated samples of all six allergen preparations were coated directly onto the microtiter plates and the results are shown relative to the positive control Bet v 1 (Figure 4). For the measurements of IgE binding to Bet v 1, Api g 1 and Dau c 1, different serum pools were used as positive controls. Comparison of MG and MQ treatments of nBet v 1 and rBet v 1 showed that recognition by the serum pool was different for rBet v 1 leading to a 50% reduction, while this was not the case for nBet v 1. A clear decrease was observed in antibody binding capacity of the serum pool to the allergens Api g 1 and Dau c 1 for both natural and recombinant preparations. Dau c 1 did not show any IgE binding for either recombinant or natural Dau c 1. Recombinant and natural Api g 1 showed a response of 30% relative to rBet v 1 and no difference for the TG sample heated to T_m. Both treatments leading to MG and MQ samples reduced the response by 50%, whereas the H2 treatment showed an equal response to the healthy control value.

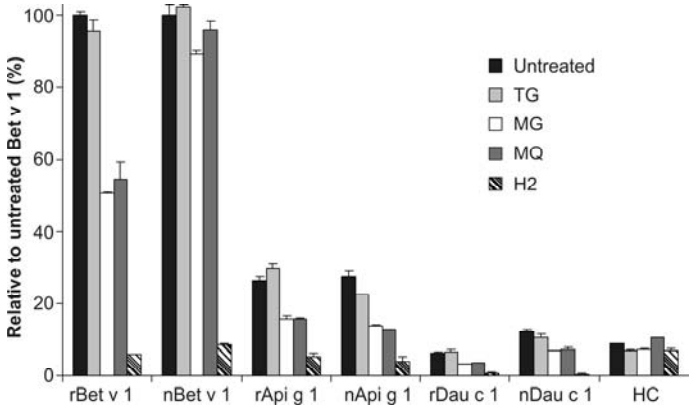

Figure 4. Indirect ELISA of thermally treated allergen coated to the plate. All values were compared relatively to the positive control by using untreated Bet v 1. A representative Healthy Control (HC) was included, which did not differ among the other used coatings. All values are means of duplicate measurements. Samples included are: Untreated; TG, heated to T_m and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; H2, heated for 2 hours at 100 °C.

In the inhibition ELISA, differences between the inhibition of 300 ng/well coated rBet v 1a and nBet v 1 were observed, when the serum was pre-incubated with the heat-treated allergens (Figure 5A and B). TG or MG and MQ heat treatment did not alter the inhibition of rBet v 1a binding of the serum pool (Figure 5A). For nBet v 1, the MQ- and MG-treated allergen decreased the inhibition by 50% when pre-incubated with 6 ng of the treated allergen (Figure 5B). The inhibition ELISA with Api g 1 showed similarly shaped inhibition curves for rApi g 1 and nApi g 1 (Figure 5C and D) for the untreated samples and the TG heated samples. The MG and MQ heated samples decreased IgE binding to nApi g 1 more than to rApi g 1, as shown by a larger decrease in the inhibition curve of nApi g 1 (Figure 5C). An inhibition ELISA for Dau c 1, by coating untreated Dau c 1 to the plate could not be performed due to the low coating efficiency of the protein and/or the low serum reactivity (data not shown).

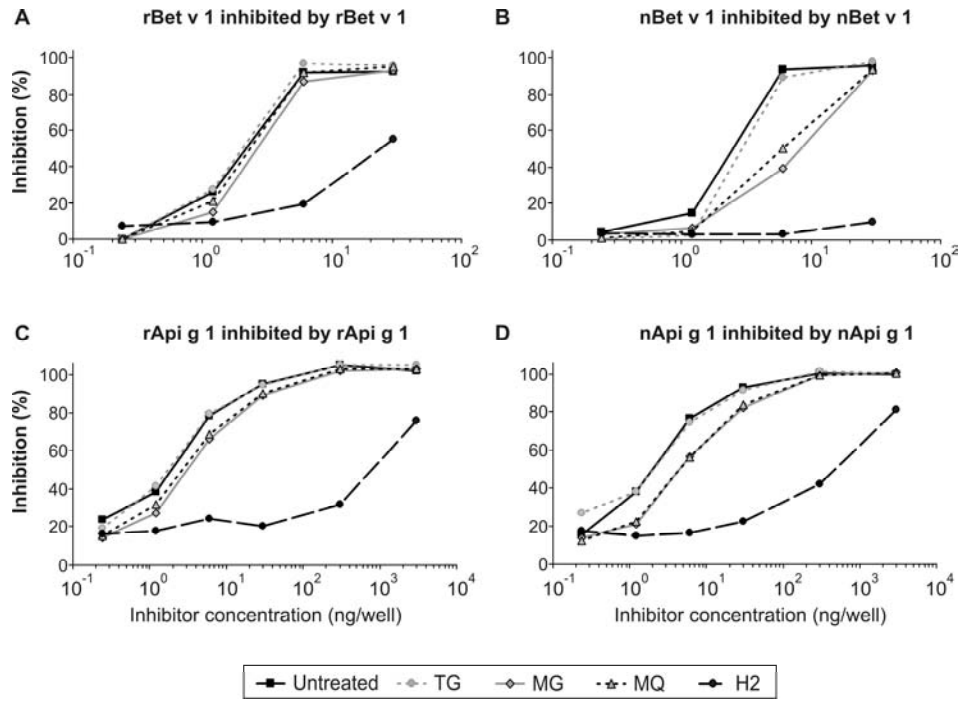

Figure 5. Inhibition ELISA with heat treated samples of rBet v 1 (**A**), nBet v 1 (**B**), rApi g 1 (**C**) and nApi g 1 (**D**) by coating with the respective untreated allergen. All values are means of duplicate measurements. Included samples are: Untreated; TG, heated to T_m and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; H2, heated for 2 hours at 100 °C.

A cross-reactivity inhibition ELISA was performed by coating rBet v 1 to the plate and pre-incubating the treated and untreated samples of natural and recombinant Api g 1 and Dau c 1 with the respective serum pools (Figure 6). The inhibition curves of rApi g 1 showed maximal 20% inhibition, which decreased to the 10% inhibition level of the H2 treatment (Figure 6A). Natural Api g 1 showed 40% inhibition, which decreased to 20% at a lower inhibitor concentration (Figure 6B). The rDau c 1 did not cause any inhibition (Figure 6C), whereas nDau c 1 was only able to completely inhibit IgE binding by pre-incubating serum with 3000 ng of untreated nDau c 1 (Figure 6D). The curves of nDau c 1 showed that the samples heated to 95 °C (MG and MQ) exhibited a 60% inhibition capacity, which was similar to the MQ and MG of nBet v 1 sample at a lower inhibitor concentration.

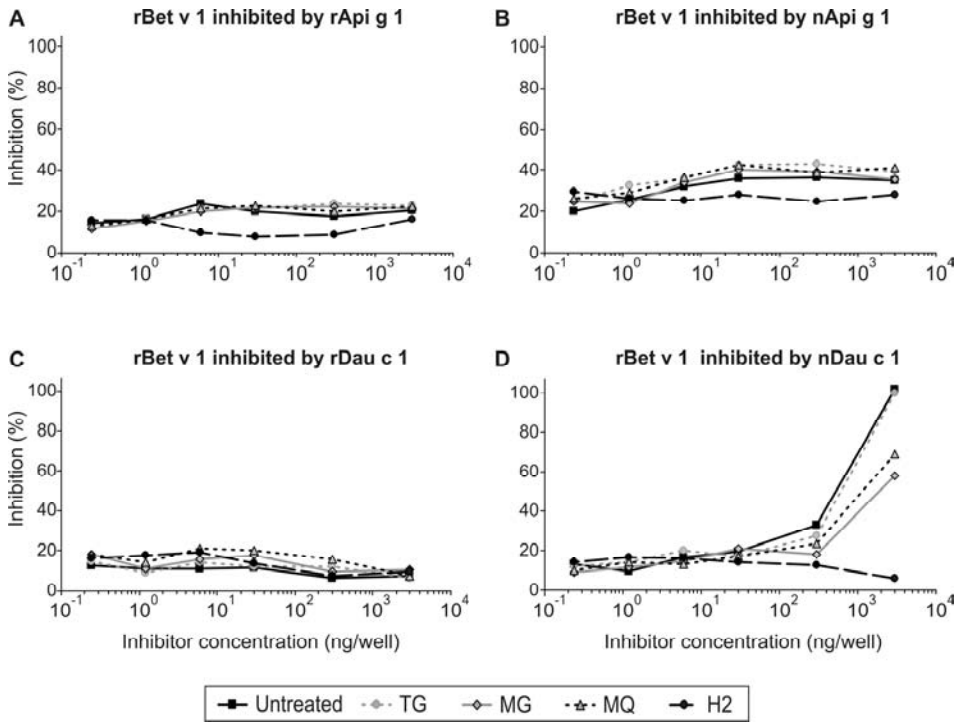

Figure 6. Cross-reactivity inhibition ELISA with rBet v 1 coated to the plate and serum preincubated with heat treated samples of rApi g 1(**A**), n Api g 1 (**B**), rDau c 1 (**C**) and nDau c 1 (**D**). All values are means of duplicate measurements. Included samples are: Untreated; TG, heated to T_m and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; H2, heated for 2 hours at 100 °C.

3.4 Discussion

In the present study, we report cytokine production profiles, T cell subset induction, and IgE binding analysis as readout parameters to investigate thermal stability and the structural consequences of purified allergens. We found small differences in thermal stability of Bet v 1 and Api g 1 for the recombinant forms, and for corresponding allergens, purified from their natural source. Striking differences were observed for recombinant and natural Dau c 1, showing a lower thermal stability than the other allergens. More profound conformational changes were observed for all the natural allergens after heating compared to the untreated samples. Particularly for the natural allergens, these conformational changes resulted in differences in allergenic potential as

measured by comparing the IgE binding ability in the indirect and inhibition type ELISA.

The T cell activation by rBet v 1 was investigated by human PBMC cultures from allergic individuals with a proven birch pollen allergy, were investigated. Besides a possible increase in cytokine production, also proliferation of the PBMC would indicate that the cells were activated by the allergen-derived peptides. However, in our study, rBet v 1 was not potent enough to induce either cytokine production or T cell proliferation. This could be explained by the low frequency of allergen-specific T cells present in the PBMC isolated from blood of selected birch pollen-allergic individuals. Also literature is scarce on Bet v 1-specific stimulation of human PBMC where often T cell clones are used [15]. Due to the fact that each T cell clone resembles the response of one single Bet v 1-specific T cell, a large range of T cell clones of one person should be obtained in order to establish the allergen-specific response of one individual. Bet v 1 changes conformation when heated, but is presented to the T cell in the form of linear peptides of approximately 12 to 18 amino acids by the antigen-presenting cell eventually leading to a predicted similar T cell response as shown with T cell clones in the study of Bohle et al. [15].

Additionally, B cell responses and resulting formation of IgE antibodies were analyzed for their effects of heat-treatment on conformational B cell epitopes, as reflected by CD and ELISA with both natural and recombinant Bet v 1, Dau c 1 and Api g 1. Bohle et al. [15] also measured thermal denaturation curves for recombinant allergens and found T_m-values similar to ours. Changes in $[\theta]_{MRW}$, as measured with CD, best represent the changes in α-helical content [28]. After heating, a conformational change was observed for all allergens resulting in the decreased α-helical content, as shown in Table 2. The structural changes were visible from the spectrum of nBet v 1 after heating (Figure 1) and also from the cooling curves which showed partial folding (Figure 2). Recombinant allergens contained only a single isoform, whereas natural allergens were composed of a mixture of isoforms. The mixture presumably induced broadening of the transition area of the thermal denaturation curve resulting in a less steep slope and a lower ΔH_{Tm}. By mass spectrometric analyses, Schenk [29] showed that the isoforms Bet v 1m, Bet v 1a and Bet v 1d were predominantly present in the natural sample of Betula pendula 'Youngii' in a ratio of about 30:45:25. In our study, the most striking differences in IgE binding capacity between the recombinant and natural

samples were observed for Bet v 1. In the indirect ELISA (Figure 4), heating to 95 °C did not affect nBet v 1, whereas the IgE binding to rBet v 1 was reduced by 50%. This can be explained by the isoform mixture of nBet v 1 containing a particular isoform that is more stable to heating, which is supported by the higher T_m-value and the decreased slope of the CD thermal denaturation curve (Figure 2A). Alternatively, the higher IgE binding affinity of a single isoform other than Bet v 1a, can become apparent after heating. Furthermore, heating to T_m did not affect the total IgE binding capacity. Heating for 2 hours at 100 °C increased the amount of in insoluble precipitate and abolished all IgE binding to all assessed PR-10 proteins.

The thermal stability of Dau c 1 was different from the other allergens. The T_m-values of recombinant and natural Bet v 1 and Api g 1 were around 65 °C, whereas 56 °C and 45 °C was determined for nDau c 1 and rDau c 1, respectively. The difference in T_m-values between nDau c 1 and rDau c 1 might be explained by the presence of a remaining His-tag attached to the N-terminus of rDau c 1, which caused a decreased thermal stability of the N-terminal β-strand that is possibly responsible for the stabilization of the C-terminal loop region and α-helix through hydrogen bonding. Another possibility is the lability of the isoform Dau c 1.0103 (Accession no. CAB03715), which was the isoform that constituted the commercially available rDau c 1. The nDau c 1 mixture contained at least two isoforms, Dau c 1.0104 (Accession no.CAB03716) and a homologue of the parsley protein PcPR1-3 (Accession no.CAA31085) [24].

Furthermore, the T_m-value for rApi g 1 resembled the T_m-value of Api g 1.01, as reported by Wangorsch et al. [30]. Besides the thermal denaturation curves of the recombinant allergen Api g 1.01, these authors also analyzed Api g 1.02. Interestingly, the thermal denaturation curve of Api g 1.02 showed a transition around 55 °C, which resembled the value of nDau c 1. Api g 1.02 has a 63% identity with the parsley homologue PcPR1-3 found in the mixture of nDau c 1, but only a 50% identity with Dau c 1.0103. Therefore, the PcPR1-3 homologue could influence the thermal stability of the nDau c 1 sample, resulting in a lower T_m-value than nApi g 1 that did not contain this isoform.

For all ELISA experiments, samples were exposed to heat-treatment, comparable to heating performed in the CD measurements, to link the results of conformational changes directly to IgE binding capacity. However, binding of an allergen to a

polystyrene microtiter plate might induce a conformational change [31], which subsequently affects the binding capacity to the plate as well as the IgE binding capacity to treated and non-treated immobilized allergens. The inhibition results of recombinant and natural Bet v 1 (Figure 5A and B) were different from the results observed in the indirect ELISA. For the indirect ELISA, rBet v 1 showed a 50% decrease in IgE binding capacity for the treated samples, but for the inhibition ELISA no decrease was observed. However, natural Bet v 1 showed the opposite effect. Again, this difference suggests differential IgE binding potentials and thermal stability by the presence of multiple isoforms. Heat-treatment of this natural isoform mixture can result in an altered IgE binding potential, which was visible in the inhibition ELISA, as this inhibition occurred in solution. However, in the indirect ELISA this difference could not be observed, as all isoforms are immobilized at the polystyrene surface, in a more or less altered conformational configuration. This observation can be complemented with the conformational differences between rBet v 1 and nBet v 1 (Figure 1 and 2A), since nBet v 1 showed a more permanent heat-induced structural loss than rBet v 1. This structural loss was increased by prolonged heating for the ELISA experiment and caused the reduced IgE binding capacity of nBet v 1. The higher affinity of heat-treated nBet v1 compared to rBet v 1, resulted in an unchanged signal for the indirect ELISA, whereas a decreased signal in the inhibition ELISA was due to the higher affinity of the untreated nBet v 1 bound to the polystyrene plate.

No effects were observed for the cross-reactivity inhibition ELISAs with rBet v 1 coated on the polystyrene plate and serum pre-incubated with rApi g 1, nApi g 1 and rDau c 1. This was probably due to the low levels of allergen-specific IgE for Dau c 1 and Api g 1. Only nDau c 1 was able to completely inhibit IgE binding to rBet v 1 at a high concentration. In contrast to the Dau c 1 response in this study, our previous ELISA experiments (chapter 2) showed similar reactivity of Dau c 1 and Api g 1 [24]. However, our current study used a phosphate containing coating buffer of pH 7.4 instead of a carbonate buffer of pH 9.6, which possibly induced conformational changes of the protein [32]. To overcome the lack of Dau c 1 plate-binding, a cross-reactive inhibition ELISA was performed by binding untreated rBet v 1 to the polystyrene plate which enabled comparison of the natural isoform mixtures with the recombinant single isoforms. The low thermal stability of Dau c 1 was reflected in the ELISA experiments as both natural and recombinant Dau c 1 did not show IgE binding in an indirect or

inhibition ELISA. In the cross-reactive inhibition ELISA, natural, but not recombinant Dau c 1, was able to inhibit rBet v 1 in solution, which emphasizes the difference between natural and recombinant Dau c 1.

The inhibition curve of rBet v 1 in this study was similar to the one published by Wagner et al. [23] who performed cross-reactivity ELISAs with hypoallergenic variants of Bet v 1 in which Bet v 1a (1.0101) was the strongest inhibitor. The other allergens, rBet v 1d (1.0401) and rBet v 1L (1.1001), were still able to inhibit the IgE binding to rBet v1a, although a higher inhibitor concentration was needed. For these inhibition experiments, Wagner et al. [23] used patient sera with a very low response to the hypoallergenic variant coated to the plate. The experiments performed in the present study showed even less reactivity of Api g 1 and Dau c 1 to IgE. It is tempting to assume that the hypoallergenic variants of Bet v 1 have a higher affinity for IgE than Dau c 1 and Api g 1. Therefore, care should be taken in adopting such recombinant isoforms as hypoallergenic proteins.

Moneo et al. described sensitization of 4 patients for carrot, who produced IgE-type antibodies that were able to recognize Dau c 1, but not Bet v 1 [33]. This observation is not in line with the common perception of pollen-fruit syndrome, starting with sensitization for Bet v 1 and later occurring cross-reactions to PR-10 containing vegetables and fruits, and suggests that Dau c 1 can be a primary sensitizer [33, 34]. As in our study, Dau c 1 was found to be less heat-stable than other PR-10 proteins and larger physico-chemical stability of Dau c 1 apparently cannot account for such observations. Likely, other (food) components play a role in the sensitization process.

3.5 Conclusion

In conclusion, heat-treatment affects the allergenic structure leading to changes in IgE binding capacity of an allergen in solution in comparison to the binding capacity of the polystyrene-bound allergen. However, no changes in the IgE binding capacity were observed for allergens heated to T_m and also no differences were observed for the thermal treatments MG and MQ considering the different cooling rates. In contrast, IgE binding capacity is largely destroyed by heating the protein for 2 hours at 100 °C. In addition, differences in heat-induced structural changes and allergenicity between natural and recombinant allergens are based on natural isoform mixtures and their differences in affinity of binding to specific IgE antibodies. Both natural and

recombinant Dau c 1 were found to be significantly less thermo-stable than Bet v 1- and Api g 1-forms, respectively. Therefore, future studies will be needed to elucidate the allergenicity of the isoforms present in the natural isoform mixtures, as these might resolve the discrepancy between negative IgE levels determined with recombinant proteins and these 'non-allergic' individuals that actually do display allergic symptoms.

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Chapter 4

Thermodynamic characterization of the PR-10 allergens Bet v 1, Api g 1 and Dau c 1 and pH dependence of nApi g 1 and nDau c 1

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Abstract

Natural and recombinant Bet v 1, the major birch pollen allergen, and homologous allergens Api g 1 and Dau c 1, from celery and carrot, respectively, were studied by CD spectroscopy under conditions of varying denaturant concentration, pH and temperature to determine fundamental thermodynamic parameters for conformational stability. Thermodynamic studies increase basic knowledge regarding differences between birch pollen related allergens and are of importance to choose processing conditions. The conformational stability determined from Guanidine Hydrochloride denaturation curves was similar for rBet v 1.0101 and rApi g 1.0101. Conformational responses to chaotropic salt were different for recombinant allergens from different species, but were similar for the natural isoform mixtures. The conformational stability of nApi g 1 and nDau c 1, was shown to be similar to rBet v 1.2801 at pH >4.4 [1], but nApi g and nDau c 1 were stable to heating at lower pH-values.

4.1 Introduction

The major birch pollen allergen Bet v 1, a pathogenesis related-10 (PR-10) protein, is known as the primary sensitizer for birch pollen-related food allergy. The structural relationship with homologous proteins in foods is the basis for cross-reactivity [2-4]. Many birch pollen allergic individuals will develop a birch pollen-related food allergy, in particular to fruits from the *Rosaceae* family, while a smaller group will respond to vegetables such as carrot and celery from the *Apiaceae* [5]. In the case of carrot and celery, the exposure route plays a role, because these foods are most commonly consumed as processed foods whereas the *Rosaceae* are mostly consumed raw. In general, the PR-10 allergens are characterized as labile proteins, in contrast to most other food allergens [6] and cooking could therefore explain reduced IgE binding to Api g 1 from celery and Dau c 1 from carrot proteins [5].

A few physico-chemical studies tested effects of processing on immune reactivity of PR-10 proteins. In several studies it was shown that the IgE binding capacity to Api g 1 of processed celery was almost completely reduced [7-9]. Pickled celery, heat sterilized at a low pH, demonstrated a remarkable reduction of the IgE reactivity [9]. Both nonenzymatic and enzymatic browning reactions on rPru av 1 from cherry and on Mal d 1 extracts from apple, caused a remarkable reduction of the IgE reactivity [10, 11]. On the other hand, 60 minutes cooking of different recombinant allergens, Bet v 1, Mal d 1, Api g 1 and Dau c 1, completely abolished IgE binding, but without a reduction of the capacity to activate allergen-specific T cells [12]. Also gastrointestinal digestion destroyed IgE binding, but not T cell activation [13].

The basis of allergen recognition is still unclear and a better understanding is needed concerning structural dynamics of the allergen. This can be accomplished by studying allergen mutants and relate structural changes of a protein to changes in IgE binding capacity [14, 15]. Nevertheless, many mutants created are not well characterized and they are different from natural isoforms, due to expression in recombinant systems. This can easily change the IgE binding capacity, resulting in limited relevance for practical situations. Furthermore, allergy research concentrates strongly on the allergen's immune reactivity and its scope is directed to the malfunction of the immune system, but is less focused on structural properties of the allergen, which could result in overlooked impact of these properties on the immune system (e.g. lipid binding [16]).

Thermodynamic studies can increase the basic knowledge regarding differences between PR-10 allergens and are of importance to choose experimental conditions during processing. A basic knowledge is obtained in terms of thermodynamic parameters such as $\Delta G_{D\text{-}N}$, T_m, ΔH_{Tm} and ΔC_P, which are derived from the assumption of the reversible two-state model $N \rightleftharpoons D$, were N is the native/folded and D the denatured/unfolded state. The conformational stability, $\Delta G_{D\text{-}N}$, is helpful in explaining differences between different PR-10 isoforms, as it is the fundamental measure for the difference of the Gibbs free energy between folded and unfolded molecules [17]. A physico-chemical parameter required for the calculation of $\Delta G_{D\text{-}N}$ is $m_{D\text{-}N}$, which is a measure for the dependence of the free energy on denaturant concentration and reflects the degree of surface area buried in the native state relatively to the denatured state [28]. The midpoint of thermal denaturation, T_m, is a stability parameter that indicates the temperature at which 50% of the protein is unfolded [17]. ΔH_{Tm}, is the enthalpy change required for a $N \rightleftharpoons D$ conversion of 1 mol of protein at T_m. This parameter is needed to calculate the heat capacity change upon unfolding, ΔC_P, which is yet another parameter to measure stability and can be used to calculate the conformational stability at any given temperature at constant pressure.

Stability measurements by circular dichroism (CD) have been performed before with rBet v 1.2801 and its mutant Tyr120Trp and also with rMal d 1, which showed relatively low values of $\Delta G_{D\text{-}N}$ and ΔC_P [1]. By isolating the allergens from their natural source under mild conditions, isoform mixtures can be obtained [18], which are closer to practical situations than recombinant proteins. These mixtures can be studied for the overall stability. The objective of this investigation was to study the thermodynamic stability of recombinant and natural Bet v 1, Api g 1 and Dau c 1 by determining the conformational stability, $\Delta G_{D\text{-}N}$ by Guanidine Hydrochloride (GuaHCl) denaturation using CD measurements. Also the effect of pH on thermal stability on nApi g 1 and nDau c 1 was studied to determine T_m, ΔH_{Tm} and ΔC_P to increase general knowledge on stability of natural isoform mixtures and their pH dependence.

4.2 Materials and Methods

4.2.1 Allergens

The natural allergens nBet v 1, nApi g 1 and nDau c 1 from birch pollen, celery tuber and carrot, respectively, were purified as isoform mixtures as described in **chapter 2**. Briefly, Bet v 1 was purified from birch pollen of *Betula pendula* 'Youngii', Api g 1 from celery tuber purchased from a supermarket and Dau c 1 from *Daucus carota* 'Narbonne'. Ammonium sulphate precipitation with the protein extracts was followed by hydrophobic interaction and size exclusion chromatography. The purified allergens were identified as isoform mixtures using Q-TOF MS/MS. The single recombinant allergen isoforms rBet v 1a (further referred to as rBet v 1.0101), rApi g 1.0101 and rDau c 1.2 (further referred to as rDau c 1.0103) were purchased from Biomay (Vienna, Austria). All allergens were dissolved in 10 mM potassium phosphate buffer, pH 7.0, buffer exchanged and concentrated on a Microsep 3K centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA). Protein concentrations were determined using the MicroBCA™ Protein Assay (Pierce, Rockford, IL, USA) with BSA as a standard.

4.2.2 GuaHCl denaturation curves

Guanidine Hydrochloride (GuaHCl) denaturation experiments were carried out with natural and recombinant Bet v 1, Dau c 1 and Api g 1 at a protein concentration of 10 µM in 10 mM potassium phosphate buffer (pH 7.0). A 6 M GuaHCl stock solution was prepared in 10 mM potassium phosphate buffer and diluted into 4, 2, 1 and 0.5 M solutions. After filtering the solution over a 0.2 µm syringe filter (Schleicher & Schuell, Dassel, Germany), the final GuaHCl concentrations were determined from refractive index measurements according to Nozaki [19] as calculated from equation 1:

$$[\mathrm{GuaHCl}] = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \qquad (1)$$

[GuaHCl] is given in mol/L and ΔN is the difference between the refractive index of the GuaHCl solution and the 10 mM phosphate buffer.

Circular dichroism (CD) spectra were recorded at 20 °C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) after allowing the GuaHCl-

protein solutions to equilibrate for two hours. Far-UV spectra were recorded from 210-260 nm with a quartz cuvette of 1 mm path length, by accumulating 10 scans at a scanning speed of 50 nm/min, using a 0.2 nm step width and 2.0 nm band width. Ellipticity values at 222 nm were corrected for buffer/GuaHCl background and plotted against the GuaHCl concentration to display protein denaturation curves. The raw CD data were converted into the mean residue weight ellipticity $[\theta]_{MRW}$ (units in deg·cm^2·dmol^{-1}) by using the following equation:

$$[\theta]_{\mathrm{MRW}} = \frac{100 \times [\theta]_{\mathrm{obs}}}{C \times l \times n} \qquad (2)$$

$[\theta]_{obs}$ is the observed signal in degrees, C is the concentration in mol/L, l is the path length of the cuvette in cm and n is the number of amino acids of the protein.

From the denaturation plots, the conformational stability, the free energy of unfolding $\Delta G_{\mathrm{D-N}}^{\mathrm{H2O}}$ of the protein in water was estimated, by assuming a two-state mechanism with a linear dependence of the pre- and post-transition baselines [20, 21]. The following equation was fitted to the data [22],

$$Y_{\mathrm{obs}} = \frac{\alpha_N + \beta_N[\mathrm{GuaHCl}] + \left(\alpha_D + \beta_D[\mathrm{GuaHCl}]\right) \times \exp\left\{\frac{m_{\mathrm{D-N}}\left([\mathrm{GuaHCl}] - [\mathrm{GuaHCl}_{50\%}]\right)}{RT}\right\}}{1 + \exp\left\{\frac{m_{\mathrm{D-N}}\left([\mathrm{GuaHCl}] - [\mathrm{GuaHCl}_{50\%}]\right)}{RT}\right\}} \qquad (3)$$

by using non-linear least squares regression with the program TableCurve (Jandel Scientific, Erkrath, Germany). Y_{obs} is the observed signal and [GuaHCl] the chaotropic salt concentration. The other six estimated parameters, from equation 3, include the slopes and intercepts of the baselines of the native (β_N) and denatured (β_D) states with the ellipticity values of the native (α_N) and denatured (α_D) state at 0 M GuaHCl. $[\mathrm{GuaHCl}_{50\%}]$ is the midpoint of denaturation from the transition state where 50% of the protein is denatured. The $m_{D\text{-}N}$ value (in kJ mol^{-1} M^{-1}) is a parameter correlating with the degree of protein surface exposed to the solvent upon unfolding. From the estimated

values of $m_{D\text{-}N}$ and [GuaHCl$_{50\%}$] the conformational stability, ΔG_{D-N}^{H2O} of the allergen in water, is calculated by using [20]:

$$\Delta G_{D-N} = \Delta G_{D-N}^{H_2O} - m_{D-N}[\text{GuaHCl}] \qquad (4)$$

The apparent free energy difference, $\Delta G_{D\text{-}N}$, is the value of ΔG_{D-N}^{H2O} in the absence of denaturant and is 0 at [GuaHCl$_{50\%}$].

4.2.3 pH stability experiments

pH stability experiments were performed with natural Api g 1 and Dau c 1 by recording far-UV CD spectra, similar to the GuaHCl denaturation experiment, and the thermal denaturation CD curves at different pH-values. Changes in ellipticity were followed at 222 nm by heating to 95 °C at a rate of 1 °C/min using a band width of 1.0 nm with 10 µM protein in different 50 mM buffers with the pH set at room temperature, similar to the method used by Mogensen et al. [1]: 100 mM HCl at pH 1.0, glycine at pH 2.0-3.5, sodium acetate at pH 4.0-5.3, MES at pH 5.5-6.5, MOPS at pH 6.5-7.5, and TRIS at pH 8.0-9.0. The thermal denaturation curves were fitted using a non-linear least squares fit method [23] from the software program TableCurve according to the relation derived from the van't Hoff equation:

$$\theta = \frac{\alpha_N + \beta_N T + (\alpha_D + \beta_D T)\mathrm{e}^{-\left[\Delta H_{T\mathrm{m}}(1-\frac{T}{T_\mathrm{m}})\right]/RT}}{1 + \mathrm{e}^{-\left[\Delta H_{T\mathrm{m}}(1-\frac{T}{T_\mathrm{m}})\right]/RT}} \qquad (5)$$

Six parameters were estimated from fitting equation 5, which includes the slopes and intercepts of the baselines of the native (β_N) and denatured (β_D) states with the ellipticity values (intercepts) for the folded (α_N) and unfolded (α_D) state. The other derived parameters were T_m, the temperature at the midpoint of denaturation, and ΔH_{Tm} the enthalpy of unfolding at T_m. After heating, the protein was cooled down to room temperature to study the refolding properties of the protein. The program CDNN was used to deconvolute the secondary structure of measured CD spectra [24].

From the linear relationship between ΔH_{Tm} and T_m at varied pH, the specific heat capacity, ΔC_P, can be obtained from the slope of the graph [25], making the assumption

that ΔC_P does not depend on pH and temperature in the thermal transition range. With the value of ΔC_P, the Gibbs energy change of unfolding at any temperature, ΔG_T, is calculated using the Gibbs-Helmholtz equation at 298.15 K [23].

$$\Delta G_T = \Delta H_{T\mathrm{m}}\left(1 - \frac{298.15}{T_\mathrm{m}}\right) - \Delta C_\mathrm{P}\left[\left(T_\mathrm{m} - 298.15\right) + 298.15\ln\left(\frac{298.15}{T_\mathrm{m}}\right)\right] \qquad (6)$$

Gibbs energy values for protein stability are obtained by taking the value of $\Delta H_{T\mathrm{m}}$ and T_m at pH 7.0. Values thus calculated can be compared with $\Delta G_{\mathrm{D-N}}^{\mathrm{H2O}}$ values of protein stability curves.

4.3 Results

4.3.1 Protein stability estimated from denaturation with guanidine hydrochloride

Conformational stability of proteins can be determined by measuring the unfolding curves in aqueous solutions with increasing concentrations of chaotropic salts like guanidine hydrochloride (GuaHCl) [20]. The parameters derived from the fit of these graphs resulted in a value for the change in Gibbs free energy, $\Delta G_{\mathrm{D\text{-}N}}$, in water ($\Delta G_{\mathrm{D-N}}^{\mathrm{H2O}}$). Protein stability curves were measured with GuaHCl denaturation for both recombinant and natural Bet v 1, Api g 1 and Dau c 1. The resulting graphs are shown in Figure 1 with the individual data points as a fraction of the unfolded protein versus the increasing GuaHCl concentration and the corresponding denaturation curves fitted with equation 3. The proteins started to unfold already at low [GuaHCl] resulting in an imprecise estimate of the parameter, β_N, for the slope of the baseline for the native protein. Therefore, a change was made in the fitting procedure by fixing this parameter to zero, resulting in similar standard errors from the regression analysis for all GuaHCl denaturation curves showing that β_N is redundant here. The GuaHCl concentration at which 50% of the protein is unfolded, [$\mathrm{GuaHCl}_{50\%}$], could be estimated from these fits, together with the parameter $m_{\mathrm{D\text{-}N}}$. These estimated parameters $m_{\mathrm{D\text{-}N}}$ and [$\mathrm{GuaHCl}_{50\%}$] are given in Table 1 with the standard deviations of the fits and the calculated $\Delta G_{\mathrm{D-N}}^{\mathrm{H2O}}$ from

equation 4. The table is supplemented with urea denaturation data adapted from Mogensen et al. [1] for rBet v 1.2801 (differs from rBet v 1.0101 at position 63 and has a Leu residue instead of Phe) and its mutant Tyr120Trp and rMal d 1.

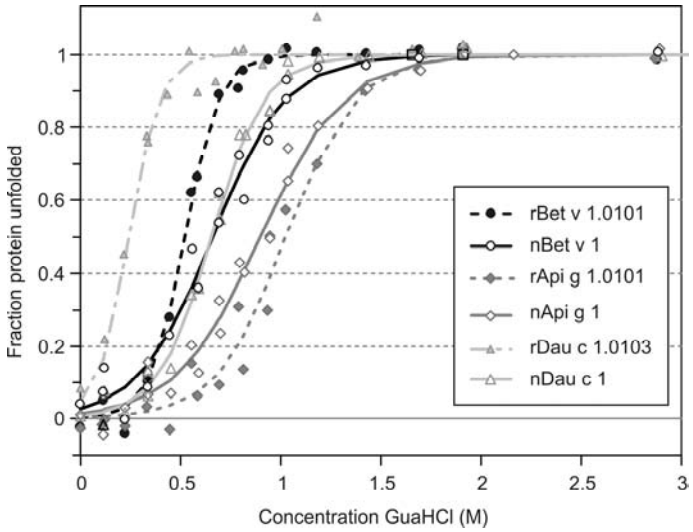

Figure 1. CD denaturation curves of natural and recombinant allergens by increasing the GuaHCl concentration. The individual measured points are displayed with the stability curves fitted using equation 3.

The stability curves of Figure 1 and the ΔG_{D-N}^{H2O} values in Table 1 clearly showed a low stability of rDau c 1.0103, which started to unfold already at low concentrations of GuaHCl, which incidentally resulted in a larger fitting error (Table 1). The sharp transition from native to denatured state was reflected in a larger value of $m_{D\text{-}N}$ for rDau c 1.0103. The conformational stability in terms of Gibbs free energy change was calculated from the two parameters [GuaHCl$_{50\%}$] and $m_{D\text{-}N}$. The generally observed trend was a lower $m_{D\text{-}N}$ value for the natural allergens and a higher ΔG_{D-N}^{H2O} for the recombinant Bet v 1 and Api g 1. Apart from rDau c 1.0103 , the [GuaHCl$_{50\%}$] values of natural and recombinant Api g 1 were 0.24-0.51 M higher than Dau c 1 and Bet v 1.

Table 1. Thermodynamic parameters of the recombinant and natural allergens of Bet v 1, Api g 1 and Dau c 1 determined from GuaHCl denaturation[a] and the pH experiment[b].

	$m_{D\text{-}N}$ (kJ/mol·M)	[GuaHCl$_{50\%}$] (M)	$\Delta G_{D-N}^{H_2O}$ (kJ/mol)	ΔC_P (kJ/mol·K)	ΔG_T (kJ/mol)	pH opt. for T_m	pH transition at 20 °C
rBet v 1.0101	28.7 ± 2.6	0.52 ± 0.01	15.0	-	-	-	-
nBet v 1	13.4 ± 2.1	0.66 ± 0.03	8.8	-	-	-	-
rApi g 1.0101	14.2 ± 2.4	1.03 ± 0.04	14.6	-	-	-	-
nApi g 1	11.7 ± 1.4	0.90 ± 0.03	10.5	5.5	19.9	6.5	4.75
rDau c 1.0103	31.4 ± 6.2	0.24 ± 0.02	7.6	-	-	-	-
nDau c 1	18.5 ± 1.6	0.66 ± 0.01	12.1	4.7	17.4	6.3-6.5	4.42
rBet v 1.2801[c]	10.1 ± 1.3	2.43 ± 0.07	24.4	5.0	18.8	6.5-7.0	~3.5
rBet v 1.2801 Y120W[c]	11.0 ± 1.1	2.46 ± 0.03	27.0	-	-	-	-
rMal d 1[c]	11.6 ± 1.3	2.61 ± 0.03	30.5	-	-	-	-

[a)] All conditions for GuaHCl denaturation are at 25 °C and pH 7.0. Errors reported are standard deviations from regression analysis. $m_{D\text{-}N}$ and [GuaHCl$_{50\%}$] values were estimated from the fits of Figure 1 and were used to calculate the conformational stability in water, ΔG_{D-N}^{H2O} from equation 4.

[b)]The value of the heat capacity change, ΔC_P, was derived from the slopes of Figure 4 and was used to calculate the conformational stability, ΔG_T, at 25 °C for pH 7.0 from equation 6. The pH optimum for reversible unfolding and the pH transition of the midpoint of the fit at 20 °C were both determined from Figure 3.

[c)]Urea denaturation and pH experimental data adapted from Mogensen et al. [1].

4.3.2 Effect of pH on thermal stability of nApig 1 and nDau c 1

Thermal stability of natural isoform mixtures of Api g 1 and Dau c 1 was determined under various pH-conditions (range 2.0-9.0) using CD spectrometry at 222 nm (Figure 2). Thermal stability was expressed in terms of T_m, the temperature values where 50% of the protein was unfolded and ΔH_{Tm}, the enthalpy of unfolding at T_m. Reproducibility of these two parameters was confirmed by duplicate measurements of buffer at pH 4.9 and by determining the differences between the two buffers MES and MOPS at pH 6.5, which were minimal (with %RSD of ≤ 1% for T_m-values and ≤ 10% for ΔH_{Tm}). Differences were observed between a 10 mM potassium phosphate and 50 mM MOPS buffers at pH 7.0 with approximately 5 °C lower T_m-values for the potassium phosphate

buffer. These differences may be caused by changes in buffer pH at higher temperature, but also by differences in ionic strength.

Thermal unfolding as a function of pH showed similar patterns for nDau c 1 (Figure 2A) and nApi g 1 (not shown). Four different relationships could be distinguished in the pH-range from 2.0-9.0: 1) no transition during heating from pH 2.0-4.0 for both nDau c 1 and nApi g 1; 2) one transition observed in the pH range 4.4-5.6 for nDau c 1 and 4.4-6.0 for nApi g 1, but most of the CD signal was lost at 95 °C due to aggregation, which prevented the protein from refolding; 3) two transitions, at about 60 °C and 85 °C for nDau c 1 at pH 6.0 and pH 6.3 for Api g 1; 4) one transition in the pH range 6.3-9.0 for nDau c 1 and 6.5-9.0 for nApi g 1 with remaining ellipticity at 95 °C that allowed refolding of the protein upon cooling.

Although a transition was not visible upon heating in the pH range 2.0-4.0 at 222 nm, changes did occur in the CD spectrum at other wavelengths. A representative example, with CD spectra measured at pH 4.0, of thermally treated nDau c 1 is displayed in Figure 2B. Compared to the CD spectrum at pH 7.0, a clear difference in shape was visible, which was apparent from the estimates of decreasing helical structure (33.4 to 20.9%) and an increase in β-sheet content (21.7 to 27.2%). When the protein was heated, a change did not occur between 210 and 260 nm, but a shift to a lower wavelength occurred in the intercept of the x-axis. Upon cooling, the protein did not refold to its starting signal and the signal at 222 nm decreased to values below its starting point with a minimum in the spectrum at 217 nm (Figure 2B). The secondary structure deconvolution program CDNN could not determine a difference between the protein spectra at 95 °C and the cooled protein, but by comparing unheated and heated protein, a decrease was found for the helical (20.9 to 18.6%) and β-sheet content (27.2 to 25.1%). For pH 3.5 and below, cooling after a 95 °C treatment resulted in an increase in the helical content and a decrease in β-sheet (data not shown).

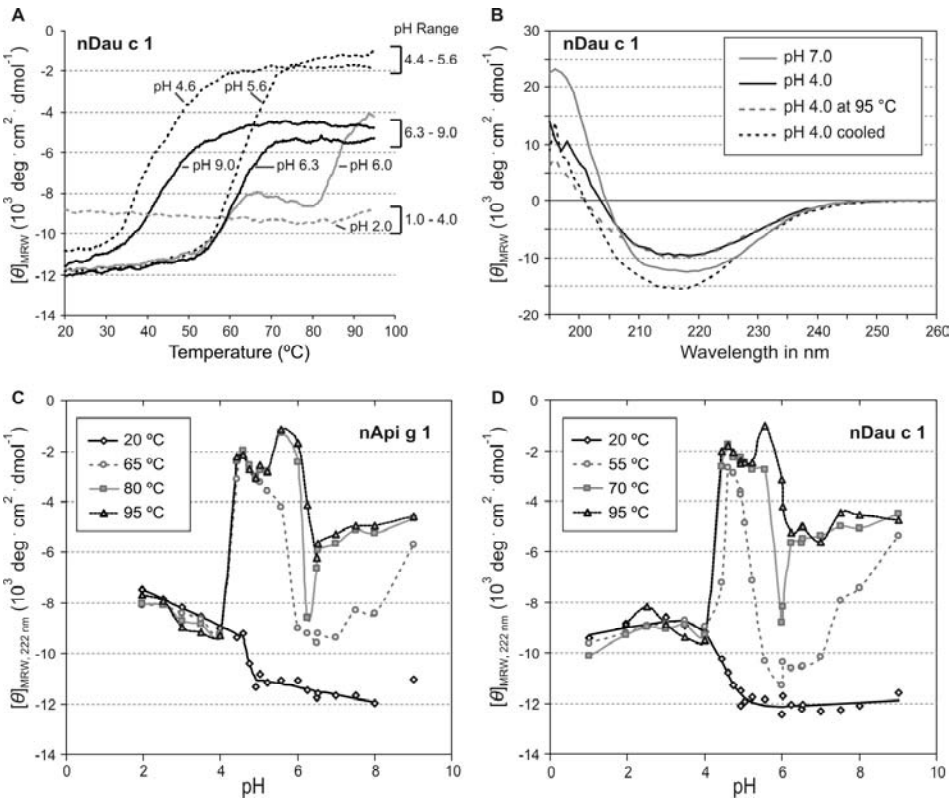

Figure 2. Effect of pH on thermal stability of nDau c 1 and nApi g 1 in the range of 20-95 °C. **A)** Thermal denaturation curves of nDau c 1 can be divided into four different groups as indicated in the graph: 1) heat stable between pH 1.0-4.0; 2) unfolding but no refolding capacity between pH 4.4-5.6; 3) both unfolding and refolding capacity between pH 6.3-9.0; 4) a double transition at pH 6.0, **B)** CD spectra of nDau c 1 at pH 7.0 and 4.0. The CD spectra at pH 4.0 were measured at 20 °C, 95 °C and at 20 °C after cooling down. **C** and **D**) CD signal at 222 nm at different pH values at four different temperatures at 20, 65, 80 and 95 °C for nApi g 1 and 20, 55, 70 and 95 °C for nDau c 1. The CD signal at 20 °C is represented with a fit to the data points at different pH.

Plotting $[\theta]_{MRW,222nm}$ at four temperatures, by taking measured points of Figure 2A at that particular temperature, against the pH (Figure 2C and D), showed differences between nApi g 1 and nDau c 1. Both plots at 20 °C showed a transition region near their pI, at 4.4 for nDau c 1 and 4.75 for nApi g 1, with a difference in the shape of the fits. Two plateaus were visible for nDau c 1 in the pH-ranges 2.0-4.0 and 6.0-8.0 while the signal for nApi g 1 was slightly decreasing. This resulted in a smaller $[\theta]_{MRW,222nm}$ change in the transition region for nApi g 1 as compared to nDau c 1. The graphs also

showed that $[\theta]_{MRW,222nm}$ was unchanged in the pH range 2.0-4.0 when heating the allergens to 95 °C. The sharp decrease in $[\theta]_{MRW,222nm}$ at 95 °C between pH 5.6-6.5 was at a point where irreversible unfolding changed into reversible folding in the higher pH range. Under the irreversible unfolding conditions, an insoluble precipitate was formed upon heating.

Differences in thermal stability between nApi g 1 and nDau c 1 were seen by plotting T_m versus pH for each thermal denaturation curve (Figure 3). The highest T_m value found for nApi g 1 was 71.9 °C at pH 6.0 and 62.8 °C for nDau c 1 at pH 5.6. The thermal denaturation curves with a double transition region at pH 6.3 and 6.0 for nApi g 1 and nDau c 1, respectively, were not taken into account. At these pH-values the allergens were more stable to heating as shown by an intermediate state visible after the first transition (Figure 2A), which continued unfolding at a temperature above 80 °C. Optimal pH stability with complete refolding ability was observed at pH 6.5 and 6.3-6.5 for nApi g 1 and nDau c 1, respectively. Furthermore, the T_m values were lowest at pH 4.6: 39.1 °C for nApi g 1 and 41.2 °C for nDau c 1. T_m increased very rapidly below pH 4.6 resulting in a higher T_m value for pH 4.4 for both nApi g 1 and nDau c 1. At a pH below 4.4 no thermal denaturation occurred, as judged from the $[\theta]_{MRW,222nm}$ signal and consequently no parameters could be calculated.

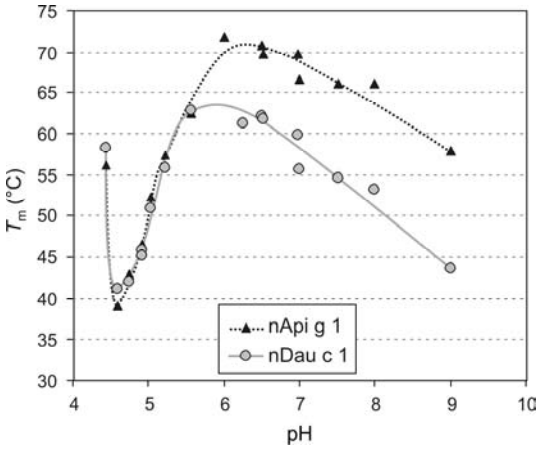

Figure 3. pH stability of nApi g 1 and nDau c 1 measured by CD. The pH is plotted to the T_m values as determined from the fits of the thermal denaturation curves of each buffer. The lines are shown to guide the eye.

The conformational stability of the allergens as determined from the GuaHCl stability curves for pH 7.0, could be derived from the proposed linear relationship of T_m and ΔH_{Tm} at different pH values (Figure 4), as was empirically established by Privalov [26]. For this approach, the calculation of ΔG_T from equation 6 required the specific heat capacity, ΔC_P as a parameter. ΔC_P is defined as the change in enthalpy with temperature and could be derived from the slope of the linear regression of ΔH_{Tm} versus T_m. All calculated ΔH_{Tm} and T_m parameters were calculated from the different buffers assuming two-state unfolding kinetics. However, data between pH 4.4-6.0 did not follow two-state unfolding kinetics and were visually divided in the plots from the pH range 6.5-9.0. All data points of nDau c 1 showed a clear linear relationship, but for nApi g 1, a linear relationship was only observed in the pH range 4.4-6.0. Therefore, the value of ΔC_P (Table 1) was derived from the linear slopes in the pH range 4.4-6.0 for nApi g 1 and the complete range for nDau c 1. The ΔG_T values were calculated at 25 °C from equation 6 by using the T_m and ΔH_{Tm} values of nApi g 1 (T_m = 66.5 °C; ΔH_{Tm} = 265.1 kJ/mol) and nDau c 1 (T_m = 55.7 °C; ΔH_{Tm} = 247.0 kJ/mol) from thermal denaturation curves at pH 7.0 (Table 1).

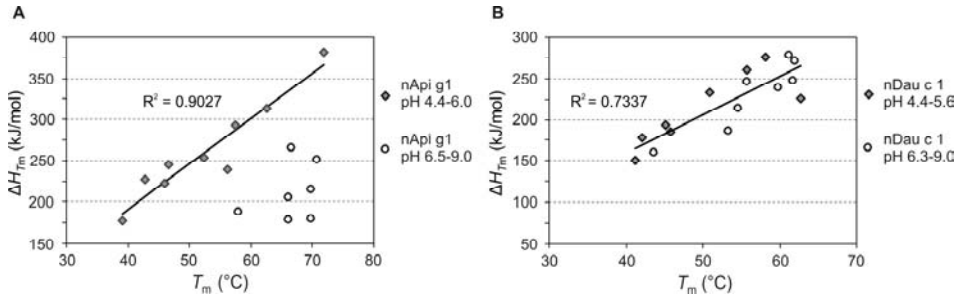

Figure 4. Relationship of enthalpy of unfolding ΔH_{Tm} and T_m of nApi g 1 (**A**) and nDau c 1 (**B**). ΔH_{Tm} and T_m were determined from the fits of the thermal denaturation curves between pH 4.4 and 9.0. The data points are split up in two series; 1) Irreversible unfolding (low pH range, diamond shapes), 2) Reversible folding (higher pH range, open circles). For nApi g 1, a linear fit is shown through the data points at low pH (diamond shapes) and for nDau c 1 a fit is shown through all data points. The heat capacity change ΔC_P was derived from the slopes of the linear fits.

4.4 Discussion

This study described differences in conformational stability of the major birch pollen allergen Bet v 1 and the vegetable allergens Api g 1 and Dau c 1 from celery and carrot, respectively. Conformational stability was determined from thermodynamic parameters, derived from CD measurements. The characterization of thermodynamic parameters is helpful in explaining differences between the folded and unfolded states of PR-10 proteins and could form a basis for explaining the severity of the allergic response to different isoforms. The recombinant single isoforms rBet v 1.0101, rApi g 1.0101 and rDau c 1.0103 were compared with isoform mixtures, purified from their natural source [18]. In general, the conformational stability of the measured allergens were low and differences observed between natural and recombinant and between the vegetable and birch protein showed how important this thermodynamic characterization could be.

In the first part of this study, differences in conformational stability were clearly visible between the recombinant PR-10 allergens. Naturally occurring globular proteins in general have a ΔG_{D-N}^{H2O} between 21-42 kJ/mol [17], but the values found by GuaHCl denaturation were below 15 kJ/mol for all allergens. By using a linear extrapolation method, the lowest estimate for ΔG_{D-N}^{H2O} was obtained, but this method in general should give the best agreement with ΔG_{D-N}^{H2O} values from urea denaturation curves [20]. The low ΔG_{D-N}^{H2O} of rDau c 1.0103 was the result of a low [GuaHCl$_{50\%}$], which was almost 3 times lower than [GuaHCl$_{50\%}$] for nDau c 1. This commercially available allergen was purchased with an attached His-tag, which likely decreased protein stability. Both rBet v 1.0101 and rApi g 1.0101 showed a similar conformational stability, but different properties in the presence of denaturant. These allergens were different in the $m_{D\text{-}N}$ and [GuaHCl$_{50\%}$] values with a 2 times higher [GuaHCl$_{50\%}$] for rApi g 1.0101 (1.03 M). A point of attention is that the parameters derived for the natural allergens were averages of a mixture, whereas the parameters determined for recombinant allergens were based on a single isoform. This resulted in reduced slopes in the transition regions for the natural allergens and therefore lower estimates of the conformational stability in GuaHCl denaturation experiments. However, [GuaHCl$_{50\%}$] values were similar between the recombinant and natural allergens of Bet v 1. For Api g 1 both [GuaHCl$_{50\%}$] and $m_{D\text{-}N}$ values were similar between natural and recombinant allergen preparations.

Most striking were the relatively high values of $m_{D\text{-}N}$ for rBet v 1.0101 and rDau c 1.0103 as compared to other denaturation studies [1, 17, 22, 27-29]. A ratio from the GuaHCl and urea denaturation data could be calculated from the $m_{D\text{-}N}$-values of rBet v 1.0101 of our data and of rBet v 1.2801 of Mogensen et al. [1]. This m(GuaHCl)/m(urea) ratio is 2.8 and describes the unfolding of the protein as polar compared to ratios of 1.6-2.3 for unfolding of non-polar sidechains [29]. Myers et al. [28] showed a linear relationship between changes in the accessible surface area (ΔASA) of a protein and the $m_{D\text{-}N}$ values measured by GuaHCl or urea denaturation. ΔASA is defined as the difference between the solvent accessible surface area of the native protein and the modelled denatured protein. From the values of ΔASA, calculated from the GuaHCl data $m_{D\text{-}N}$ (Table 2), an estimate could be made of the theoretical number of amino acid residues, ΔC_P and $m_{D\text{-}N}$ of urea denaturation. The $m_{D\text{-}N}$ value of rBet v 1.0101 calculated for urea denaturation corresponded to the $m_{D\text{-}N}$ value of Mogensen et al [1] and was slightly higher, because the linear relationship did not correct for polar unfolding. The number of amino acid residues was overestimated for rBet v 1.0101 and rBet v 1.2801 for the GuaHCl and urea experiments, respectively (not shown for data of Mogensen et al. [1]), whereas the number of amino acid residues for nBet v 1 and nApi g 1 were underestimated, due to lower $m_{D\text{-}N}$ values as a result of isoform mixtures. These theoretically derived values showed the possibility that rBet v 1.0101 does not follow two-state unfolding, whereas rApi g 1.0101 does. rBet v 1.0101 was shown to be able to form dimers (and oligomers) in solution [30]. In folding experiments by Mogensen et al. [1], a misfolded intermediate state was detected for rBet v 1.2801 whereas Mal d 1 folded without intermediates.

The second part of this study described the pH dependence of nApi g 1 and nDau c 1 as another method to estimate the conformational stability, which could be compared with a similar study with rBet v 1.2801 [1]. This method also showed the effect of pH on thermal stability of the natural allergens. According to this method, the conformational stability of nApi g 1 and nDau c 1 was similar to rBet v 1.2801, but higher than the conformational stability determined in the GuaHCl denaturation experiment. However, the pH characteristics of nApi g 1 and nDau c 1 were different from rBet v 1.2801.

For the thermal denaturation curves, two-state folding kinetics was assumed, which is a completely reversible process meaning that the unfolded protein is able to refold

Table 2. Theoretical derived parameters from the linear relation of $m_{D\text{-}N}$ values and the changes in accessible surface area.

	ΔASA[a] (Å²)	Number of AA-residues[b]	ΔC_P[c] (kJ/mol·K)	Urea $m_{D\text{-}N}$[d] (kJ/mol)
Theoretical protein[e]	13,880	**159**	9.4	7.9
rBet v 1.0101	27,459	305	17.6	12.7
nBet v 1	10,641	124	7.4	6.0
rApi g 1.0101	11,550	134	8.0	6.4
nApi g 1	8,823	105	6.3	5.3
rDau c 1.0103	30,186	334	19.3	13.8
nDau c 1	16,095	183	10.7	8.2

All parameters were calculated from the $m_{D\text{-}N}$ values of GuaHCl denaturation (Table 1) from the empirically established linear correlations described by Myers et al. [28].

[a] The change in accessible surface area, ΔASA was calculated from; $m_{D\text{-}N(GuaHCl)} = 859 + 0.22(\Delta ASA)$

[b] The number of amino acid residues was calculated from; $\Delta ASA = -907 + 93(\#res)$

[c] The heat capacity change, ΔC_P, was calculated from; $\Delta C_P = -336 + 0.66(m_{D\text{-}N(GuaHCl)})$

[d] The theoretical value for urea denaturation, Urea $m_{D\text{-}N}$, was calculated from; $m_{D\text{-}N(GuaHCl)} = -110 + 2.3(m_{D\text{-}N(urea)})$

[e] A theoretical protein was included in the table with the parameters calculated for 159 amino acid residues.

N.B. All linear correlations are given in Cal.

completely. From the described linear relationship [26] between ΔH_{Tm} and T_m the heat capacity change was determined by linear fitting of the data points of thermal denaturation curves at different pH without using a calorimeter [25, 26]. For both nApi g 1 and nDau c 1, a pH range was observed without two-state kinetics (simplest representation; $N \rightleftharpoons D \longrightarrow$ Aggregation) showing irreversible unfolding between pH 4.4-6.0 and pH 4.4-5.6, respectively. However, a linear relationship was observed for nDau c 1 for both reversible and irreversible unfolding data points fitted to a two-state equation, showing more correlation than rBet v 1.2801 [1]. For nApi g 1 a linear relationship was only visible for the data points corresponding to irreversible unfolding (pH 4.4-6.0). For reversible unfolding, a non-correlated cluster was visible and a linear relationship was only fitted to the ΔH_{Tm} and T_m values at low pH. This division of data points was caused by the T_m-values of nApi g1, which were 10 °C higher than for nDau c 1 above pH 6.0, but below pH 6.0 T_m-values were similar (Figure 3). ΔH_{Tm} was comparable for both allergens (ratio $\Delta H_{Tm,\text{Api g 1}}/\Delta H_{Tm,\text{Dau c 1}}$) = 0.75-1.4) in the complete

pH range. The linear relationship between ΔH_{Tm} and T_m was likely affected by non-two state kinetics and not by the presence of multiple isoforms in the measured solution. Nevertheless, a general trend was observed for the natural allergens, which allowed us to study the overall protein stability.

The slopes of the linear fits of nApi g 1 and nDau c 1 from our study showed ΔC_P values, which were similar to rBet v 1.2801 observed by Mogensen et al [1]. The parameter ΔC_P is related to $m_{D\text{-}N}$ and gives indications for exposure of the non-polar accessible surface area by unfolding. The low values of ΔC_P found in this experiment, indicated that the protein is not strongly hydrophobic, which corresponds to polar unfolding established by $m_{D\text{-}N}$ [17]. The conformational stability parameter, ΔG_T, of rBet v 1.2801 at pH 7.0 was 18.6 kJ/mol, a value close to the ΔG_{D-N}^{H2O} of the GuaHCl curve of rBet v 1.0101 (Table 1) and close to the ΔG_T values of both natural allergens (Table 3). By using the pH dependence as a model system for determining the conformational stability instead of GuaHCl denaturation, the influence of the isoform mixture could be averaged and results in better estimates of ΔG.

Besides differences in thermodynamic parameters, differences were also observed in the pH stability of the natural allergens Api g 1 and Dau c 1 between rBet v 1.2801 [1]. A midpoint for a pH transition was visible at pH 3.5 for rBet v 1.2801 which was approximately 1 pH unit lower than for nApi g 1 and nDau c 1 and 2 units lower than Bet v 1's isoelectric point. The optimal pH-values of about 6.4 with the highest T_m were similar for the three allergens (Table 1, Figure 3). The most remarkable difference was the observation that both nApi g 1 and nDau c 1 were stable to heating below pH 4.4 whereas rBet v 1.2801 showed unfolding at pH 3.5-4 [1]. In a different study, it was shown that rBet v 1.0101 was completely unfolded at pH 2.2 [30]. The Bet v 1 homologue, rPru p 1 from peach, showed reversible unfolding at neutral pH when heated to 95 °C. At pH 3, rPru p 1 was completely unfolded at room temperature [31]. The natural allergens Api g 1 and Dau c 1 in our study therefore contradict the general statements that Bet v 1 homologues are more susceptible to denaturation at low pH than at neutral pH [31].

In conclusion, our study has shown that thermodynamic characterization gives useful information about allergens. Such information could be helpful in interpreting behavior of allergens in relation to food processing as well as in relation to immunological response.

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Chapter 5

Differences of PR-10 allergens and implications for predicting cross-reactivity of birch pollen-related food allergens

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Abstract

The major allergen from birch pollen, Bet v 1, is responsible for cross-reactivity to PR-10 allergens from especially fruits from the *Rosaceae* and vegetables from the *Apiaceae*. It is believed that cross-reactivity is based on similarities of the tertiary structure of PR-10 allergens, but as such cross-reactivity is poorly understood. The present study shows that IgE binding is not easily predicted on the basis of surface topology alone and that the differences in amino acid properties, surface charge and intermolecular interactions, all contribute to IgE. On the basis of structural properties of amino acids and secondary structure, the *Rosaceae* PR-10 were characterized as positively charged/polar proteins and the *Apiaceae* as negatively charged/hydrophobic proteins. Electrostatic potentials were calculated for structural models of PR-10 sequences to determine the charge distribution on the surface, which shows large charge differences among PR-10 sequences compared to Bet v 1. This study also takes into account the effect of low pH of fruit resulting in protonation of amino acid side chains and the resulting changes in the electrostatic potential.

5.1 Introduction

One of the major challenges in allergy research is to predict the allergenic potential of a protein. Molecular structure and biological function are highly important factors with regard to the identification of proteins as allergens. Only a few of the protein families that are described in the Pfam-database are known to contain allergens. In general, their biological functions are limited to hydrolysis of proteins, polysaccharides and lipids; binding of metal ions and lipids; storage; and cytoskeleton association [1, 2]. Besides having a limited number of biological functions, allergens have none or very few bacterial homologues, whereas a randomly selected group of control proteins have hundreds of bacterial homologues [3]. Furthermore, a relationship exists between allergenicity and the evolutionary distance from human homologues, showing that proteins are rarely allergenic when their amino acid sequence identity to human homologues is higher than 62% [4].

Allergy to the major birch pollen allergen Bet v 1 is the primary cause for birch related allergies to foods of especially the *Rosaceae* and *Apiaceae* families. The high amino acid similarity among these homologues provides a molecular basis for cross-reactivity [5]. Structural characterization, by X-ray crystallography and NMR studies, yields important information about the tertiary fold of the ubiquitous PR-10 protein family. However, observed differences in IgE binding to different naturally occurring isoforms of Bet v 1 can not be directly explained from this conserved three dimensional structure alone as both allergenic and hypoallergenic isoforms are structurally similar [6-8]. Over 50 unique Bet v 1 isoforms from various birch species have already been identified [9]. It is unclear whether all isoforms of Bet v 1 are relevant for cross-reactivity to fruit and vegetables as the IgE reactivity of numerous Bet v 1 isoforms remains unknown. Selected on the basis of its high IgE reactivity, Bet v 1a (Bet v 1.0101 or Bet v 1.2801 with mutation F62L) has become the most studied PR-10 isoform. This isoform is highly abundant among several birch species [10], although the other potential allergenic isoforms might be equally important for the induction of an allergic response.

A highly conserved region in the primary structure of PR-10 proteins is a glycine-rich loop, referred to as the phosphate loop (P-loop, 45-EGNGGPGT-52 for Bet v 1). The Fab fragment of a monoclonal murine IgG1 antibody BV16, which inhibits the binding of human IgE to Bet v 1a, was crystallized in complex with the structure of Bet

v 1, showing that this antibody binds to Bet v 1 in the P-loop. Residue Asn43, Glu45, Gly46, Asn47, Pro50 and Gly51 of Bet v 1 were involved in hydrogen bonding to the Fab fragment [11]. Substitution of the acidic Glu45 residue in the P-loop by a polar serine, resulted in a 50% reduction of IgE binding capacity and no binding to the monoclonal BV16 antibody, suggesting that the P-loop is an important epitope. The 3D-structure of the serine mutant and the wildtype Bet v 1 were identical as determined by X-ray crystallography [12].

Ever since, residue Glu45 is considered a critical amino acid for human IgE binding [12], but cannot be the only factor involved. The Bet v 1L isoform is hypoallergenic [6, 7] and shows virtually no affinity to human IgE in *in vitro* studies [8]. However, the 3D-structure of Bet v 1L is identical to Bet v 1a and its P-loop is 100% identical to all other Bet v 1 isoforms. Moreover, Pru av 1, Api g 1.01, and two isoforms of Mal d 1, were not able to bind to BV16, whereas residue Glu45 is conserved for Pru av 1 and Mal d 1 [13, 14]. This indicates that other residues are important for binding of BV16 and for human IgE as well. The amino acids Asn43, Asn47 and Pro50, which are forming hydrogen bonds between Bet v 1a and BV16, are substituted by Ile43, Asp47 and Val50 in case of Mal d 1 and could be of comparable interest. Furthermore, the P-loop region is not completely conserved for the celery allergen Api g 1, which has a positively charged Lys at this position. An increase in IgE binding was observed for the substitution of this residue into the acidic residue Glu [14], but the mutation of a positively into a negatively charged amino acid could also cause an intramolecular rearrangement in the proteins as observed for mesophilic and thermophilic proteins [15].

Potential IgE binding sites can be determined by site-directed mutagenesis studies of Bet v 1. However, such studies were driven by the creation of hypoallergenic mutants more than by the wish to elucidate the nature of cross-reactivity [16]. Other mutant studies are based on the different amino acids of Bet v 1a and the hypoallergenic isoforms Bet v 1d and Bet v 1L [17]. Many of the conclusions are drawn on the basis of immunoblotting or direct ELISA and for both methods the sensitivity may be influenced by conformational changes of the allergens [17-19]. Furthermore, these mutant studies are transferred to the cross-reactive allergens of the *Rosaceae* and *Apiaceae*. For these allergens it is mostly unknown if cross-reactivity is affected by changes in tertiary structure or protein stability.

The PR-10 protein family contains several proteins that are known as allergens, while some members are hypoallergenic or have not been described as such. Previous studies have focused on identifying IgE epitopes on the basis of similarities between known allergens. In contrast, the present study focuses on differences and similarities between PR-10 proteins, including those of the *Rosaceae* and *Apiaceae* family, but also those which are not known as allergens. From this comparison, it becomes evident that prediction of IgE-binding cannot be based on surface topology of the putative binding site alone, and that such structure-function relationships are much more complex. Differences in amino acid composition of PR-10 proteins may lead to changes in their electrostatic potential. In our approach, the relevance of the charge state of the PR-10 structure and its influence on IgE binding is considered. For this purpose, we searched the GenBank database for ESTs or mRNAs of PR-10 sequences that have been recovered from edible parts of fruits, nuts, legumes and vegetables. Some of these have been identified as allergen, while others are potential allergens.

5.2 Materials and methods

5.2.1 Database search

Bet v 1 homologues were recovered from GenBank/EMBL/DDJB by searching with MegaBLAST for entries with more than 25% sequence identity to Bet v 1a (X15877).
This search was performed in April, 2007. We selected only mRNA sequences and ESTs that had been recovered from edible plant parts for further analysis. PR-10 proteins are present as a multigene family in many plants. Different PR-10 gene family members display a high degree of similarity within a particular plant species and to avoid redundancies, one complete sequence was randomly selected among the recovered databank entries (Table 1).

5.2.2 Amino acid properties

Sequences were aligned to the sequence of Bet v 1a (X15877) using ClustalW and sequence identity was calculated. Next, a data table was created on the basis of Bet v 1's secondary structure of the protein entry in the Protein Data Bank (PDB-entry; 1bv1) as assigned by the DSSP-algorithm in the graphical viewer Visual Molecular Dynamics (VMD) [20]. The protein was divided into nine structural elements (Table 2) by taking

one loop and/or random coil region together with the subsequent β-strand or α-helix. Among the PR-10 proteins, the chain length differs by a few amino acids at the C-terminal end (residues 156 to 160). Therefore, residues 156 to 160 were not taken into consideration. The two short α-helices were taken together as these structures interact with all parts of the structure and are, not for all sequences, partially shielded by the same residues from the C-terminal end. The discussed P-loop region is included in the element that is represented by the third β-strand.

The amino acid residues were distinguished per element by four different characteristics: 1) positively charged/basic (His, Arg and Lys), 2) negatively charged/acidic (Asp and Glu), 3) hydrophilic/polar (Asn, Gln, Gly, Ser, Thr and Tyr), and 4) hydrophobic/non-polar (Ala, Cys, Ile, Leu, Met, Phe, Pro, Trp and Val). These characteristics are chosen on basis of the representation by VMD. The number of amino acids per characteristic and element were combined in a data table, which was subjected to a principal component analysis with Pirouettte 4.0 (Infometrix Inc, Bothel, WA, USA).

5.2.3 Structural modeling and energy minimization

To calculate the net charge and protonation states of PR-10 sequences, 3D protein models were built using SWISS-MODEL [21] for PR-10 sequences of grape (*Vitis vinifera;* CV096864 and EC931728), clementine mandarin (*Citrus clementina;* DY260937), Valencia orange (*Citrus sinensis;* EH406480), bell pepper (*Capsicum annuum;* CO912259), Ara h 8 from peanut (*Arachis hypogaea;* AY328088), Dau c 1.01 from carrot (*Daucus carota;* Z81361) and Mal d 1, Mal d 1.06A02 and Mal d 1.06A03 from apple (*Malus domestica;* X83672, AY789248, AY827701). These PDB-structures were submitted to energy minimization and a short equilibration (100 ps) in a water-box in the VMD interface using NAMD molecular dynamics [22]. The original PDB-entries of Bet v 1a (1bv1), Bet v 1L (1fm4), Pru av 1 (1e09) and Api g 1 (2bk0A) were taken from the RCSB protein data bank as well and treated similarly to rule out any effects of constraints. For this procedure, hydrogen atoms were added to the amino acid side chains of the PDB-files, followed by solvation of the protein in a water box with 10 Å of water on each end of the molecule. The parameters used for the configuration file are described on the VMD website in the NAMD tutorial file (http://www.ks.uiuc.edu/Training/Tutorials/). CHARMM forcefield parameters were

included at a simulation temperature set to 310 K. A 2 fs/step timestep with rigid bonds (fixed protein) and periodic boundary conditions were used with all molecules wrapped (wrapAll) at constant pressure. The protein was minimized for 1,000 steps to lower the potential energy, reinitialize the velocities to 310 K and eliminate bad initial contacts such as steric hindrance, bond length and bond angles. The equilibration was run for 100 ps. The last frame of the trajectory file was considered as the energy minimized and equilibrated protein. Under similar conditions, the equilibration of Bet v 1L was run for 2 ns to determine differences between the long and short equilibrations.

5.2.4 Electrostatic potential

Electrostatic potentials of the proteins were calculated for the energy minimized and equilibrated PDB-structures. PDB-files were converted to a PQR format by using PDB2PQR (version 1.3.0) [23] with a CHARMM forcefield [24]. PROPKA (Prediction of protein pKa values) Web interface [25] was used to assign protonation states in the pH range 3.0-8.0. The conversion to a PQR-file determines the side chain pKa values, places missing hydrogen atoms, optimizes the protein for favourable hydrogen bonding and assigns charge and radius parameters from the CHARMM forcefield. The electrostatic potentials were calculated by using APBS 0.5.0 (adaptive Poisson-Boltzmann solver [26]) as a plug-in in VMD. A system temperature of 298.15 K was chosen with 0.15 M of mobile ions. The dielectric constants were set to 1.0 for the protein and 78.54 for the solvent (water). The electrostatic potentials were visualized in VMD and represented by their isosurfaces (blue as positive and red as negative charge). Pictures were rendered using Tachyon version 0.98, a program included in VMD.

5.3 Results and Discussion

5.3.1 Primary structure properties

PR-10 genes are transcribed in many food sources other than the plant species for which cross-reactivity by Bet v 1 homologues has been demonstrated. Table 1 lists the EST or mRNA sequences of PR-10 proteins, recovered from GenBank/EMBL/DDJB, which are transcribed in edible parts of plant foods. Not all plant foods, listed in Table 1, have been described to cause birch-pollen related food allergy in which Bet v 1 acts as the primary sensitizer. For example, grape, mandarin and orange are regularly consumed fruits, but it is unknown whether these PR-10 proteins are expressed at low concentrations or whether cross-reactive epitopes are absent. The cross-reactivity of *Rosaceae* fruits, such as apple and cherry, and *Apiaceae* vegetables, such as carrot and celery, has been extensively studied. Table 1 shows the Genbank accession numbers, the allergen code, and the percentage of amino acid identity to Bet v 1a. The *Rosaceae* have an identity of approximately 55-60 % and are more related to Bet v 1a than the *Apiaceae* with an identity of 35-40 %.

Bet v 1a has been identified as an isoform with a high IgE-reactivity [6]. This isoform was shown to encompass approximately 40% of the total Bet v 1 content in pollen of *Betula pendula* Youngii [10]. Other abundant isoforms (20%) are highly similar to the hypoallergenic Bet v 1d or L (Bet v 1.0401 and Bet v 1.1001). However, the IgE binding capacity of the other 40% of the Bet v 1 content has not been determined or was shown to be intermediate. Little is known beyond the allergenic properties of Bet v 1a and Bet v 1L and there is also little information regarding the impact of individual isoforms on cross-reactivity. The differences in cross-reactivity between isoforms of Mal d 1 among different apple cultivars have recently been emphasized in a study of Gao et al. [27]. The isoforms of the iso-allergen Mal d 1.06A were shown to be associated with differences in allergenicity among 14 apple cultivars. Isoform variants 01 and 03 were shown to be associated with a higher allergenicity, while variant 02 was associated with a lower allergenicity as measured in skin prick tests. However, Mal d 1.0108 (indicated as Mal d 1) is the most frequently studied isoform. Therefore, sequences of Mal d 1.06A (and also Mal d 1.04) were also included for further analyses besides the sequence of Mal d 1 (Table 1).

Table 1. PR-10 sequences transcribed in fruits, vegetables, grains, nuts, legumes and seeds sorted by the overall amino-acid sequence identity to Bet v 1a (X15877).

Source	EST or mRNA from edible part	Allergen code (Genbank Acc No)	Sequence identity to Bet v 1a
Beech nut (*Fagus sylvatica*)	AJ130889	-	69%
Hazelnut (*Corylus avellana*)	AF136945	Cor a 1.04 (AF136945)	67 %
Apple (*Malus domestica*)	AY789242	Mal d 1.04 (AY789242)	60 %
Apricot (*Prunus armeniaca*)	U93165	Pru ar 1 (U93165)	60 %
Cherry (*Prunus avium*)	U66076	Pru av 1 (U66076)	59 %
Peach (*Prunus persica*)	DQ251187	Pru p 1 (DQ251187)	59 %
Grape 1 (*Vitis vinifera*)	EC958497	-	58 %
Pear (*Pyrus communis*)	AF057030	Pyr c 1 (AF057030)	57 %
Apple (*Malus domestica*)	X83672	Mal d 1 (X83672)	56 %
Apple (*Malus domestica*)	AM283501	Mal d 1.06A (AM283501)	55 %
Strawberry (*Fragaria ananassa*)	AM236319	Fra a 1 (AM236319)	54 %
Grape 2 (*Vitis vinifera*)	CV096864	-	53 %
Grape 3 (*Vitis vinifera*)	EC931728	-	53 %
Cassava (*Manihot esculenta*)	DV457043	-	52 %
Valencia orange (*Citrus sinensis*)	EH406480	-	46 %
Soybean (*Glycine max*)	-	Gly m 4 (X60043)	46 %
Peanut (*Arachis hypogaea*)	AY328088	Ara h 8 (AY328088)	45 %
Cluster bean (*Cyamopsis tetragonoloba*)	EG987166	-	44 %
Sweet potato (*Ipomoea batatas*)	CO499980	-	44 %
Sesame (*Sesamum indicum*)	BU668133	-	44 %
Cowpea (*Vigna unguiculata*)	CK151444	-	43 %
Oca (*Oxalis tuberosa*)	AF333436	-	43 %
Tomato (*Solanum lycopersicum*)	AK427718	-	43%
Mungbean (*Vigna radiata*)	AY792956	Vig r 1 (AY792956)	42 %
Mandarin (*Citrus reticulata*)	DY260838	-	40 %
Celery (*Apium graveolens*)	Z48967	Api g 1.01 (Z48967)	40 %
Bell-pepper (*Capsicum annuum*)	CO912259	-	39 %
Yam-bean (Pachyrhizus erosus)	AY433943	-	39 %
Celery (*Apium graveolens*)	Z75662	Api g 1.02 (Z75662)	39 %
Clementine mandarin (*Citrus clementina*)	DY260937	-	38 %
Carrot (Daucus carota)	AF456481	Dau c 1.02 (AF456481)	38 %
Parsley (*Petroselinum crispum*)	X98688	-	38 %
Carrot (Daucus carota)	Z81361	Dau c 1.01 (Z81361)	36 %
Onion (Allium cepa)	CF452005	-	34 %
Asparagus (*Asparagus officinalis*)	AJ132612	-	33 %
Bread Wheat (*Triticum aestivum*)	CJ856226	-	31 %
Barley (*Hordeum vulgare*)	BI778636	-	31 %
Sorghum (Sorghum bicolor)	U60764	-	28 %

In general, PR-10 proteins have a highly similar tertiary structure, while having several differences at the amino acid level. Therefore, the amino acid properties of the secondary elements, α-helix, β-strands and loops (Table 2), of the PR-10 sequences were investigated in detail studying the properties of the amino acid side chains in our analysis (positively charged, negatively charged, hydrophilic (polar) or hydrophobic). A principal components analysis (PCA) was performed on the properties per secondary element and the first two dimensions explained 40.5 % of the total variance (Figure 1). Several clusters were observed in the scores-plot of the PCA (Figure 1A). In general, PR-10 sequences within plant families, *Betulaceae*, *Fabaceae* (legumes), *Rosaceae* and *Apiaceae*, cluster together. The plot shows that the PR-10 sequences of the *Fabaceae* or legumes are more closely related to Bet v 1 than those of the *Rosaceae* and *Apiaceae*. Allergy to legume PR-10 is rather uncommon, although severe reactions have been observed for the soy allergen Gly m 4. Legumes are normally consumed as processed foods and the antibody binding capacity is depending on the rate of processing, which decreases with fermentation and heating time [28]. PR-10 sequences of asparagus, bread wheat, onion, sorghum and barley form a separate cluster, which is visible when the third dimension is taken into account. These sequences also have the lowest sequence identity to Bet v 1a. In conclusion, based on the properties of the amino acid side chains and secondary structure, we found no indication why PR-10 proteins in the *Rosaceae* family are more involved in Bet v 1-related allergies than those in the *Fabaceae*.

Table 2. Sequence of Bet v 1 divided into secondary elements.

Name	Amino acid residues	Loop and/or random coil	α-Helix or β-strand
β1	1-12	1-2	3-12
α1+2	13-34	13-14	15-34
β2_L1	35-46	35-40	41-46
β3_P-loop	47-58	47-52	53-58
β4_L2	59-76	59-67	68-76
β5_L3	77-88	77-80	81-88
β6_L4	89-107	89-95	95-107
β7_L5	108-123	108-112	113-123
α3_L6	124-155	124-130	131-155

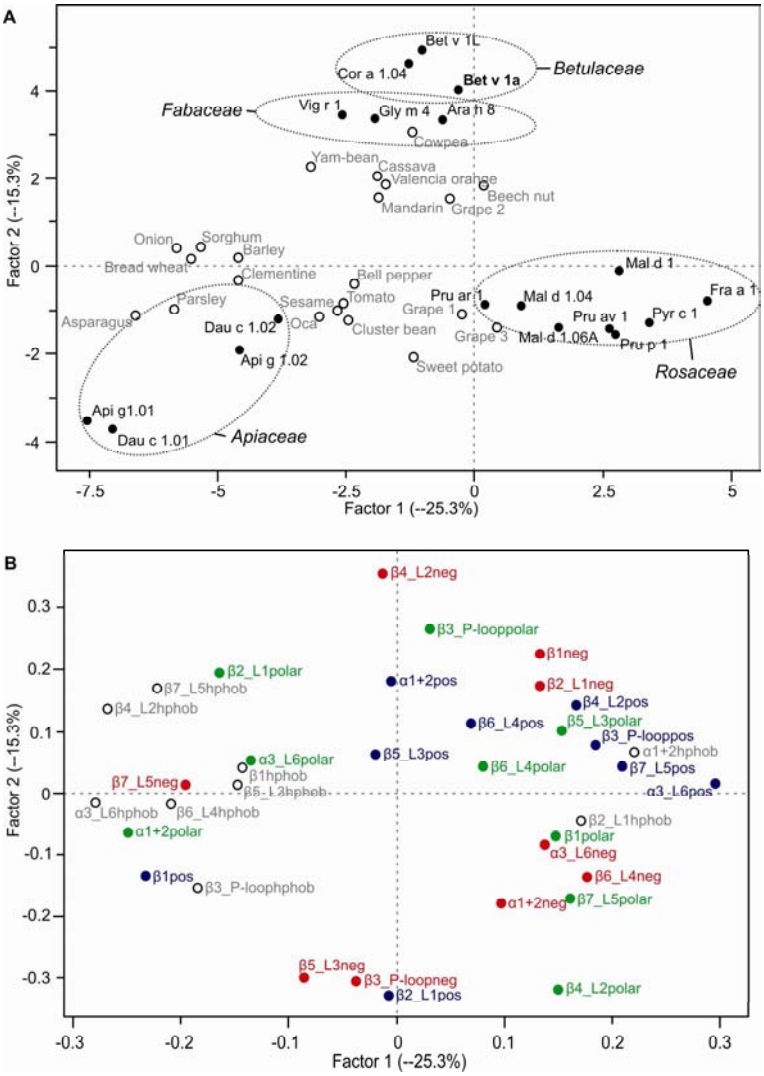

Figure 1. PCA plot of the amino acid characteristics of the secondary structure regions (Table 2) of PR-10 sequences, including both allergens and proteins that have not been described as such. **A**) Scores plot of the first two dimensions with the sources of PR-10 sequences with cross-reactive plant families indicated by ellipses. PR-10 sequences identified as allergens are shown in black with filled circles. Sequences of PR-10 proteins that have not been described as allergens are displayed in gray with open circles. **B**) Loadings plot of the first two dimensions of the variables, of the secondary structure components divided into the amino acid characteristics: positively charged (blue), negatively charged (red), polar (green) and hydrophobic (hphob, gray and open circles).

The loadings plot (Figure 1B) can be used to quickly characterize the differences between PR-10 clusters and similarities in properties of PR-10 clusters per sequence region. The largest difference is observed for the region α3_L6, for which most *Rosaceae* PR-10's contain more positively charged residues than Bet v 1a and for which the *Apiaceae* PR-10's contain more hydrophobic residues and a reduced number of positively charged residues. The α1+2 region, especially α1, is shielded by the C-terminal end (residue 155-160) of α3 for most PR-10 sequences, resulting in an increased number of hydrophobic residues. In general, the PR-10 sequences of *Apiaceae* are more hydrophobic than the *Rosaceae*, which contain more positively charged and polar residues. Api g 1 and Dau c 1 are especially more hydrophobic in the β5_L3, β6_L4, β7_L5 and α3_L6 regions, whereas these regions are more polar for Mal d 1, Pru av 1, Pyr c 1 and Pru p 1.

For region β4_L2 of Api g 1 and Dau c 1, two positively and three negatively charged residues have been substituted by polar residues compared to Bet v 1a. For *Rosaceae*, this region shows an increase in polar residues and is reduced by two negatively charged residues. The buried amino acids of this region are important for ligand binding as shown for several PR-10 proteins [8, 29]. The negatively charged residue Asp69 in the interior of Bet v 1 is not present in Api g 1 and is replaced by the hydrophobic residue Leu69. Mal d 1 and Pru av 1 have a positively charged residue His at this position. It is unknown whether a bound ligand has an effect on IgE binding, but the different residues indicate that PR-10 proteins of *Rosaceae* and *Apiaceae* may have different ligand binding properties. This may in turn have an effect on IgE binding as the interaction of the ligand changes the electrostatic potential of the protein surface.

In total, Api g 1 and Dau c 1 have eight positively and two negatively charged residues less than Bet v 1, which are substituted by mainly hydrophobic and polar residues. Mal d 1, Pru av 1, Pyr c 1 and Pru p 1 have an overall increased number of positively charged residues (two or three) and polar residues, which makes the *Rosaceae* PR-10 protein group more polar. Plotting the total number of hydrophobic residues versus the ratio of the number of positively and negatively charged residues (Figure 2) shows that Bet v 1a has a similar number of positively and negatively charged residues as the ratio is 1.0. Regions, indicated in this plot, show different characteristics: positively charged and polar; positively charged and hydrophobic; and negatively charged and hydrophobic. The *Apiaceae* are located close to some sequences

of the *Fabaceae* as opposed to the PCA analysis. The *Rosaceae* are more closely related to Bet v 1, especially the sequences of Mal d 1.04 and Mal d 1.06A. Several PR-10 proteins which are not known to be involved in Bet v 1-related food allergies have intermediate characteristics between Bet v 1 and allergens of the *Apiaceae* and *Rosaceae* families as shown in Figure 1 and 2. Therefore, these sequences could be potential allergenic proteins as their structural characteristics are more related to Bet v 1.

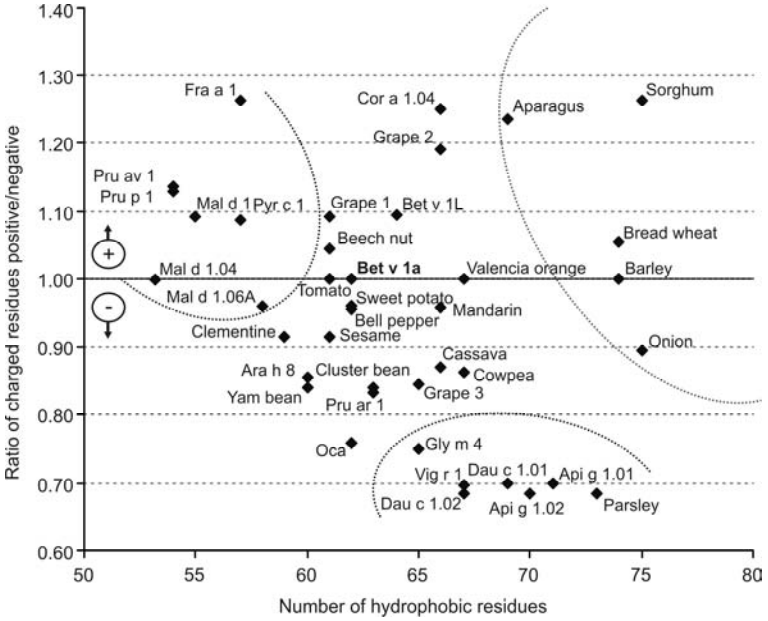

Figure 2.Characteristics of PR-10 sequences from Table 2 based on the total amino acid count for AA 1-155. The total number of hydrophobic residues is plotted versus a ratio, which is the number of positively charged residues divided by the number of negatively charged residues. PR-10 sequences are considered positive above 1.00 and negative below 1.00. The dotted lines indicate sequences with similar properties.

5.3.2 Structural modeling

Three dimensional models of different PR-10 sequences were constructed by using existing PDB entries. All models, including the PDB structures from the PDB databank (Bet v 1a, 1bv1; Bet v 1L, 1fm4, Pru av 1, 1e09; and Api g 1.01, 2bk0A), were submitted to an energy minimization and a short equilibration in an aqueous environment. The short equilibration time can be used to study some of the dynamical processes, such as

hydrogen bond and salt bridge formation, of the protein in water. It is not within the scope of this study to add ions to the system, as this requires longer simulation time (at least 2 ns) in order to reach equilibrium between ion binding to and release from the protein. During the minimization and equilibration step, the polypeptide backbone and amino acid side chains have moved in the aqueous environment. The root mean square deviation (RMSD) was calculated per residue (Figure 3) to determine the movement of the minimized structures Bet v 1a, Mal d 1, Pru av 1 and Api g 1, as compared to the original PDB-file of Bet v 1. This was accomplished by a multiple structure alignment in VMD, which results in an overlay of all structures. Residues in the β-strands are the most stable in the structure and show little movement as observed from the RMSD-plot. A larger RMSD is observed for the α-helices, with α3 showing an inward movement into the cavity as a result of the simulation (Figure 3, insert). The difference in RMSD for α2 of Api g 1 and Bet v 1a is observed in the original PDB-file [30] and is not a result of the simulation. The loop regions are the most flexible regions, and consequently show a high RMSD. The RMSD per residue is smallest for the equilibrated Bet v 1 structure. The inward movement of the α-helix can be explained by the absence of physiological ligands or other stabilizing molecules, such as water or ions, inside the cavity.

Interestingly, these short simulations show that the two salt bridge forming residues, Asp27 and the buried residue Lys54, are key residues, which are conserved among most PR-10 sequences. The formation and breaking of this salt bridge is influenced by the buried residue Asp69, a non-conserved residue which is important for ligand binding, as mentioned in the previous part. The ability of Bet v 1L to form salt bridges is influenced by Lys28, a residue which is replaced by Asn28 in Bet v 1a. In a 2 ns simulation of Bet v 1L, a salt bridge formed between Asp25 and Lys28. This prevents the formation of the Asp67-Lys54 salt bridge. The Asp27-Lys54 salt bridge can be broken, resulting in the formation of a salt bridge between Asp27 and Lys28, which allows the formation of the Asp69-Lys54 salt bridge. However, Bet v 1a is able to form both salt bridges. Furthermore, the formation of salt bridges is a dynamic process and responsible for changes in the electrostatic potential and could therefore affect antibody binding [31]. The salt bridge formation in *Apiaceae* PR-10 is reduced as a result of a reduced number of charged amino acids.

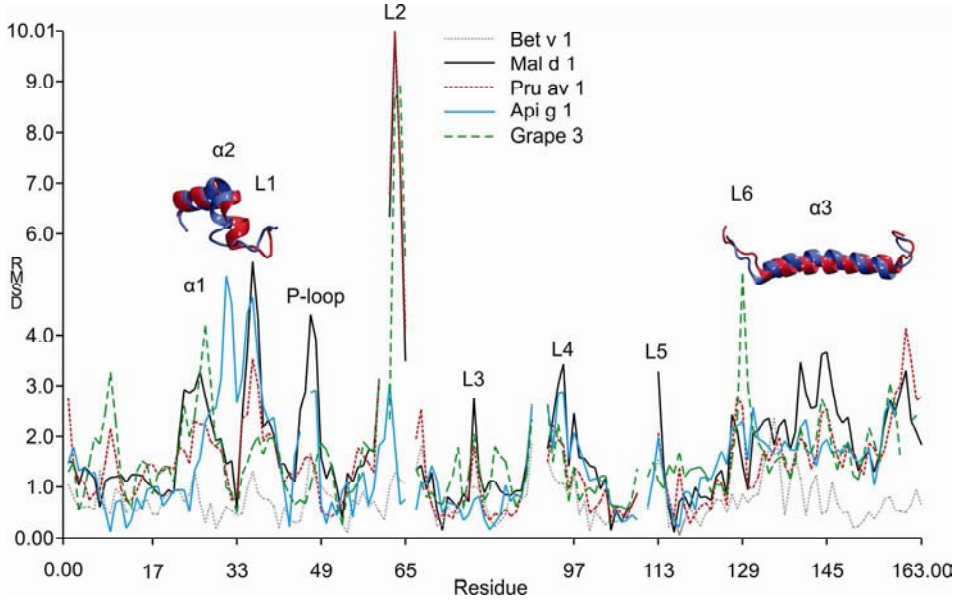

Figure 3. RMSD of the aligned and energy minimized PDB models of Bet v 1, Mal d 1, Pru av 1, Api g 1 and Grape 3 compared to the non-simulated PDB-file of Bet v 1, 1bv1. The variable α-helix regions are shown as inserts for 1bv1 (red) and Api g 1 (blue, α1+2 with L1) and 1bv1 (red) with Mal d 1 (blue, L6 with α3).

5.3.3 Differences in electrostatic potentials

The modeled and equilibrated structures were used to calculate the electrostatic potential of the protein. Small changes in electrostatic properties can change the interaction with antibodies as the formation of an antibody-allergen complex requires both a high degree of complementarity in shape and chemical complementarity [32]. This chemical complementarity is determined by hydrophobic interactions, electrostatic interactions and proton donors and acceptors for hydrogen bond formation [33]. The overall net charge at neutral pH is negative for all PR-10 proteins (Figure 4). Dau c 1 and Api g 1 have the most negative net charge as supported by the observed difference between the positively and negatively charged residues (6 to 7 more negatively charged residues). Mal d 1.06A02 has a similar negative net charge, but a different ratio in positively and negatively charged residues.

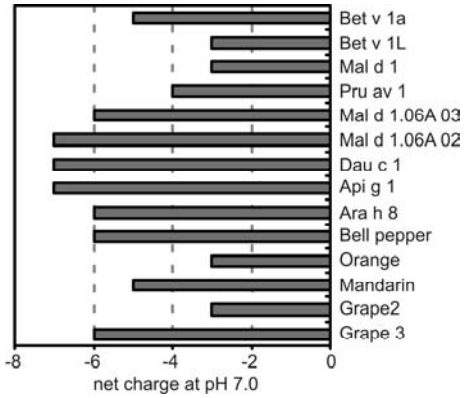

Figure 4. PR-10 net charge of energy minimized PDB-files (Bet v 1a, Bet v 1L, Pru av 1 and Api g 1) and structural models at pH 7.0.

By mapping the electrostatic potential on the surface of a protein, it is possible to better understand the interaction of IgE and the PR-10 protein via charged residues that play an important role as electron donors [34]. A general idea of important binding sites can be established by looking at PR-10 sequences of similar plant families. However, when electrostatic potentials of PR-10 proteins from different plant families are compared, more differences than similarities are observed. These differences are emphasized by the electrostatic potential as shown in Figure 5 for Bet v 1 (A), Api g 1 (B), Dau c 1 (C), Mal d 1 (D) and a grape PR-10 (E). The electrostatic potentials are depicted as isosurfaces, showing the full range of the charge, and showing the positively (blue) and negatively (red) charged areas with the P-loop facing forward. These pictures show that charges around the P-loop are different among the PR-10 sequences, but within plant families, such as Api g 1 and Dau c 1 from the *Apiaceae*, the electrostatic potentials are similar. Therefore, cross-reactivity is hard to predict and the different mutant studies do not contribute to a better understanding.

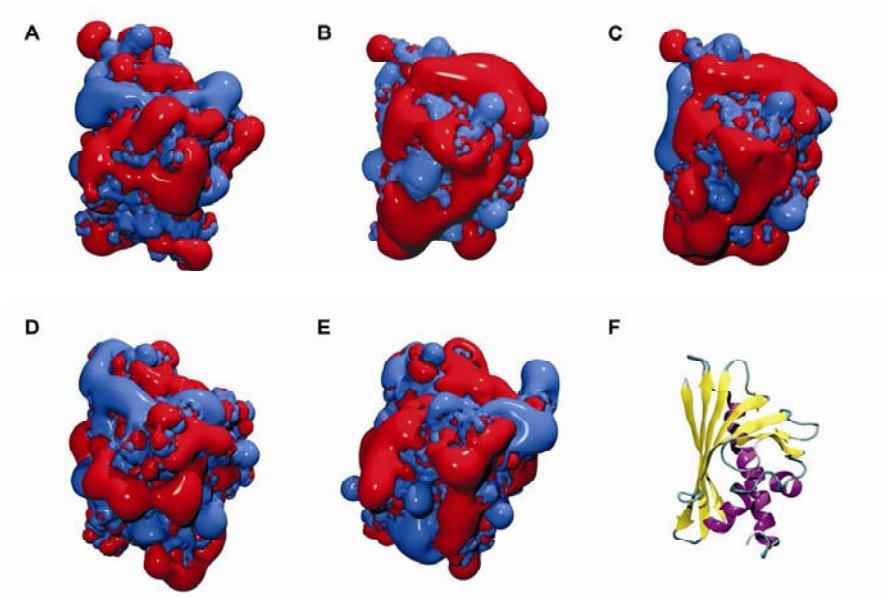

Figure 5. Electrostatic potentials of PR-10 proteins Bet v 1 (**A**), Api g 1 (**B**), Dau c 1 (**C**), Mal d 1 (**D**) and grape 3 (**E**). The blue and red isosurfaces represent the positive and negative charges, respectively. A cartoon representation of the Bet v 1 structure (**F**) is shown oriented with the P-loop facing forward.

5.3.4 The P-loop

The assignment of Glu45 as the most important amino acid of the IgE- epitope in the P-loop has led to mutational IgE-epitope analyses in allergens as Pru av 1 and Api g 1 whereas these proteins are different in their overall charge. For Pru av 1, residue 45 was substituted by Trp, creating a mutant with different properties (aromatic residue) and large steric hindrance. Although the 3D-structure is similar, as shown by NMR spectroscopy, IgE binding to this mutant is reduced [14]. In a mutant study of Api g 1.01, the positively charged residue Lys44 was substituted by the negatively charged Glu, which resulted in increased, but also decreased IgE binding capacity [35]. This substitution changes the electrostatic properties at this part of the protein and can have an effect on long range ion pairs without affecting the structure [31]. Furthermore, β3 of Api g 1 is different from Bet v 1, lacking a residue in the sequence, 42-EVK-44 compared to 42-ENIE-45. The surface exposed Ile44 residue in Bet v 1 forms hydrogen bonds with the surface exposed Glu45, whereas Val43 is a buried residue in Api g 1 and Lys44 is in closer proximity to the negatively charged Glu42. When Lys44 is replaced

by a negatively charged residue, this would rather result in repulsion of charges on the surface, which may also affect IgE-binding considerably.

5.3.5 Effect of pH on the electrostatic potential

Electrostatic interactions are important for protein-antibody interactions and membrane binding. A study by Mogensen et al. [36], showed that Bet v 1 and Mal d 1 were bound to membranes in a pH dependent manner. The ability to bind to membranes was first discovered for the steroidogenic acute regulatory protein (StAR), which is a structural homologue of Bet v 1. StAR associates with lipid membranes of mitochondria at a pH around 3.5-4.0 by undergoing a conformational change [37]. It was suggested that binding of Bet v 1 and Mal d 1 is encouraged by electrostatic attractions at low pH when the protein is positively charged. A conformational change of the protein was visible upon *in vitro* membrane binding, observed as an increase in α-helix by circular dichroism, and a loss of tertiary structure by 1D-NMR [36].

In diagnostics and research studies, the effect of pH on the IgE binding to proteins in food products is generally not taken into account. The tested allergens are generally purified and dissolved in a buffer of neutral pH. PR-10 proteins have been described as cytosolic proteins and the pH of the cytosol is in general neutral. When fruits of the *Rosaceae* are eaten, the PR-10 sequences are released from the cytosol into an acidic environment, which has a pH of pH 3.3-4.0. This may cause protonation of negatively charged amino acid side chains. It takes approximately 30 seconds to reduce the pH in the oral cavity to 4.0 when eating acidic products [38]. The allergens will come into contact with the acid before they bind to antibodies. Protonation itself leads to an increase in the positive charge of the electrostatic potential and between pH 3.5- 4.5 the total charge of most PR-10 proteins will be positive. Vegetables of the *Apiaceae* have a higher pH varying from pH 5.7 to 6.4 (http://www.cfsan.fda.gov/~comm/lacf-phs.html), which was shown to minimally affect the overall charge. The positive charge can affect IgE binding as well as binding of the PR-10 to mucosal membranes.

To illustrate the effect of a physiologically relevant pH on the electrostatic potential, the electrostatic potentials were calculated for the original PDB-entries of Bet v 1a (pH=7.0), Api g 1.01 (pH=6.0) and Pru av 1 (pH=4.0), accordingly. Figure 6 shows the difference between PR-10 allergens in both food groups. Negatively charged areas are observed for Api g 1, whereas Pru av 1 is mostly positively charged. In the context

of membrane binding, Pru av 1 and other fruits could cause oral allergy symptoms as a direct effect of membrane binding, whereas Api g 1 and other *Apiaceae* do not have this effect. This might explain differences between the IgE binding capacity of *Apiaceae* and *Rosaceae*, apart from the fact that most *Apiaceae* are eaten as cooked foods.

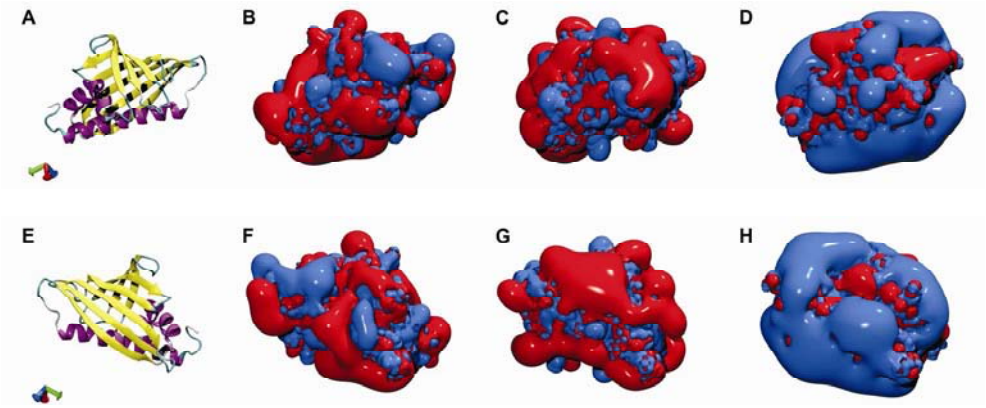

Figure 6. The effect of pH changes on the electrostatic potential of PR-10 allergens. A cartoon representation of Bet v 1a (**A**, **E**) shows the orientation of the PR-10 sequences presented by their electrostatic potential: Bet v 1a at pH 7 (**B**, **F**); Api g 1 at pH 6 (**C**, **G**); and Pru av 1 at pH 4 (**D**, **H**).

The charge and polar characteristics of Mal d 1.06A02 and Mal d 1.06A03 are more similar to the sequence of Bet v 1a than Mal d 1 (Figure 2). For these sequences models were created, by using the 3D-structure of Pru av 1 (PDB-entry: 1e09) as a template, to investigate the difference of amino acid Val12 of variant 02 and Ile12 of variant 03 and the difference between Mal d 1. Between pH 5.0-7.0, the calculated overall net charge shows a more negatively charged character of Mal d 1.06A02 than Mal d 1.06A03, which are both more negatively charged than Mal d 1. At pH 4, the isoforms are positively charged and Mal d 1.06A03 has the highest positive charge. Furthermore, Mal d 1.06A03 is able to form more hydrogen bonds than Mal d 1.06A02 at position 12 with the surrounding amino acids Ile13, Glu147 and Val151, because of the longer side chain of Ile12.

The charge predictions depend fully on the prediction of the pKa-values and the correctness of the energy minimized conformation of the protein in a completely aqueous solution. The prediction of pKa-values depends not only on the position of

ionisable groups (buried or surface exposed), but also on interactions via hydrogen bonds [25]. These pKa-values are important as they can be used to determine differences in deprotonation or protonation of side chains at a specific pH, which can give an extra indication for differences in antibody binding sites. Therefore, differences in IgE binding between Bet v 1a and Bet v 1L and between Mal d 1.06A02 and Mal d 1.06A03 could be partially explained by charge differences caused by side chain pKa-values of the negatively charged residues and histidine. These pKa-values are affected by intramolecular interactions, which can be decisive for the charge state of the allergen at acidic pH.

5.4 Concluding remarks

By characterizing the secondary structure by properties of amino acids, clear differences are observed for the PR-10 plant families of the *Rosaceae* and *Apiaceae*. The *Rosaceae* are more related to the *Betulaceae*, but are in general more positively charged and polar. The *Apiaceae* are more negatively charged and hydrophobic. The *Fabaceae* are closely related on the basis of the amino acid side chain properties of the secondary structure, but the overall negative charge is comparable to *Apiaceae*. Moreover, the characteristics of the PR-10 sequences of foods for which PR-10 cross-reactivity has hardly been described, are less pronounced in characteristics and are in closer proximity to Bet v 1 than the *Rosaceae* and *Apiaceae*. Nevertheless, allergic responses of this group are rarely reported and could imply that these PR-10 sequences are expressed at very low amounts as was shown for studies on grape allergens. Furthermore, for grape it is unknown what the effects are of the high tannin (polyphenol) content on the protein structure and concentration during eating [39, 40]. Enzymatic effects on polyphenols have been described to decrease the IgE-binding capacity of Mal d 1 [41].

Electrostatic complementarity is one of the driving forces in protein-antibody binding. However, the electrostatic potentials, shown on the 3D-structure of Bet v 1 cross-reactive PR-10 sequences, indicate that it is troublesome to draw consistent conclusions on conformational epitopes. The difference in charge and hydrophobic residues between Bet v 1 and other allergens is responsible for the IgE-binding capacity, depending on the surface topology of IgE-antibodies. To understand the differences in cross-reactivity of PR-10 proteins, the different characteristics of amino acid side chains should be taken into account, when a site-directed mutagenesis study is performed. The

influence of the pH of fruit on PR-10 allergens is unknown, but suggests that IgE-binding could be directed to a positively charged surface of *Rosaceae* PR-10 and a negatively charged surface of *Apiaceae* PR-10. A lower pH and changes in the electrostatic potential could also induce membrane binding to mucosal areas or could introduce conformational changes in the protein that affect IgE-binding. Furthermore, molecular dynamics could be well suited to explain differences between Bet v 1a and Bet v 1L including the effect of introduced mutations, but requires experimental evidence. A well suited analytical approach to determine possible IgE epitopes of Bet v 1 and differences among groups of plant families, would be amino acid shaving, for which residues are only substituted by Alanine [42]. A first step is to understand the differences in IgE-binding capacity between Bet v 1 isoforms before the transfer is made to cross-reactive allergens.

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Chapter 6

General Discussion

6.1 Introduction

Phylogenetic analysis results in overall topology of the relationships between the amino acid sequences of PR-10 related proteins with a proven allergenicity, and provides a tool to predict possible relationships at the amino acid level and protein structure. By constructing a phylogenetic tree based on the available PR-10 allergen sequences in the UniProt database (Table 1), the different PR-10 sequences grouped according to large families from the *Betulaceae, Rosaceae*, *Apiaceae* and the *Fabaceae* (Figure 1). Apparently, a large discrepancy exists between the amino acid sequence and the final allergenicity of the protein, as illustrated by the sequence identity between the allergenic sequence Bet v 1a and the hypoallergenic sequence Bet v 1L despite their largely different capacity in IgE binding. Alternatively, Dau c 1 and Api g 1 sequences are situated far apart from the Bet v 1, with low sequence identity, but share a profound IgE cross-reactivity. The allergenic potential of the various PR-10 protein family members is thus not only based upon the primary amino acid relationships. Therefore, studies into the functional properties of these proteins, such as structure-function and stability studies are required to explain the allergenic potential of these PR-10 family members.

The first aim of this study was therefore to characterize and identify differences between PR-10 proteins as compared to the major birch pollen allergen Bet v 1. For this purpose the allergens Api g 1 and Dau c 1 were purified from their natural sources. Subsequently, thermodynamic stability studies were performed on the natural allergen mixtures and the most commonly used recombinant isoforms: Bet v 1.0101, Api g 1.0101 and Dau c 1.0103. The second aim was to find a relation between the physico-chemical stability of PR-10 proteins as differences in the allergen's structural properties and stability could explain the differences observed for the IgE binding capacity. The same factors are likely to predict the allergenic nature of other homologous PR-10 proteins. By investigating PR-10 proteins, using a bioinformatics approach and including pH-effects demonstrated the distinction between the two major families, *Rosaceae* and *Apiaceae*, of birch pollen related cross-reactive food allergens. In general, this study has applied a different approach in allergen characterization, which can lead to improved understanding of the relationship between (structural) stability and differential IgE binding of the various isoforms to which people are exposed.

Table 1. PR-10 allergen sequences from *Betulaceae* trees, *Rosaceae* fruits, *Apiaceae* vegetables and *Fabaceae* legumes sorted by the overall amino-acid sequence identity to Bet v 1a (P15494).

Source	Family	UniProt Accession No	Allergen code	Sequence identity to Bet v 1a
Birch pollen (*Betula pendula*)	*Betulaceae*	P15494	Bet v 1a	100 %
Birch pollen (*Betula pendula*)	*Betulaceae*	P43177	Bet v 1d	96 %
Birch pollen (*Betula pendula*)	*Betulaceae*	P43185	Bet v 1L	94 %
Alder (*Alnus glutinosa*)	*Betulaceae*	P38948	Aln g 1	81 %
Hornbeam (*Carpinus betulus*)	*Betulaceae*	P38949	Car b 1	73 %
Hazel (*Corylus avellana*)	*Betulaceae*	Q08407	Cor a 1.01	73 %
Hazelnut (*Corylus avellana*)	*Betulaceae*	Q9SWR4	Cor a 1.04	67 %
Apple (*Malus domestica*)	*Rosaceae*	Q4VPL0	Mal d 1.04	60 %
Apricot (*Prunus armeniaca*)	*Rosaceae*	O50001	Pru ar 1	60 %
Cherry (*Prunus avium*)	*Rosaceae*	O24248	Pru av 1	59 %
Peach (*Prunus persica*)	*Rosaceae*	Q2I6V8	Pru p 1	59 %
Pear (*Pyrus communis*)	*Rosaceae*	O65200	Pyr c 1	57 %
Apple (*Malus domestica*)	*Rosaceae*	P43211	Mal d 1	56 %
Apple (*Malus domestica*)	*Rosaceae*	B0B0M5	Mal d 1.06A	55 %
Strawberry (*Fragaria ananassa*)	*Rosaceae*	Q2565S2	Fra a 1	54 %
Raspberry (*Rubus idaeus*)	*Rosaceae*	Q0Z8U9	Rub i 1	fragment
Soybean (*Glycine max*)	*Fabaceae*	P26987	Gly m 4	46 %
Peanut (*Arachis hypogaea*)	*Fabaceae*	Q6VT83	Ara h 8	45 %
Mungbean (*Vigna radiata*)	*Fabaceae*	Q2VU97	Vig r 1	42 %
Celery (*Apium graveolens*)	*Apiaceae*	P49372	Api g 1.01	40 %
Celery (*Apium graveolens*)	*Apiaceae*	P92918	Api g 1.02	39 %
Carrot (Daucus carota)	*Apiaceae*	Q8SAE7	Dau c 1.02	38 %
Carrot (Daucus carota)	*Apiaceae*	O04298	Dau c 1.01	36 %

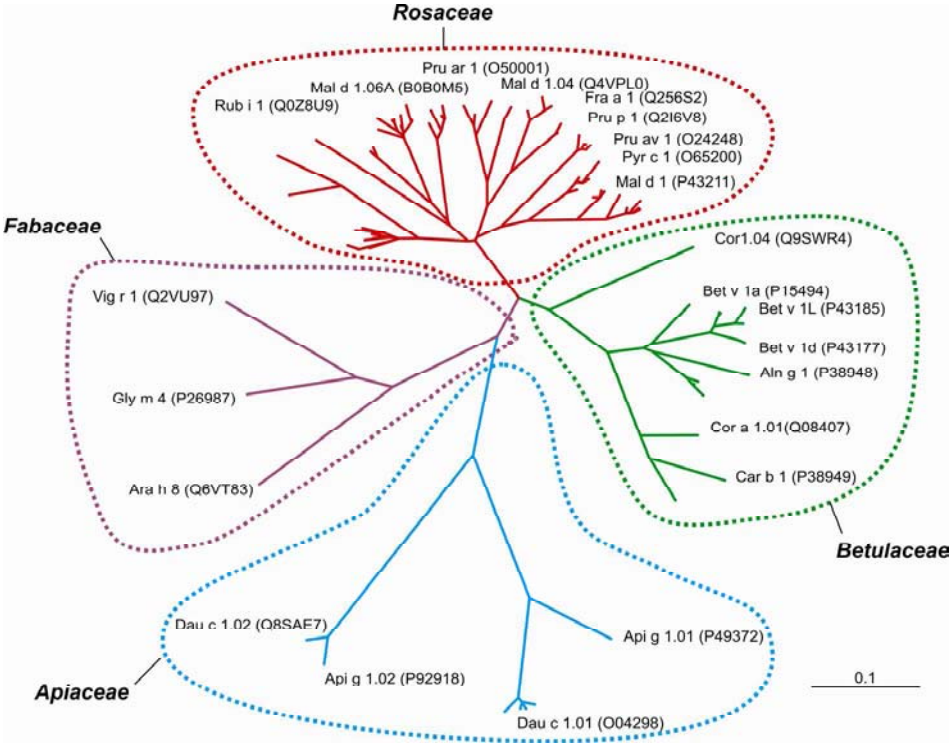

Figure 1. Phylogenetic tree of PR-10 allergens from the *Betulaceae*, *Rosaceae*, *Fabaceae* and *Apiaceae*. The multiple sequence alignment and neighbour joining was carried out with ClustalW2 at the EMBL-EBI website.

6.2 PR-10 protein stability

6.2.1 Mal d 1 and Gly m 4

The general purification protocol, as described in chapter 2 allows the purification of the PR-10 allergens Api g 1 and Dau c 1 and can also be used for the purification of Bet v 1. However, when applying the same protocol to purify Mal d 1 from apple and Gly m 4 from soybean results were less satisfactory. In apples, Mal d 1 is present in very low concentrations, resulting in low yields or complete loss of the allergen during purification. Therefore, we discontinued the purification of Mal d 1, as affinity chromatography has been shown to perform better [1], although it is unknown

whether the antibodies used for affinity chromatography are selective for certain isoforms. Gly m 4 is present in a completely different matrix since soybeans need to be defatted before making a protein extract. The major allergenic soy protein are the seed storage proteins such as legumins, vicilins, glycinin and β-conglycinin. Gly m 4 is only a minor part of the protein fraction. One of the subunits of glycinin has the same molecular weight as Gly m 4 and was interfering on SDS-PAGE gels [2]. Therefore, the protocol in chapter 2 is not suitable for purification of Gly m 4.

For the low yield of purified nMal d 1, the CD spectrum was similar to that for nApi g 1 (Figure 2A). It is expected, as described in chapter 2, that different Mal d 1 isoforms are present in the purified Mal d 1 solution. The heat denaturation curve was obtained after heating to 95 °C at 1 °C/min followed by cooling down at the same rate (Figure 2B). A midpoint of thermal denaturation, T_m, was determined around 75 °C and the protein was able to refold for at least 80%. The concentration used to measure thermal denaturation for Mal d 1 was at least 2 times lower (below 5 μM) than for the other measured allergens and resulted in increased noise in the curve. As a result of a higher T_m and increased noise, a two-state denaturation curve, to determine ΔH_{Tm} as shown in chapter 4, could not be fitted to the data points. Nevertheless, T_m of the natural allergen is different from rMal d 1.0108 as shown by Bohle et al. [3], which did not show a clear thermal transition. The T_m measured for nMal d 1 is 10 °C and 5 °C higher than those obtained for rBet v 1 and rApi g 1, respectively. The recombinant Mal d 1.0108 of Bohle et al. [3] did not have the ability to refold, whereas a study by Ma et al. [4] showed the ability of rMal d 1.0108 to refold completely. Bohle et al. [3] used a 5-10 times higher protein concentration, which could have influenced thermal unfolding. The difference in refolding capacity could also be caused by differences in folding of recombinant proteins during production and purification. These observations suggest that stability studies may be especially useful to determine differences between batches of recombinant allergens.

6.2.2 Thermal stability

The differences in thermal stability between different PR-10 allergens can be partially explained from the differences between homologous proteins from mesophiles and thermophiles. Mesophiles are organisms with an optimal growth temperature between 20 to 40 °C and thermophiles live at temperatures above 55-60 °C [5]. Thermophilic

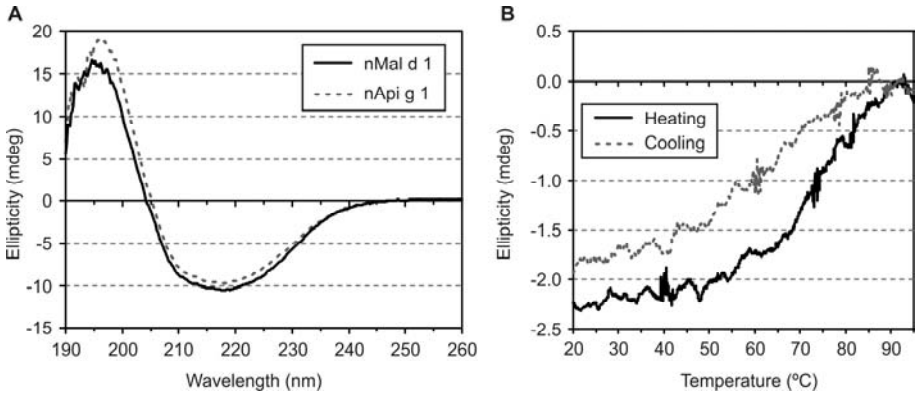

Figure 2. CD spectra of purified nMal d 1 and nApi g 1 (A) and thermal denaturation curve of nMal d 1 encompassing the heating and cooling patterns, measured at 222 nm (B).

proteins are built to function optimally under extremely high temperatures, and are protected from denaturation by differences in amino acid composition as compared to mesophilic proteins [6, 7]. Large data sets of structurally aligned mesophilic and thermophilic proteins, and others for 3D-structures are available (e.g. the PDB-databank) which may be combined to explain the differences in stability. Only small differences in thermal stability were found between Bet v 1 and Api g 1 (chapter 3), but their amino acid composition and hydrophobic and charge properties are different (chapter 5). In contrast, the heat stability of Api g 1 was quite different from that of Dau c 1 while both allergens showed 80.5 % identity in overall amino acid composition.

In general, the proportion of charged residues, especially Glu and Lys, and the hydrophobic residues Ile and Val are increased in thermophilic proteins. Some of the residues are less abundant in proteins that are biologically active at high temperatures, such as Ala, Met, Cys, Asn and Gln. At elevated temperature Met and Cys , due tot the presence of a sulphur group, can undergo oxidation and Asn and Gln can be deamidated [6, 8]. The relative amino acid composition of Bet v 1a, Bet v 1L, Mal d 1, Mal d 1.06A, Api 1 and Dau c 1 is shown in Table 2 with the averages for proteins in general [9]. Amino acids of the respective PR-10 allergens that are less or more abundant than for average proteins, have been indicated with light grey and dark grey, respectively (difference >1 %). Bet v 1 and the Mal d 1 isoforms have an increased fraction of charged amino acids, in particular Lys and Glu, as compared to Api g 1 and Dau c 1

(chapter 5). This could be important for an increased stability, especially because these residues are able to form salt bridges and hydrogen bonds. Figure 3 shows the differences in charged residues and hydrogen bonds on the surface of the β-sheet of Bet v 1a and Api g 1. Most of these charged residues can be found on the C- or N-terminal ends of the β-strands of Bet v 1a and can therefore increase stability of the β-sheet. Api g 1 does not have these charged residues and consequently can not form hydrogen bonds and salt bridges to the same extent as Bet v 1a.

Api g 1 and Dau c 1 have an increased Ile and Val content, compared to proteins in general. These amino acids can stabilize the protein as they do in thermophilic proteins. Bet v 1a and Api g 1 are stabilized by the high proportions of charged and hydrophobic residues, respectively, both resulting in similar T_m values. On the other hand, Dau c 1 is less thermo stable, which could be a result of fewer hydrophobic interactions due to the higher content of the small Ala residues. Other, less pronounced stability factors in thermophilic proteins are a low proportion of the polar Ser and Thr residues [7]. Thus, the higher proportion of these residues in Dau c 1 than for Api g 1 could explain the lower stability of the former. Furthermore, a higher T_m is observed for (purified) Mal d 1 [3] as compared to Bet v 1a and this increase in thermal stability could be caused by a higher proportion of charged and/or hydrophobic residues, e.g. Ile (and Val for Mal d 1.06A). The amount of polar residues is also higher in Mal d 1, which could have a negative effect on stability, giving a possible explanation for the inability of rMal d 1.0108 to refold under certain conditions. The differences and similarities in protein stability can also be explained by thermodynamic stability, as described by Pace [10] and Myers and Pace [11]. In the first study the contribution of the hydrophobic effect to globular protein stability is described. Pace found that proteins gain 5.4 kJ/mol in ΔG for each buried -CH_2 group of a hydrophobic residue [10]. In the second study Myers and Pace [11] concluded that uncharged hydrogen bonding of polar residues stabilizes proteins by 4.2 to 8.4 kJ/mol per intramolecular hydrogen bond. Therefore, differences in stability between PR-10 proteins can be readily explained by differences in proportion of polar (charged and uncharged) and hydrophobic residues.

Table 2. Amino acid composition of the major birch pollen allergen Bet v 1 and homologous allergens

	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
Allergen (Genbank Acc No)	8.3%	5.7%	4.4%	5.3%	1.7%	4%	6.2%	7.2%	2.2%	5.2%	9%	5.7%	2.4%	3.9%	5.1%	6.9%	5.8%	1.3%	3.2%	6.6%
Bet v 1a (X15877)	6.9	1.9	5.6	5.6	0.0	1.3	8.8	8.8	2.5	8.1	5.6	9.4	1.3	5.0	5.0	6.9	5.6	0.0	4.4	7.5
Bet v 1l (X77273)	7.5	1.9	6.3	5.0	0.6	1.3	8.8	8.8	2.5	6.9	5.0	10.0	1.9	4.4	5.0	5.6	5.0	0.0	4.4	9.4
Mal d 1 (X86372)	6.3	1.3	3.1	5.0	0.6	1.9	9.4	8.8	3.8	10.7	6.3	10.1	0.6	3.8	3.8	8.2	6.3	0.0	6.3	3.8
Mal d 1.06A (AY789242)	7.5	0.6	4.4	6.9	0.0	0.6	8.8	8.8	3.1	9.4	6.9	10.7	0.6	1.9	3.1	7.5	3.8	0.0	6.9	8.2
Api g 1 (Z48967)	8.4	0.6	3.9	6.5	0.6	1.9	6.5	9.7	1.9	9.7	8.4	6.5	1.3	3.9	4.5	4.5	9.1	0.0	2.6	9.1
Dau c 1 (Z81361)	11.0	1.3	2.6	6.5	0.0	1.3	6.5	8.4	1.9	10.4	5.2	5.8	1.9	3.2	3.2	7.8	11.0	0.0	1.9	9.7

The amino acids are shown by their three letter codes and the overall amino acid composition in %.

Sequences were taken from the Genbank database.

Average protein composition is based on reference Creighton et al. [9].

The dark grey color shows the amino acids below average and the light grey above average. White is unchanged (margins + or – 1%).

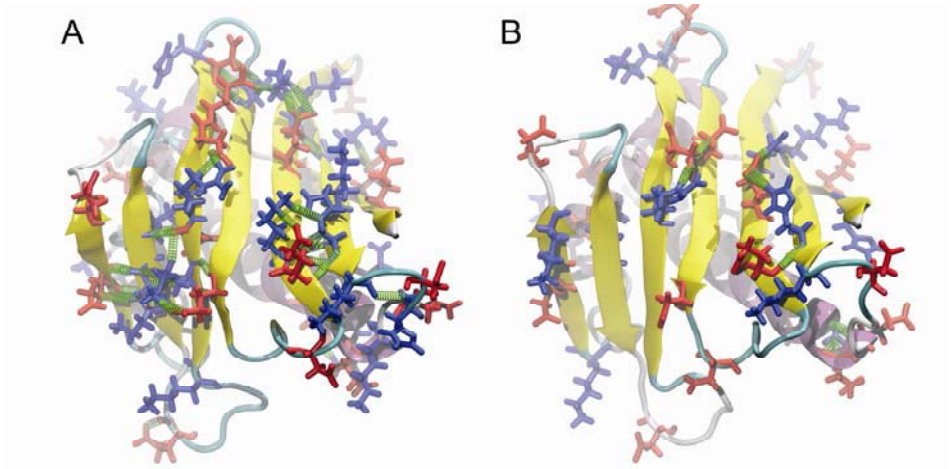

Figure 3. Hydrogen bonds between charged residues on the surface of Bet v 1 (**A**, PDB-entry; 1bv1) and Api g 1 (**B**, PDB-entry; 2bk0). Negatively charged residues are shown in red and positively charged in blue. Hydrogen bonds are shown as green wires (cutoff values: distance 4.0 Å and bond angle 30°).

6.2.3 Stability towards denaturants and pH

In chapter 4, it was shown that the natural isoform mixtures of Api g 1 and Dau c 1 are stable to heating at a low pH, an observation different from rBet v 1 and also rPru p 1 [12, 13], but similar to pH dependence properties as found for patatin [14], a soluble protein from potato tuber also known as the allergen Sol t 1. This protein maintains its secondary structure at low pH and does not unfold between 20 and 80 °C at pH 2, 3 and 4 [14]. Patatin showed both unfolding and precipitation at pH 6, which was also observed for nApi g 1 and nDau c1 in the pH range of 4.4-6.0. The conformation, most stable to heating, is attained for all three proteins at approximately pH 6.0, which is close to their iso-electric points [14]. Api g 1, Dau c 1 and patatin have an iso-electric point below 5.0 and proteins are generally more stable near their iso-electric point [15]. The allergen's electrostatic charges are neutral around its pI. This probably leads to increased exposure of hydrophobic sites causing the protein to precipitate. In general, proteins are more soluble at a pH more remote from the iso-electric point, because of the lower hydrophobic exposure, which explains the refolding capacity observed at a pH above 6.0. At a pH below the iso-electric point, small changes in the secondary structure are observed, which can be explained by altered electrostatic interactions.

Aspartic acid and glutamic acid residues are protonated at low pH and this will change the overall net charge from negative to positive resulting in the stabilization of the structure of Api g 1 and Dau c 1 [9].

As mentioned in chapter 4, differences in protein stability in Guanidine hydrochloride experiments, can be ascribed to differences in charged and hydrophobic residues. This property is apparent in chapter 5 and is confirmed by the denaturation experiments in chapter 4. Recombinant Bet v 1.0101 showed polar unfolding as illustrated by the high value of $m_{D\text{-}N}$ value and the steep slope in the transition (chapter 4; Table 1 and Figure 1). Recombinant Dau c 1 has a polar His_6-tag and this may have resulted in unfolding behaviour similar to polar unfolding. Api g 1 is most stable towards denaturant, which is probably related to a more optimal hydrophobic packing compared to Bet v 1 and Dau c 1. The difference between the denaturant stability of Api g 1 and Dau c 1 can therefore be similarly explained as for thermal unfolding: Dau c 1 has more polar (uncharged) residues and less hydrophobic residues.

6.3 The Bet v 1 superfamily and membrane binding

Classification of allergens on the basis of structure and function showed that 707 allergens belong to 134 different allergen families, which together contain 184 protein family domains. This is only 2% of the 9318 known protein families [16]. The biological function of allergens remains unclear in many cases, although certain protein families are involved in biochemical functions such as hydrolysis of proteins, polysaccharides and lipids; metal and lipid binding; transport; storage; and cytoskeleton association [16]. The tertiary fold of Bet v 1 has been shown to be distributed throughout all three biological superkingdoms (Archaea, Bacteria and Eukarya). These related proteins show low sequence similarity but many of them are involved in the binding of hydrophobic ligands such as membrane lipids. Bet v 1 has also been shown to bind various ligands [17].

Bet v 1 is not only able to bind ligands, but was also shown to bind and permeabilize membranes [18]. This property was discovered for one of the structurally related proteins, the steroidogenic acute regulatory protein-related lipid transfer (START) domain MLN64 from human and StarD4 from mouse, which both have an extra N-terminal α-helix and two additional β-strands in the β-sheet as compared to Bet v 1 [19]. The structure of the START domain of the steroidogenic acute regulatory

protein (StAR) is predicted to be closely related to MLN64 and StarD4 and is responsible for the transport of cholesterol from the outer to the inner mitochondrial membrane. Different studies have shown that StAR acts exclusively on the outer membrane of mitochondria in the cytoplasm. A conformational change of StAR at pH 3.5 was characterized as a molten globule state, a partially unfolded protein which has lost some of its tertiary structure but retained its secondary structure. This allowed the protein to associate with membranes. When StAR is exposed *in vivo* to protonated phospholipids of the mitochondrial membrane, the hydrophobic residues are exposed to the StAR surface, the protein changes conformation and the C-terminal α-helix of StAR is partially inserted into the membrane [19-21]. Moreover, in the presence of membrane lipids the C-terminus of the protein is protected from digestion by pepsin or trypsin [19]. Furthermore, most of the StAR characteristics seem to apply to Bet v 1.

A membrane binding study was performed with Bet v 1 and Mal d 1 and both allergens were shown to bind phospholipid membranes with the ability to change conformation and the property to protect against proteolysis [18]. Bet v 1 was shown to form an enriched α-helical structure at pH 3.3 in the presence of sonicated, zwitterionic lipid vesicles. This structural change was even more pronounced at pH 6.5 and below in the presence of anionic lipid vesicles. In the presence of micelle-forming lysophospholipids, an increase in α-helix was observed throughout the pH range 2.3-9.3. Together with the α-helix content, also the proportion of random coil increased, indicating that the membrane binding states were partially unfolded. Furthermore, the conformational change protects the protein from proteolytic degradation which may be due to shielding of the N- instead of the C-terminal part as shown for StAR [18].

A major difference between Bet v 1 and both Api g 1 and Dau c 1 was the unfolding of Bet v 1 at acidic pH with a midpoint around pH 3.6 whereas Api g 1 and Dau c 1 were shown to be stable below pH 4.0 (chapter 4). Bet v 1 clearly showed unfolding to a denatured state at low pH without the formation of a stable intermediate [12, 18]. It can be speculated that Api g 1 and Dau c 1 are able to form a molten globule state at low pH similar to the properties of StAR, whereas Bet v 1 lacks this ability. A molten globule state was not confirmed in our study, but it is very likely that membrane binding also occurs for Api g 1 and Dau c 1.

Similar membrane binding properties were shown for β-lactoglobulin [22] and α-lactalbumin [23], which are major cow milk allergens from the whey fraction [24]. The

α-helix content in both milk proteins increased when the proteins bound to anionic membranes when lowering the pH. Similar to Bet v 1 [18], a decrease in tertiary structure was observed for α-lactalbumin. These membrane binding interactions were shown to be driven by electrostatic and hydrophobic interactions [22]. Bet v 1, β-lactoglobulin and α-lactalbumin have a pI of 5.4, 5.3 and 4.2-4.5, respectively, and the net charge of a protein becomes positive at pH values below pI. In case of StAR, a mitochondrial proton pump in the membrane is associated with the activity of StAR. This proton pump creates a local pH, which is needed for membrane binding. In living cells, Bet v 1 and PR-10 proteins are located in the cytoplasm (cytosol) where the pH is neutral and therefore a pH gradient at membranes would be necessary for zwitterionic membrane association [21]. The StAR membrane binding mechanism thus suggests that negatively charged membranes are not a prerequisite for conformational changes whereas at the same time a pH gradient is created by the proton pump. The influence of pH and the charge distribution on the protein can be important for fast membrane binding and yet unknown protein-antibody interactions, as was shown in chapter 5. Protein membrane binding is even more important as it may result in membrane disruption and cell leakage, thereby permitting the start of the immunological sensitization process.

6.4 Fibril aggregation of allergenic proteins

An allergen is a normally innocuous protein, which is considered harmful when the immune response is aberrant. It is not well understood what structural features of allergenic proteins induce the development of allergic diseases, but many allergenic proteins contain large hydrophobic portions, which are prone to aggregation. These proteins include animal lipocalins, lipid transfer proteins, pathogenesis related proteins and seed storage proteins [25]. Many food proteins are known to form soluble or insoluble aggregates, but little is known about the immunogenicity of these aggregates and whether or not this property is important in the development of allergies.

Under appropriate conditions, proteins form aggregates, which result in the formation of highly ordered β-structures, also known as amyloid fibrils. Amyloid fibrils are known to cause diseases such as Alzheimer's disease, Parkinson's disease, type 2 diabetes and prion disorders (Creutzfeldt-Jakob disease) [26]. Simple variations in physico-chemical conditions such as temperature, protein concentration and ionic

strength can lead to the formation of fibrils. Mechanical influences such as agitation can have a similar effect [27]. The formation of amyloid fibrils can, in a variety of proteins, be triggered by membranes containing negatively charged phosphatidylserine. An instantaneous formation of fibrils is observed for lysozyme, insulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, transthyretin, cytochrome *c*, histone H1 and α-lactalbumin [28]. These proteins have been shown to be cytotoxic or involved in the induction of apoptosis. Similar to Bet v 1 and β-lactoglobulin, they can bind membranes containing negatively charged phospholipids. A search in the Pubmed database shows that all these proteins, except for histone H1, have been described as allergens. Increases in the α-helix content for protein binding at the membrane could be of importance for the transition into the amyloid β-sheet structure as this was observed for the islet amyloid polypeptide and medin [29, 30].

The ability to form amyloid fibrils is also common for other allergens. It is a dynamic process, which can be influenced by different factors. For example, the interaction of κ-casein from milk with phospholipid membranes increased fibril formation, but fibril formation was inhibited when membrane binding was enhanced upon the addition of α_S-casein and β-casein [31]. The milk allergen α-lactalbumin formed amyloid fibrils at low pH where it adopts a molten globule state. This allergen is more susceptible to fibrillation when present in the disordered form, called S-(Carboxymethyl)-α-lactalbumin, with three out of four reduced disulfide bonds [32]. Amyloid fibril formation in ovalbumin, an allergen from fresh egg-white, was observed in heat denaturation experiments where it forms soluble aggregates together with heat denatured ovalbumin [33]. On the other hand, formation of ovalbumin amyloid aggregates was inhibited when heme was added during heating [34]. It is unknown if fibril aggregates are involved in the development of allergies, but when they are in the range of available conformational structures of homologous allergens will increase significantly.

6.5 Allergenicity of modified allergens

An important effect of processing is the post-translational modification of allergenic proteins by a variety of reactions created by factors such as pH, enzymatic and non-enzymatic reactions. Proteins can also denature and form soluble or insoluble aggregates. Most food allergens are known as stable allergens, but birch pollen related food allergens from the PR-10 family are generally regarded to be unstable. In several studies it has been shown that the IgE binding capacity of celery was almost completely lost by various processing technologies [35-37] and IgE reactivity of rPru av 1 from cherry and Mal d 1 extracts from apple were remarkably decreased by enzymatic and non-enzymatic browning [38, 39].

The reduction of the IgE binding capacity as mentioned above is influenced by the complexity of the matrix and is not translatable to allergens in aqueous solution. A small reduction of IgE reactivity by heating for 30 minutes at 95 °C of natural isoform mixtures and recombinant isoforms of Bet v 1, Api g 1 and Dau c 1 has been described in chapter 3. Heating for 2 hours at 100 °C was more effective and showed a more than 500-fold reduction. On the other hand, 60 minutes cooking of different recombinant allergens, Bet v 1, Mal d 1, Api g 1 and Dau c 1, completely abolishes IgE binding, but without a reduction of the capacity to activate allergen-specific T-cells [3]. The stability of natural Api g 1 and Dau c 1 at low pH, as shown in chapter 4, could be due to the exclusion of the matrix. As mentioned above, isolated patatin has properties similar to natural Api g 1 and Dau c 1 at low pH, but van Koningsveld et al. [40] observed precipitation of patatin in protein extracts below pH 4. This was explained by the ability of proteins to form stable soluble or insoluble complexes with plant polyphenols, which influenced precipitation. Moreover, heat-induced aggregation of patatin resulted in irreversible unfolding of patatin with a reduction of the IgE-binding capacity by 25-110 fold [41]. This reduction in IgE binding is also expected for the precipitate formed for heated Api g 1 and Dau c 1 between pH 4.4-5.6. The effect of other proteins and matrix components on Api g 1 and Dau c 1 is as yet unknown.

Also gastrointestinal digestion generally destroys IgE binding, but not T cell activation, though activation is less for digested than for undigested allergens, especially for rApi g 1.0101 [42]. It has been shown that aggregation of allergens can lead to protection of the allergen against pepsin and result in no or incomplete digestion, followed by higher proliferation of activated T-cells [42]. Protective effects to digestion

are also observed upon membrane binding of rBet v 1.0101 and rMal d 1 [18], while other PR-10 allergens are generally considered to be susceptible to pepsin digestion [43]. The protective effect against digestive enzymes makes it possible to explain several reported cases on allergic mono-sensitization to Dau c 1[44, 45].

6.6 Food systems and systems biology

Macromolecular crowding has an effect on biochemical reactions in the cytoplasm of living cells that take place at a high concentration of macromolecules (50-400 mg/ml) where they are susceptible to nonspecific interactions. These nonspecific interactions are of biological importance and influence various cellular processes, but are missed in *in vitro* studies [46]. Many food allergens are present in a matrix with a low water content. For *in vitro* or *ex vivo* measurements the allergen is removed from its natural environment and dissolved in aqueous solutions where it can adopt an activity or conformation that is energetically favorable. In the context of the flexibility of allergenic proteins, it is unclear if and how this can influence the sensitization process and IgE binding, whereas the native state of the protein is only known as the aqueous state. The structure of allergens may change by food processing, storage, consumption, the stomach, the gut and in case of aero-allergens the lungs, nose and eyes. Systems biology would be a solution to learn more about all complex interactions and factors involved in the mechanism of allergic sensitization.

One approach in systems biology is by combining all information of discovered mechanisms to start the creation of a general pathway. For house dust mite allergens, a general pathway is built, which forms the molecular basis of allergenicity for the biologically intact Der p 1 allergen in the human host. Der p 1 is an allergen with protease activity showing proteolytic activity in lung tissues, which is likely to be influenced by concentration, pH, stability and inactivation by anti-Der p 1 antibodies. The allergen has been shown to facilitate its own passage and the passage of other allergens across the epithelial barrier by digestion of tight junction proteins. This passage is followed by proteolytic activity of Der p 1 on the receptors CD23 on B-cells, CD25 on T-cells and CD40 on dendritic cells of the adaptive immune system favoring a T helper cell 2 (Th2) response. This may lead to uncontrolled IgE production and allergy [47]. Another house dust mite allergen, Der p 2, mimics the role of a human structural homologue, MD-2-related lipid binding protein, by interacting with the Toll-

like receptor 4 (TLR4). Der p 2 promotes TLR4 signaling by binding lipopolysaccharide, which is related to the development of allergic asthma [48]. This kind of mimicry could also apply to the biological function of Bet v 1 and could give an explanation for the differences in the observed isoform allergenicity.

A different approach in systems biology is by transcriptomics analyses to follow the expression of different proteins involved in the induction and development of allergy. One of these studies reported the involvement of different proteins in the binding and transport of Bet v 1 through conjunctival epithelium (eye epithelium). The binding and transport only occurred in allergic patients and not in healthy individuals and was accompanied by the expression of receptors associated with lipid rafts/caveolae [49]. The processes involved with caveolae formation include endocytosis, exocytosis, cholesterol homeostasis and signal transduction. Human diseases, such as prion disease and tumorigenesis are affected by changes in caveolae function [50]. Interestingly, if a transport mechanism exists in conjunctival epithelium, it can also exist elsewhere such as the nose, lungs or mouth. Uptake of cross-reactive food allergens in the oral cavity could thus be a mechanism for cross-sensitization.

6.7 Allergen isoforms

As discussed in chapter 5, allergen isoforms are generally not well characterized. First of all, a systematic approach is needed to better understand the differences in single allergen isoforms and their differential impact on sensitization and on IgE binding capacity. Most studies concentrate on the isoform Bet v 1a, while other isoforms can be of similar interest [51]. Mutational studies, with single amino acid substitutions in Bet v 1a, show patient-dependent changes in IgE binding capacity. A stronger effect in the decrease of IgE binding is obtained by replacing several residues [52-54], but it is unknown if similar effects are obtained by replacing several residues on a random basis. The best characterized birch pollen related food is apple for which it has been shown that a large variety of Mal d 1 isoforms are present. The Mal d 1.0108 is used in most research studies, but the isoform Mald 1.06A, with three different variants 01, 02 and 03, accounts for differences in allergenicity among different apple cultivars [55]. Decreases in IgE binding capacity in different mutants of Bet v 1 are poorly understood and lead to unfounded statements in case of cross-reactive allergens, illustrating large differences in amino acid properties.

A second biological example is the importance of Mal d 1 isoforms and the changes in their expression due to postharvest storage. Freshly picked apples might induce fewer symptoms in allergic individuals as the amount of expressed Mal d 1 is lower than in stored apples [56]. Storage effects were also observed for the carrots used in this study. Carrots were stored for 2 years at -20 °C and afterwards, the extract mainly showed a Dau c 1 protein band on SDS-PAGE gels. Furthermore, differences were observed in IgE-binding capacity in our study of purified Bet v 1 from birch pollen of *Betula pendula* 'Youngii' trees in two different Dutch cities. No IgE binding was detected on a dot-blot membrane, using sera from birch pollen allergic individuals for one of the cities, while in the other sample a strong response was observed. This observation can be explained by the release of different isoforms in early or late flowering trees.

6.8 Future perspectives

It can be hypothesized that various biological activities of Bet v 1 are required in the human body to eventually result in allergic sensitization. When this hypothesis is true, different functional and structural aspects of the allergen should be taken into consideration. The first aspect is the conformation of the Bet v 1 allergens and their propensity to bind to membranes. The second aspect is the charge state of the protein, where the pI value can serve as a molecular switch at which the protein changes from a negatively to a positively charged state. This can affect membrane binding and recognition by IgE antibodies. The third aspect is ligand binding which can be necessary for the intrinsic function of a PR-10 protein. Different PR-10 proteins may have different ligand specificities. The fourth aspect is the presence of allergen isoforms with different IgE binding capacity, membrane binding, ligand binding or other activities. The fifth aspect is cross-reactivity, which depends on the first four aspects and can explain differences observed between the PR-10 allergens from *Rosaceae* and *Apiaceae.* For example, transport through membranes in the oral mucosa may be facilitated by the low pH of the fruit, which changes the membrane binding property. *Apiaceae* are mainly consumed after cooking, which inactivates the membrane binding activity. In its active form, membrane binding would be difficult for *Apiaceae* PR-10 allergens as they are mainly negatively charged. The pH of the food is too high to be

supportive, but when the allergic symptoms deteriorate, more receptors could be expressed on the surface of the membranes that also allow binding of these allergens.

Such a mechanism would imply that the importance of gastro-intestinal enzymes is highly overrated and that a T cell response against food allergens is not induced from the gut. It is unknown whether the allergen influences the T cell response directly through interactions in the adaptive immune response. IgE binding studies only detect Bet v 1 in its aqueous conformation and these assays are therefore of lower biological relevance. It would be meaningless to screen for IgE epitopes on the surface of the aqueous conformation, because the hydrophobic residues exposed to the surface under specific physiologically conditions will be equally important.

However, if the hypothesis that biological activity of Bet v 1 is needed in the human body for sensitization is untrue and IgE binding does depend on the aqueous conformation of the protein, a different approach would be required. Most research with site-directed mutagenesis show a far-UV CD spectrum to conclude that the protein is in a folded state with a similar secondary structure to Bet v 1. A CD spectrum does not give information about the tertiary structure, soluble aggregates, and monomeric or multimeric states. In mice, for example, it has been shown that dimerization is important for *in vivo* IgE-cross linking [57]. Differences in thermodynamic parameters of PR-10 allergens can be easily determined by stability studies with CD and can be used to find a relationship between IgE binding capacity to explain differences in the allergenic potential of processed foods.

Only a minority of studies intends to draw conclusions on allergenicity by analyzing physical properties of allergenic proteins and their IgE binding capacity. Many of the (bio) physical properties of Bet v 1 and other PR10 allergens apply also to milk allergens of the whey fraction. The most extensively studied allergenic proteins in literature are β-lactoglobulin and α-lactalbumin with its structural homologue lysozyme. The stability characteristics of these proteins could reveal important information in relation to their allergenicity. Most mutant studies have been performed on lysozyme and the obtained structural and functional characteristics could serve as a model for other allergens. Milk is the best studied food system worldwide and could therefore be a good starting point for analyzing interactions between allergens and matrix compounds.

6.9 Concluding remarks

In the context of the research reported, a general trend is observed for allergen stability, structural differences and their relation to IgE binding capacity in aqueous solutions. Bet v 1 is the primary allergen of birch pollen related allergies and shows the highest IgE binding potential. The allergenic potential decreases in the order Mal d 1, Api g 1 and Dau c 1, in accordance with their amino acid sequence identity. Bet v 1 cross-reactive IgE antibodies preferably bind to the charged and polar residues of Mal d 1. Api g 1 appears to be more stable than Dau c 1 as the result of a tighter hydrophobic packing. However, the thermodynamic stability of Api g 1 is similar to that of Bet v 1, but the higher proportion of hydrophobic residues and the reduced proportion of charged residues are responsible for the lower IgE binding capacity.

Thermodynamic characterization of PR-10 allergens leads to a better understanding of differences in conformational stability between isoforms. However, a single recombinant isoform allows a more precise determination of the thermodynamic parameters. Amino acid changes lead to altered stability, biological function, electrostatic potential and packing of the protein and all of these can be important for facilitating protein-protein interactions in terms of explaining differences between the IgE binding capacity of Bet v 1 isoforms. Furthermore, the natural PR-10 allergen mixtures do contain different isoforms for which the IgE binding capacity is not well characterized. These mixtures can be used to screen for differences between the natural and the recombinant isoforms to obtain a better insight in stability and IgE binding of different plant foods that more closely resembles actual and realistic conditions and situations as encountered in tangible food products.

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Summary

The allergen Bet v 1 is known as the primary sensitizer for birch pollen-related food allergy and its general characteristics are described in **chapter 1**. Bet v 1 is a member of the pathogenesis related-10 protein family (PR-10) and can be found in a variety of plant groups such as other trees, fruit, vegetables, nuts and legumes. Cross-reactivity to other PR-10 proteins mainly occurs for trees of the *Betulaceae,* fruit from the *Rosaceae* and vegetables from the *Apiaceae* families.

This thesis describes the differences between PR-10 proteins, concentrating on Api g 1 from celery and Dau c 1 from carrot in comparison to Bet v 1. The aim of this research was to purify and characterize PR-10 allergens in natural isoform mixtures in an attempt to relate structural characteristics to their physico-chemical stability and differences in allergenicity. The structural changes of the PR-10 allergens were mainly studied by circular dichroism (CD), a spectroscopic method, which can be used to study thermodynamic stability.

Chapter 2 describes the purification and characterization of natural isoform mixtures of Bet v 1, Api g 1 and Dau c 1. The allergens were purified under mild conditions using a standardized protocol, which comprised three major steps. In the first step, water-soluble proteins in the extract were fractionated by an ammonium sulphate precipitation. The PR-10 protein fraction was further purified by hydrophobic interaction chromatography and size exclusion chromatography. The isolated allergens were characterized by Q-TOF MS/MS showing that the allergen was purified as an isoform mixture with minor impurities. Using Q-TOF MS/MS, approximately 41-63 % of the amino acid sequences of the allergens could be determined, revealing not only earlier reported, but also new isoforms. At least three isoforms of Bet v 1, two isoforms of Api g 1 and three isoforms of Dau c 1 were detected. All allergen mixtures showed a characteristic CD spectrum of PR-10 allergens with a similar secondary structure composition and gave a similar IgE response compared to single, commercially available recombinant isoforms.

These natural isoform mixtures were used to find a structural relationship between thermal treatment and the immune response by the T cell and the IgE binding capacity, described in **chapter 3**. Therefore, thermal denaturation curves were recorded for natural (n) and recombinant (r) Bet v 1, Api g 1 and Dau c 1. The midpoint of thermal denaturation, T_m, was determined for all allergens. Heat treatment of the allergens, for

the immune experiments, was performed at a similar heating rate as for CD by gradually heating to 95 °C or to T_m followed by a 30 minutes incubation at these temperatures after which the protein sample was cooled down gradually or quickly. Human PBMC cultures were used to assess the T cell response and were only tested for Bet v 1 and thermally treated Bet v 1. However, Bet v 1 was not capable to induce cytokine production or T cell proliferation, possibly related to the low frequency of allergen-specific T cells in the peripheral blood. The IgE binding capacity was tested for all three natural and recombinant PR-10 allergens in different ELISA experiments and showed that heating to T_m does not have an effect on IgE binding, as the allergens are able to refold completely. Heating to 95 °C resulted in a decrease in IgE binding capacity, although the effect was minor and independent of the cooling rate. However, heating to 100 °C for 2 hours destroyed IgE binding capacity. Moreover, the small structural differences observed in the CD spectra of the allergens before and after thermal treatment were accompanied by only minor differences in IgE binding capacity between recombinant and natural isoforms. Dau c 1 was shown to be least thermo-stable and was not able to bind IgE.

The allergens were further characterized in **chapter 4** in a thermodynamic CD study to determine thermodynamic parameters for conformational stability. The Gibbs free energy change, $\Delta G_{D\text{-}N}$, was determined for both natural and recombinant Bet v 1, Api g 1 and Dau c 1 by Guanidine Hydrochloride (GuaHCl) denaturation. The determined $\Delta G_{D\text{-}N}$ values were more precise for single isoforms and showed an equal stability of rBet v 1 and rApi g 1. The stability of rDau c 1 was very low, which was also observed for the thermal denaturation curve. The $\Delta G_{D\text{-}N}$ values of the natural isoforms were affected by the mixture composition, as the midpoint of denaturation, [$GuaHCl_{50\%}$], differed for various PR-10 proteins. Natural and recombinant Api g 1 had a [$GuaHCl_{50\%}$], which was almost 2 times higher than the other isoforms. Only rDau c 1 was unfolding at very low concentrations of denaturant.

A better approximation of the conformational stability of the natural isoform mixtures Api g 1 and Dau c 1 was obtained by measuring the thermal stability at different pH, for which similar ΔG_T values were obtained as for rBet v 1. Moreover, different pH characteristics were found for nApi g 1 and nDau c 1 as compared to rBet v 1. Both nApi g 1 and nDau c 1 showed secondary structure at pH-values below their pI, whereas rBet v 1 showed unfolding to a denatured state at low pH. Furthermore, below

pH 4.4, nApi g 1 and nDau c 1 were stable to heating, but above this pH, the allergens precipitated upon heating. At pH 6.0-6.3, a double transition was observed during unfolding and above this pH both allergens were able to refold after heating. Both nApi g 1 and nDau c 1 showed similar characteristics, although the T_m-values for the pH dependence of nDau c 1 were 10 °C lower.

Differences in structural characteristics of PR-10 proteins of fruits from the *Rosaceae* and vegetables from the *Apiaceae* compared to the primary allergic sensitizer Bet v 1 from the *Betulaceae*, are described in **chapter 5** using a bioinformatics approach. Cross-reactivity of birch-pollen related allergens is poorly understood and site-directed mutagenesis studies described in literature do not contribute to a better understanding. The primary and secondary structure of PR-10 proteins clearly showed different properties between the *Rosaceae* and *Apiaceae*, which were characterized as positively charged/polar proteins and negatively charged/hydrophobic, respectively. These differences have consequences for IgE-binding, because protein-antibody interactions are driven amongst others by electrostatic complementarity. The contribution of the electrostatic charges is affected by the physiological pH of the food product and can have an, as yet unknown, effect on the IgE-binding capacity. For example, the acidic pH of *Rosaceae* fruit can result in protonation of negatively charged amino acids changing the PR-10 proteins from a negatively into a positively charged state.

The differences in structural characteristics of PR-10 proteins in relation to their physico-chemical stability were further discussed in the general discussion (**chapter 6)**. The similar stability found between Bet v 1 and Api g 1, can be explained by the difference in amino acid composition of the polar (charged and uncharged) Bet v 1 and the hydrophobic Api g 1. Bet v 1 is stabilized by charged salt bridge forming residues on the protein surface, whereas Api g 1 is stabilized by its hydrophobic packing. The difference in thermo-stability between Api g 1 and Dau c 1, can be explained from a reduction in hydrophobic interactions in Dau c 1 due to a higher proportion of the small alanine residue and polar serine and threonine residues.

Furthermore, the ability of Bet v 1 and Mal d 1 to bind and permeabilize membranes is considered an important feature of the allergens in the sensitization mechanism. The allergen changes conformation upon binding to membranes, especially when they are anionic, and is then protected from proteolytic digestion. This membrane binding property is observed for various other negatively charged food allergens and could be a

general feature for allergic sensitization. However, this suggests that IgE could bind to an unknown conformation of the allergen, which exposes the buried amino acids to the surface of the protein. The ability to bind to anionic membranes could be induced by the physiological pH of food, especially for fruits of the *Rosaceae*. The low internal pH in these fruits gives the PR-10 allergen a positive charge, which would subsequently increase membrane association and IgE-binding.

In conclusion, thermodynamic characterization leads to a better comprehension of differences in conformational stability and structural properties between allergens. Amino acid differences, which occur in all mixtures of natural isoforms, lead to altered stability, biological function, electrostatic potential and packing of the protein. All of these properties can have an effect on protein-protein interactions and could explain differences between the IgE binding capacity of PR-10 isoforms.

Samenvatting

Het allergeen Bet v 1 staat bekend als de primaire sensibilisator bij berkenpollen gerelateerde voedselallergie. De algemene eigenschappen van Bet v 1 zijn beschreven in **hoofdstuk 1**. Bet v 1 is een eiwit uit de PR-10 familie, die aanwezig is in een variëteit aan plantengroepen zoals in andere bomen, fruit, groenten, noten en peulvruchten. Kruisreactiviteit tegen andere PR-10 eiwitten treedt voornamelijk op bij bomen van de *Betulacea*e, fruit van de *Rosaceae* en groenten van de *Apiaceae* families.

Dit proefschrift beschrijft de verschillen tussen PR-10 eiwitten, waarbij de focus ligt op Api g 1 uit knolselderij en Dau c 1 uit wortel in vergelijking met Bet v 1. Het doel van dit onderzoek was de zuivering en karakterisering van PR-10 allergenen in natuurlijke isovorm mengsels in een poging een verband te leggen tussen de structurele eigenschappen met de fysisch-chemische stabiliteit en verschillen in allergeniciteit. De structurele veranderingen van de PR-10 allergenen werden voornamelijk bestudeerd met behulp van de spectroscopische techniek circulair dichroisme (CD), welke kan worden gebruikt om de thermodynamische stabiliteit te bestuderen.

Hoofdstuk 2 beschrijft de zuivering en karakterisering van natuurlijke isovorm mengsels van Bet v 1, Api g 1 en Dau c 1. De allergenen werden gezuiverd onder milde omstandigheden waarbij gebruik werd gemaakt van een algemeen protocol dat bestond uit drie belangrijke stappen. De eerste stap bestond uit een fractioneringstap, waarbij wateroplosbare eiwitten in het extract werden geprecipiteerd met ammoniumsulfaat. De PR-10 eiwitfractie werd verder gezuiverd met behulp van hydrofobe interactie chromatografie en gelpermeatie chromatografie. De geïsoleerde allergenen werden gekarakteriseerd met behulp van Q-TOF MS/MS, waarbij werd aangetoond dat het allergeen gezuiverd was als isovormmengsel met slechts kleine verontreinigingen. Met Q-TOF MS/MS kon 41-63% van de aminozuursequentie van de allergenen bepaald worden, waarbij niet alleen eerder gepubliceerde isovormen werden gedetecteerd, maar ook nieuwe. Tenminste drie isovormen werden gevonden van Bet v 1, twee isovormen van Api g 1 en drie isovormen van Dau c 1. Alle allergeenmengsels hadden een karakteristiek CD spectrum voor PR-10 allergenen met een vergelijkbare samenstelling in secundaire structuur en vergelijkbare IgE-reactiviteit als bij de commercieel verkrijgbare recombinante isovormen.

Deze natuurlijke isovormmengsels werden gebruikt om een structurele relatie te vinden tussen een thermische behandeling en de immuunrespons op basis van de T-cel-

reactiviteit en IgE-bindingscapaciteit, zoals beschreven in **hoofdstuk 3**. Hiervoor werden, met CD, thermische ontvouwingscurves opgenomen voor natuurlijk (n) en recombinant (r) Bet v 1, Api g 1 en Dau c 1. Het thermodenaturatie middelpunt, T_m. werd bepaald voor alle allergenen. Voor immuunreactiviteitsexperimenten werd de hittebehandeling van de allergenen hetzelfde uitgevoerd als bij CD, door middel van het geleidelijk verhitten tot 95 °C of T_m, gevolgd door een incubatie van 30 minuten bij deze temperatuur, waarna het eiwitmonster geleidelijk of snel gekoeld werd. Menselijke PBMC-cellen werden gebruikt om naar de T-cel-respons te bestuderen, maar werd alleen getest voor Bet v 1 en temperatuur-behandeld Bet v 1. Bet v 1 veroorzaakte geen productie van cytokines en leidde niet tot T-cel-proliferatie, wat waarschijnlijk veroorzaakt werd door het lage aantal van allergeen specifieke T-cellen in het perifere bloed. De IgE-bindingscapaciteit werd bepaald voor alle drie natuurlijke en recombinante PR-10-allergenen in verschillende ELISA-experimenten, waarbij werd aangetoond dat verhitten tot T_m geen effect heeft op IgE-binding, zolang het eiwit in staat is om volledig terug te vouwen. Verhitten tot 95 °C resulteerde in een afname van de IgE-bindingscapaciteit, hoewel dit effect klein was en onafhankelijk van de koelingsnelheid. Verhitten gedurende 2 uur bij 100 °C was voldoende om de IgE-bindingscapaciteit te vernietigen. Daarnaast gingen de kleine structurele verschillen in de CD-spectra van de allergenen voor en na verhitting gepaard met slechts kleine verschillen in de IgE-bindingscapaciteit tussen recombinante en natuurlijke isovormen. Dau c1 was het minst thermostabiel en was niet in staat om IgE te binden.

De allergenen werden verder gekarakteriseerd in hoofdstuk 4 door middel van een thermodynamische CD-studie om de thermodynamische parameters voor conformationele stabiliteit te bepalen. De verandering van de Gibbs vrije energie, $\Delta G_{D\text{-}N}$, werd bepaald voor natuurlijk en recombinant Bet v 1, Api g 1 en Dau c 1 middels Guanidine Hydrochloride (GuaHCl) denaturatie. De $\Delta G_{D\text{-}N}$ waarden kunnen nauwkeuriger worden bepaald voor enkelvoudige isovormen en die toonden een gelijke stabiliteit voor rBet v 1 en rApi g 1. De stabiliteit van rDau c 1 was laag, wat ook al naar voren kwam bij de temperatuurdenaturatiecurve. De $\Delta G_{D\text{-}N}$ waarden van de natuurlijke isovormen werden beïnvloed door de samenstelling van het mengsel, waarbij het denaturatiemiddelpunt, $[GuaHCl_{50\%}]$, verschilde voor de verscheidene PR-10-eiwitten. Natuurlijk en recombinant Api g 1 hadden 2 keer hogere $[GuaHCl_{50\%}]$ dan de andere isovormen. Alleen rDau c 1 ontvouwde bij hele lage denaturant concentraties.

Een betere benadering van de conformationele stabiliteit van de natuurlijke isovormmengsels van Api g 1 en Dau c 1 werd verkregen bij het bepalen van de thermische stabiliteit bij verschillende pH-waarden, waarbij vergelijkbare ΔG_T waarden werden gevonden als voor rBet v 1. Daarnaast werden andere pH-afhankelijkheidseigenschappen gevonden voor nApi g 1 en nDau c 1 dan bekend is voor rBet v 1. Beneden het isoelectrisch punt van nApi g en nDau c 1, kon nog steeds secondaire structuur worden aangetoond, waar rBet v 1 ontvouwen was in een gedenatureerde toestand. Verder waren nApi g 1 en nDau c 1 stabiel bij verhitten bij een pH lager dan 4,4, maar boven deze pH precipiteerden deze allergenen bij verhitting. Bij pH 6,0-6,3 was een dubbele transitie zichtbaar in de thermische ontvouwingscurve en boven deze pH vouwden de allergenen volledig terug na verhitting. Hoewel de T_m-waarden, voor de pH-afhankelijkheid van nDau c 1, 10 °C lager lagen, vertoonden nApi g 1 en nDau c 1 beide dezelfde pH-afhankelijkheidseigenschappen.

Verschillen in structurele eigenschappen van PR-10-eiwitten van fruit van de *Rosaceae* en groenten van de *Apiaceae* werden vergeleken met de primaire allergische sensibilisator Bet v 1 van de *Betulaceae*, als beschreven in **hoofdstuk 5** met behulp van een bioinformatica aanpak. Kruisreactiviteit van berkenpollen-gerelateerde allergenen wordt niet volledig begrepen en *site-directed* mutagenese studies, beschreven in de literatuur, dragen niet bij aan een beter inzicht. De primaire en secundaire structuur van PR-10-eiwitten zijn duidelijk verschillend tussen de *Rosaceae* en *Apiaceae*, die respectievelijk gekarakteriseerd werden als positief geladen/polair en negatief geladen/hydrofoob. Deze verschillen hebben consequenties voor IgE-binding, omdat de drijfveer bij eiwit-antilichaam-interacties onder andere wordt veroorzaakt door elektrostatische complementariteit. De bijdrage van de elektrostatische lading wordt beïnvloed door de fysiologische pH van het voedselproduct en kan, een tot nu toe onbekend, effect hebben op de IgE-bindingscapaciteit. De zure pH van *Rosaceae* fruit kan bijvoorbeeld resulteren in de protonering van negatief geladen aminozuren, waarbij de negatief geladen toestand van een PR-10-eiwit verandert in een positief geladen toestand.

Het verschil in structurele eigenschappen van PR-10-eiwitten in relatie to hun fysisch-chemische stabiliteit werd verder bediscussieerd in de algemene discussie (**hoofdstuk 6**). De vergelijkbare stabiliteit die gevonden werd voor Bet v 1 en Api g 1 kan worden verklaard door het verschil in de aminozuursamenstelling van het polaire

(geladen en ongeladen) Bet v 1 en hydrofobe Api g 1. Bet v 1 wordt gestabiliseerd door geladen zoutbrug-vormende residuen op het eiwitoppervlak, waarnaast Api g 1 wordt gestabiliseerd door de hydrofobe pakking. Het verschil in thermische stabiliteit tussen Api g 1 en Dau c 1 kan worden verklaard vanuit een afname in hydrofobe interacties in Dau c 1 vanwege een hoger gehalte aan het kleine apolaire alanine-residu en de polaire serine en threonine-residuen.

Verder wordt de membraanbindende en –doordringbare eigenschap van Bet v 1 en Mal d 1 in beschouwing genomen, wat belangrijk kan zijn voor het sensibilisatiemechanisme. Het allergeen verandert van conformatie wanneer het aan het membraan bindt, met name aan anionische membranen, en wordt dan afgeschermd voor enzymatische afbraak door verteringsenzymen. Deze membraanbindende eigenschap is aanwezig voor een verscheidenheid aan andere negatief geladen voedselallergenen, wat zou kunnen betekenen dat dit een belangrijk kenmerk is voor allergische sensibilisatie. Dit wekt de suggestie dat IgE in staat is om aan een onbekende conformatie van het allergeen te binden, waarbij de verborgen aminozuren aan de oppervlakte van het eiwit worden blootgesteld. De mogelijkheid tot het binden van anionische membranen kan worden versterkt door de fysiologische pH van voedsel, in het bijzonder bij *Rosaceae* fruit. De lage interne pH van deze vruchten geven de PR-10-allergenen hun positieve lading, waarop membraanassociatie en IgE-binding wordt verhoogd.

Samenvattend leidt een thermodynamische karakterisering tot een beter begrip van verschillen in conformationele stabiliteit en structurele eigenschappen tussen allergenen. Aminozuurverschillen, die in alle natuurlijke isovormmengsels aanwezig zijn, leiden tot een verandering in stabiliteit, biologische functie, elektrostatische potentiaal en pakking van het eiwit. Al deze eigenschappen kunnen een effect hebben op eiwit-eiwit-interacties en zouden verschillen kunnen verklaren tussen de IgE-bindingscapaciteit van PR-10-isovormen.

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List of abbreviations

APBS	Adaptive Poisson-Boltzmann solver
CD	Circular dichroism
CE	Cellfree extract
GuaHCl	Guanidine hydrochloride
HIC	Hydrophobic interaction chromatography
IEF	Isoelectric focusing
IL	Interleukin
m/z	Mass-to-charge ratio
$m_{D\text{-}N}$	Measure for the dependence of $\Delta G_{D\text{-}N}$ on denaturant concentration
Mr	Relative molecular mass
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal components analysis
PDB	Protein data bank
Pfam	Protein family
P-loop	Phosphate-loop, motif rich in glycine
PR-10	Pathogenesis related family 10
PROPKA	Prediction of protein pKa-values
Q-TOF MS	Quadrupole time of flight mass spectrometry
RMSD	Root mean square deviation
SEC	Size exclusion chromatography
StAR	Steroidogenic acute regulatory protein
START	Steroidogenic acute regulatory lipid transfer domain
TBS	TRIS buffered saline
Th2	T helper 2 cell
T_m	Thermal denaturation midpoint
VMD	Visual molecular dynamics
ΔC_P	Heat capacity change
$\Delta G_{D\text{-}N}$	Gibbs free energy change
ΔH_{Tm}	Enthalpy change at T_m

Curriculum Vitae

Mirko Abraham Bollen was born in Hoogwoud on the 3rd of February, 1979. In 1997 he passed his VWO exam at the Atlas college location Copernicus SG in Hoorn. In the same year he started a study chemistry at the University of Amsterdam and graduated with a master degree in Biomolecular Chemistry in 2003. He did a short thesis subject at the biocatalysis researchgroup and did his main thesis subject at the molecular microbiology researchgroup. In 2003 he started a PhD study at the Product Design and Quality Management Group of Wageningen University. The research topic was about birch pollen-related food allergies as a part of the Allergy Consortium of Wageningen and formed the basis of this thesis entitled "Stability of the Bet v 1 cross-reactive allergens Api g 1 and Dau c 1, a biophysical approach".

Publications

Bollen, M.A., Schenk, M. F., Wichers, H.J., Helsper, J.P.F.G., Savelkoul, H.F.J., and van Boekel, M.A.J.S., Differences of PR-10 allergens and implications for predicting cross-reactivity of birch pollen-related food allergens. *Submitted for publication*

Bollen M.A., Wichers H.J., Helsper J.P.F.G., Savelkoul H.F.J., van Boekel M.A.J.S., Thermodynamic characterization of the PR-10 allergens Bet v 1, Api g 1 and Dau c 1 and pH dependence of nApi g 1 and nDau c 1. *Submitted for publication*

Bollen M.A., Jeurink P.V., Wichers H.J., Helsper J.P.F.G., van Boekel M.A.J.S., Savelkoul H.F.J., Allergenicity of natural and recombinant allergens in relation to their thermal stability. *Submitted for publication*

Bollen M.A., García A., Cordewener J.H.G., Wichers H.J., Helsper J.P.F.G., Savelkoul H.F.J., van Boekel M.A.J.S., Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. *Mol Nut. Food Res* **2007**, 51 (12), 1527-1536.

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Overview of completed PhD training activities

Discipline specific activities

Courses

Reaction kinetics in Food Science, VLAG, 2004
Food Related Allergies and intolerances, WUR, Wageningen, 2004
Food Enzymology, VLAG, 2005
Industrial proteins, VLAG, 2006
RBL-assay workshop, PEI, Langen, 006
Bioinformatics, VLAG & EPS, 2007

*Conference*s

Conference Allergy Matters!, WUR, Wageningen, 2004
European Allergy and Clinical Immunology congress, EAACI, Amsterdam, 2004
World Allergy Congress, EAACI, Munich, 2005
International Symposium on Molecular Allergology, 2008

General courses

VLAG PhD week, Bilthoven, 2004
Scientific writing, CENTA, Wageningen, 2005
Presentation Skills, CENTA, Wageningen, 2005
Philosophy and Ethic of Food Science & Technology, VLAG, 2006
Career perspectives, Wageningen Graduate Schools, 2007

Optionals

Preparation PhD research proposal
Product design and quality management PhD trip, USA, 2007
Allergy Consortium Project meetings, 2003-2007