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Identification and characterization of almond proteins homologous to Bet v 1 and their contribution to almond allergy associated with birch pollinosis

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D A N K S A G U N G

Diese Masterarbeit wurde am Institut für Pathophysiologie und Allergieforschung der Medizinischen Universität Wien durchgeführt.

Die wissenschaftliche Betreuung im Zuge der praktischen und theoretischen Arbeit erfolgte durch Frau Frau Assoc. Prof. Priv.-Doz. Mag. Dr. Merima Bublin.

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Preface

Parts of the results of this thesis have been published in: “Kabasser, S., Crvenjak, N., Schmalz, S., Kalic, T., Hafner, C., Dubiela, P., ... & Bublin, M. (2022). Pru du 1, the Bet v 1-homologue from almond, is a major allergen in patients with birch pollen associated almond allergy. Clinical and Translational Allergy, 12(8), e12177. The following results obtained within this thesis are included in the publication: the comparison of the circular dichroism spectra of Bet v 1 and rPru du 1.06A, results obtained by analysis of thermostability, cross-reactivity to Bet v 1 and rPru du 1.06A.

The publication also has been cited and included in the reference list.

Abstract

Introduction: Almond allergy associated with birch-pollinosis strongly suggests the presence of a cross-reacting Bet v 1- homologue in almond. The aim of the study was to identify Bet v 1-homologues from almond and to investigate its allergenic properties.

Methods: Almond extract was analyzed by tandem mass spectrometry to identify the protein sequences matching seven previously identified *Pru du 1* genes. The identified isoform Pru du 1.06A was expressed in *Escherichia coli* and characterized by MALDI-TOF and circular dichroism spectroscopy. IgE-specific to rPru du 1.06A was determined using a quantitative ELISA and sera from almond allergic subjects. Cross-reactivity to Bet v 1 and other Bet v 1 homologous were tested by dose-dependent IgE ELISA. Its ability to induce mast cell activation was tested using a human FceRI-transfected rat basophil cell line.

Results: rPru du 1.06A has a molecular mass of 18,090 Da, 56% amino acid sequence identity and nearly identical structure to Bet v 1. Thermostability analysis revealed denaturation of rPru du 1.06A at 55 °C. rPru du 1-spezific IgE was detected in 82% of the tested sera. rBet v 1 inhibited IgE-binding to rPru du 1 by 100%, while vice versa by 40%. Cross-reactivity to related allergens from peanut, peach or apple was in a range of 36-81%. Maximum cell activation was achieved at a concentration of 1 µg/ml of rPru du 1.06A and 0.1 ng/ml of Bet v 1.

Conclusion: This study provides the first experimental conformation of a cross-reactive Bet v 1 homologue in almond.

Zusammenfassung

Einleitung: Die mit der Birkenpollinose verbundene Mandelallergie deutet stark auf das Vorhandensein eines kreuzreagierenden Bet v 1-Homologs in Mandeln hin. Ziel der Studie war es, Bet v 1-Homologe aus Mandeln zu identifizieren und ihre allergenen Eigenschaften zu untersuchen.

Methoden: Mandelextrakt wurde mittels Tandem-Massenspektrometrie analysiert, um die Proteinsequenzen zu identifizieren, die mit sieben zuvor identifizierten Pru du 1-Genen übereinstimmen. Die identifizierte Isoform Pru du 1.06A wurde in Escherichia coli exprimiert und durch MALDI-TOF und Zirkulardichroismus-Spektroskopie charakterisiert. Das für rPru du 1.06A spezifische IgE wurde mit einem quantitativen ELISA und Seren von Mandelallergikern bestimmt. Die Kreuzreaktivität mit Bet v 1 und anderen Bet v 1-Homologen wurde mit einem dosisabhängigen IgE-ELISA getestet. Seine Fähigkeit, eine Mastzellenaktivierung auszulösen, wurde mit einer humanen FceRI-transfizierten Rattenbasophilen-Zelllinie getestet.

Ergebnisse: rPru du 1.06A hat eine Molekularmasse von 18.090 Da, 56 % Aminosäuresequenzidentität und eine nahezu identische Struktur wie Bet v 1. Die Thermostabilitätsanalyse ergab eine Denaturierung von rPru du 1.06A bei 55 °C. rPru du 1-spezifisches IgE wurde in 82 % der untersuchten Seren nachgewiesen. rBet v 1 hemmte die IgE-Bindung an rPru du 1 zu 100 % und umgekehrt zu 40%. Die Kreuzreaktivität zu verwandten Allergenen aus Erdnuss, Pfirsich oder Apfel lag in einem Bereich von 36-81%. Die maximale Zellaktivierung wurde bei einer Konzentration von 1 µg/ml von rPru du 1.06A und 0,1 ng/ml von Bet v 1 erreicht.

Schlussfolgerung: Diese Studie liefert die erste experimentelle Konformation eines kreuzreaktiven Bet v 1-Homologs in Mandeln.

Table of content

1 Introduction

The term allergy was firstly invented by the Viennes pediatrician Clemens von Pirquet (1874–1929) in 1906 [1]. He invented the word allergy from Greek *allos* meaning "other" and *ergon* meaning "work".

1.1 Hypersensitivity reactions (or responses)

Hypersensitivity reactions are immunological reactions occurring in response to an antigen or allergen. These are further classified based on the type of immune reaction and effector mechanisms. Until now, four different hypersensitivity reactions are described: immediate (Type 1) hypersensitivity, antibody-mediated (Type II) hypersensitivity, immune complex-mediated (Type III) hypersensitivity, and T cell-mediated (Type IV) hypersensitivity [2].

1.2 Immediate (Type 1) hypersensitivity reaction

The immediate type 1 hypersensitivity reaction, also named as atopic, allergic, or immediate reaction, is the most prevalent [3]. The reaction is triggered by an antigen (allergen) leading to sensitization of the individual followed by an immediate and late-phase response (Figure 1) [2]. Antigens that induce immediate hypersensitivity are called allergens. Typical allergens comprise proteins in pollen, animal dander, house dust mites, drugs, and food. The first encounter of an individual to an allergen is described as allergic sensitization and it is characterized by development of IgE memory B-cells and allergen-specific memory T cells. Characteristics of the epithelial barrier, the stability of the allergen, nonallergenic factors of the food matrix and adjuvants define the allergic sensitization process [4]. Both, intrinsic (e.g., genetic factors) and exogenous factors (alcohol, anti-inflammatory drugs, stress, and pathogens) can decrease the barrier property of the intestinal epithelium and promote sensitization [5]. Sensitization can occur through oral, cutaneous or airway routes. Allergens can be further divided into class 1 and class 2 food allergens. Class 1 allergens (e.g., egg, milk

or peanut) induce allergic sensitization by the gastrointestinal tract resulting in systemic reactions. On the other hand, class 2 food allergens (e.g., apple, celery, and pollen homologues proteins) do not induce sensitization but are known to provoke cross reactivity with inhalative allergens as pollen allergens resulting in oral allergy syndrome [6]. New studies proposed that additionally to cutaneous sensitization also airway sensitization routes may take place referred as dual allergen exposure hypothesis. The study showed that airway sensitization to peanut and household dust as adjuvant induced an allergic reaction. On the other hand, exposure to dust or peanut extract alone did not lead to sensitization [7]. Additionally, other studies also showed sensitization to food allergens as buckwheat and sunflower seed through inhalation followed by anaphylaxis after oral ingestion [7,8]. Furthermore, antigen presenting cells (APC) e.g., dendritic cells (DCs) take up the allergens processing them further into peptides. These are presented by the MHC class II complex to naïve CD4+ T-cells leading to their activation and further differentiation into specific T_H2-cells and follicular helper cells (T_{FH}). Both cells secrete cytokines like interleukin-4 (IL-4) and IL-13 that further support immunoglobulin-class switching of B cells into allergen specific IgE antibody. Allergen-specific IgE in allergic patients is mainly generated by IgE+ memory B cells that need further help from allergen-specific T cells for activation [2]. Produced IgE further binds to high-affinity IgE-receptors (FcεRI) on the surface of mast cells and basophils. The immediate-phase reaction begins within minutes after antigen challenge in a previously sensitized individual inducing inflammation, vasodilatation, bronchoconstriction, and/or edema. It is accomplished by activation of mast cells by cross-linking of FcεRI molecules, which occurs by binding of multivalent antigens to the IgE molecules that are attached to Fc receptors. This further results in three types of biologic responses: degranulation, secretion of lipid mediators and secretion of cytokines. Lipid mediators (e.g. prostaglandins, leukotriene), biogenic amines (e.g. histamine), cytokines (e.g. IL-1, IL-4, IL-5, IL-6, IL-13, tumor necrosis

factor) and enzymes (e.g. tryptase) induce the pathologic reaction. The immediate reaction is always continued by much slower inflammatory late-phase reactions with symptoms occurrence between 2-24 hours after allergen exposure. It is accompanied by accumulation of inflammatory leukocytes (neutrophils, eosinophils, basophils) T_H2-cells and corresponding secreted cytokines (IL-4, IL-5, and IL-13). Cytokine IL-4 induces endothelial VCAM-1 expression that promotes induction of eosinophils into tissues where IL-5 induces eosinophil activation. On the other hand, IL-13 stimulates epithelial cells to produce increased amounts of mucus. Symptoms of the late phase reaction involve pain, oedema, warmth, and erythema of the skin as well as mucus hypersecretion.

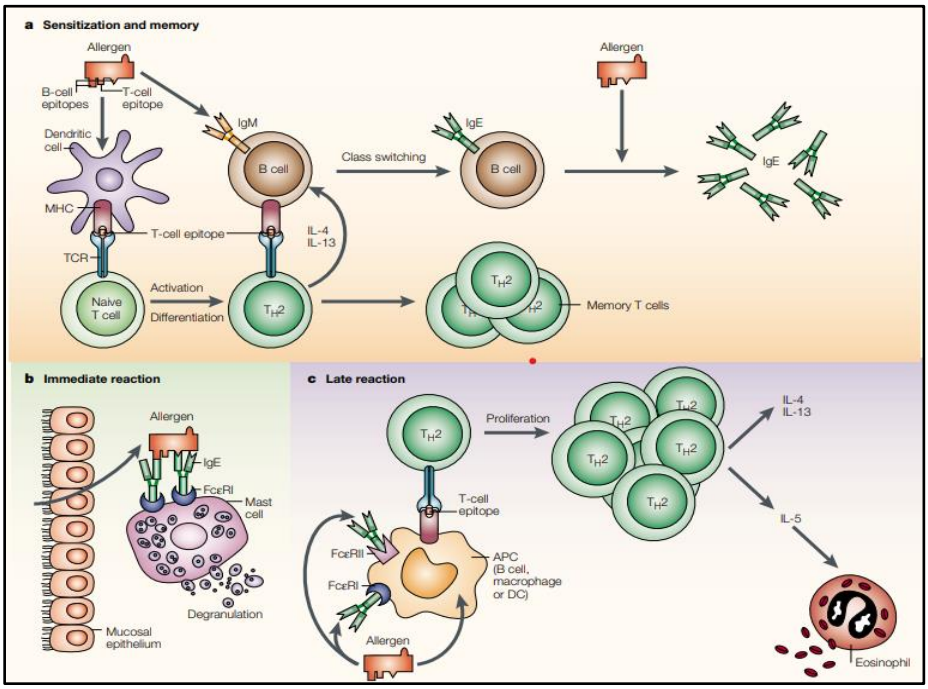

Figure 1: *Three different stages of immediate type 1 hypersensitivity.* ***a) Sensitization and memory:*** *after initial exposure, allergen get uptaken by dendritic cells or specific B cells that further leads to production of interleukin (IL-4) and IL-13. These further induce immunoglobulin class switching in B cells and further generation of memory B cells and allergen-specific memory T cells.* ***b) Immediate reaction****: after allergens crosslink with IgE antibodies bound to effector cells degranulation occurrs* ***c) Late reaction:*** *allergens presentation to T cells results in their activation, proliferation and release of cytokines as IL-4, IL-5 and IL-13. Figure taken from [9]. I have made every effort to identify all copyright holders and obtain their consent to use the images in this work. However, if a copyright infringement is still discovered, please inform me.*

1.2.1 Immediate (type 1) hypersensitivity diseases

Immediate hypersensitivities stimulate atopic and allergic diseases. Atopy refers as a genetic predisposition to generate allergic diseases like bronchial asthma, atopic dermatitis (eczema), allergic rhinitis and food allergies. The exaggerated immune response is associated with production of allergen-specific IgE antibodies to the recognized foreign allergen [10].

The prevalence of food allergies, conjunctivitis, dermatitis, asthma, and allergic rhinitis appears between 20-30% of the general population [2]. Bronchial asthma affects approx. 20 million individuals in the US and is treated by antihistamines (H1 antagonist) or glucocorticoids to block the generation of cytokines and reduce inflammation. Individuals with significant symptoms undergo immunotherapy to reduce IgE levels in patient sera. For example, the anti-IL-13 antibody is implemented for lingering the type 2 response [11]. On the other hand, allergic rhinitis (also known as hay fever) is the most common atopic disease caused by pollen or house dust mites [12]. It manifests as coughing, sneezing, difficulty in breathing and mucus secretion. As well, allergic conjunctivitis (with itchy eyes) and nasal polyps are associated with rhinitis. For temporary lingering of symptoms, topical nasal or optical decongestants are used. Atopic dermatitis (known as eczema) is a common skin disorder resulting in defective skin barrier function. The prescribed therapy usually use of corticosteroids that inhibit cytokine synthesis. On the other hand, food allergies have become a global public health issue as it influences individuals of all ages, ethnicities, and socioeconomic levels. Data from the US demonstrated the five most common food allergies reported by individuals were shellfish, peanut, milk, tree nuts and fish. In general, data indicated that beyond 10% of the US population experiences at least one IgE-mediated food allergy [13]. Urticaria, rhinitis and mild bronchospasm are associated with food allergy where also anaphylaxis can occur leading to fatal consequences [14]. Anaphylactic shock results in mediators released from mast cells and is defined by restriction of airways leading to cardiovascular collapse resulting in shock that

can have fatal consequences. It is usually triggered by tree nuts, peanut, fish, shellfish, milk, eggs, penicillin family antibiotics and many other foods, drugs, and environmental pollutants [15]. The shock occurs within seconds to an hour after allergen exposure where epinephrine (adrenaline) is usually used as therapy. Theories also indicate that potential genetic predisposition play a role in the development of allergies, but the gene-environmental interactions are very complex and poorly understood [16]. Related individuals are more likely to develop allergies than unrelated individuals even when not sharing the same environment. Different environmental factors aside from exposure to allergens e.g., air pollution and exposure to microbes (in industrialized societies) have intense influence on allergy development [17]. The tendency in developing allergies is influenced by inheritance of several genes. A susceptibility locus for allergy on chromosome 5q was identified close to site of the gene cluster encoding for IL-4, IL-5, IL-9, and IL-13 and the IL-4 receptors [2]. As well polymorphisms on IL-33 gene have been mapped and have strong association with asthma [18]. The risk of developing allergies is supported by the hygiene hypothesis [19]. It states that early-life and even prenatal exposure to gut commensal reduces risk of allergy development in later life.

1.2.2 Immunoglobulin E (IgE) antibody

An important role in immediate hypersensitivity reaction plays the IgE antibody. IgE is an isotype containing ε heavy chain binding to Fc receptors on mast cells, basophils and activated eosinophils. IgE synthesis depends on the tendency of an individual to produce allergen-specific type 2 T follicular helper (Tfh2) cells that further produce cytokines IL-4 and IL-13[2]. These cytokines stimulate B cells undergoing heavy chain class switching to produce IgE antibodies. The development of the two mentioned cytokines depends on inherited genes, history of antigen exposure and the nature of antigens. IgE antibodies circulate as bivalent antibodies at a

concentration below 240 ng/ml in healthy individuals. Mast cells and basophils express high-affinity Fcε receptor type I (FcεRI), which binds IgE. The affinity of FcεRI binding to IgE is very high (dissociation constant [Kd] of approximately 1×10^{-10} M), higher than that of any other Fc receptor for its antibody ligand [20]. FcεRI receptor is composed of an α chain that binds the Fc region of IgE, a β chain and two γ chains that are responsible for signalling (Figure 2). The α chain includes two Ig-like domains as IgE binding site. The β chain of FcεRI consists of a single immunoreceptor tyrosine-based activation motif (ITAM). The two γ chain polypeptides are linked by a disulphide bond and the cytoplasmic portion of each γ chain contains one ITAM [21]. When an allergen binds to IgE cross-linking occurs with the FcεRI leading to phosphorylation of ITAMs of FcεRI β and γ chains in the cytoplasm [2].

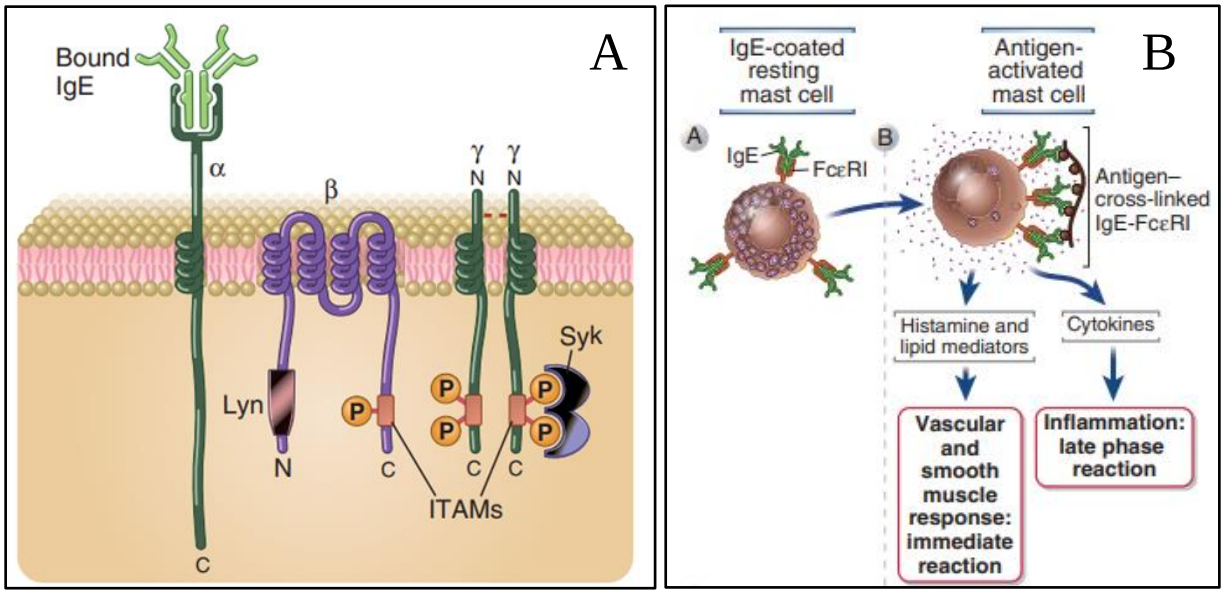

Figure 2: *A) Structure of the high affinity Fcε receptor type I (FcεRI). B) Mast cell activation by an antigen. Figures taken from [2]. I have made every effort to identify all copyright holders and obtain their consent to use the images in this work. However, if a copyright infringement is still discovered, please inform me.*

1.2.3 Diagnosis and therapy

Several different diagnostic methods are used for diagnosis of food allergies. These can be subdivided into extract-based diagnosis and modern molecular allergy diagnosis. Either way,

the diagnosis starts with the medical history of the subject. In traditional extract-based diagnosis, allergen extracts are used for further serological and provocation tests. If the provocation test results negatively, oral food challenge (OFC) is carried out. Oral food challenge (OFC) is another method of diagnosis of food allergies. Since OFC can possibly lead to fata anaphylaxis, lip dose challenge (LDC) is suggested as an alternative [22]. Skin prick test (SPT) and atopy patch testing (APT) are two types of skin tests used in allergy diagnosis. By using SPT, mast cell degranulation is measured where in contrast, APT estimates IgE-independent T cell–mediated activation [23]. On the other hand, molecular allergy diagnosis is characterized by IgE serology to different allergens [24]. In molecular allergy diagnosis, recombinant purified allergens are used to define IgE sensitization of allergic subjects, also known as component-resolved diagnostics (CRD). Different assays have been developed in order to detect serum IgE to a single allergen (singleplex assay) or multiple allergens (multiplex assay) [25]. Today, a chip containing 170 allergen molecules (natural purified and recombinant allergens) has been developed for the European MeDALL research project [26]. Almond allergens can additionally be detected by allergen microarrays as the MeDALL allergen-chip. An advantage of this method depends on the simultaneous detection of several allergens by using a small quantity of subject sera. Additionally, it is useful for tracking food allergies and implementation of personalized therapies [27]. Basophil activation test (BAT) is another method applied for in vitro allergy diagnosis. Here, the degree of basophil degranulation after stimulation with an allergen is measured.

Allergen-Specific Immunotherapies (AIT) represents the future in preventing allergen causing diseases. This treatment leads to a reduction in specific IgE levels by hindering IgE binding through allergen-specific IgG and IgA antibodies [2,28]. The long-term effect results by the perseverance of high-affinity and functional allergen-specific IgG_4 antibodies [29]. Nevertheless, the limitations of implementation of AIT are proper identification of the disease-

causing allergens for appropriate vaccine administration. Besides, this approach is difficult to implement in individuals with polysensitization's since multiple vaccines may induce side effects. Another hindrance is the optimal timing for proper AIT begin, as well as dosing intervals for continues administration [30]. Still, implementation of AIT leads to progression of mild symptoms against severe symptoms where progression of allergic rhinitis toward asthma was successfully prevented by this method [31].

1.2.4 Cross-reactivity in Type 1 allergy

The capacity of IgE antibodies to recognize secondary allergens after the individual has been already primarily sensitized is defined as cross-reactivity [32]. The sensitization process is triggered by a primary initiator allergen via T_H2 response inducing allergen specific IgE antibodies (see section 1.2.1) that can further recognize conformational or linear allergen epitopes [33]. Furthermore, when individuals are exposed to the secondary allergen that can be a homologous allergen, cross-reactivity occurs through cross-reactive epitopes resulting in basophil and mast cell activation [34]. Cross-reactivity demands up to 70% sequence identity, where proteins sharing less than 50% sequence identity are rarely cross-reactive [32]. Cross-reactivity has been observed between homologous allergens from phylogenetically related grasses, serum albumins from vertebrates and IgE antibodies to food allergens. The development of immunoblotting procedure in the early 1980s helped tremendously in recognition of two different types of cross-reactivity namely, one due to proteins and the other due to cross-reacting carbohydrate determinant (CCD) [35]. Clinically relevant cross-reactivity between plant allergens has been described for Bet v 1 homologues, nsLTPs and profilins [36]. Studies have demonstrated that four significant IgE epitopes of Pru p 3 are shared by LTPs of fruits from the *Rosaceae* family [36]. Patients suffering from nsLTP syndrome repeatedly exhibit symptoms with various plant-derived foods due to the prevalent distribution of these

panallergen [37]. On the other hand, cross reactivity was long time believed to only occur between homologous proteins, however there is accumulating evidence that this also happens between non-homologous proteins. Findings have demonstrated that high cross-reactivity occurs between unrelated seed storage proteins in peanut (Ara h 1-3) and cashew (Ana o 1-3) [38,39]. Furthermore, it is demonstrated that IgE cross-reactivity among unrelated allergens occurs as well in hazelnut but to a lesser degree compared to cashew nut and peanut [40]. Furthermore, these findings emphasize that protein sequence identity alone cannot precisely predict cross-reactivity. Many individuals can be sensitized to an allergen (possessing allergen specific-IgE) but not exhibiting clinical reactivity. This makes the diagnosis and management of allergic cross-reactivity between food allergens difficult [39].

1.3 Food allergy and allergens

Food allergies are immunological reactions mediated by IgE, which are distinct from other types of food sensitivities (food intolerance or toxic food reactions) affecting 2 % of the adult population in industrialized countries and about 6-8 % of infants and children [41]. According to the findings of numerous studies, the prevalence appears to have increased over the past 20 years and play a serious strain on the public's health [42]. Over 180 allergenic food sources have been discovered by far and are further classified under eight most important allergen sources: milk, egg, fish, crustaceans, peanuts, soybeans, wheat, and tree nuts [43]. Only few protein families have the possibility to sensitize predisposed individuals and induce an immunological reaction. Furthermore, there are features typical for food allergens such as: resistance to pH and digestive enzymes of the gastrointestinal tract (GIT) and thermal stability. GIT is the largest immunological organ of the human body, and it frequently gets exposed to a high diversity of different ingested food. For that reason, food allergens possess these features as protection from digestion enzymes present in the GIT. Plant food allergens have been classified into a few conserved and universal families and superfamilies based on their

structural characteristics. Those include for example prolamin superfamily, cupin superfamily, and other families such as profilins, defensins and oleosins. In the following three most frequent protein groups involved in food allergy are described.

The prolamin superfamily

Allergens of the prolamin superfamily are defined by a low molecular weight and containing 8-10 cysteine residues in their sequence . These cysteines are engaged in the formation of four or five intra-chain disulphide bonds required for the 3D structure. Allergens of this superfamily are highly resistant to heat, low pH, and gastrointestinal enzymes. Different allergenic seed storage proteins belong to the prolamin superfamily comprising 2S albumins, nonspecific lipid transfer proteins (nsLTPs), cereal α-amylase inhibitors and protease inhibitors. Numerous monocotyledonous and dicotyledonous species contain 2S albumins, which have some protective effects on plants against fungal invasion [43]. On the other hand, nsLTPs make up 4% of all soluble proteins in higher plants and belong to major food allergens present in *Rosaceae* fruits like peach (Pru p 3), apple (Mal d 3), apricot (Pru ar 3), and plum (Pru d 3). NsLTPs can also be found in foods like grains, vegetables, and nuts [24].

The cupin superfamily

Cupin protein family is widely present in higher plants, animals, and fungi [24] . It comprises at least six different β-barrel structures that give the name to this superfamily, since *cupa* in Latin means barrel. This superfamily can be further subdivided into various cupin subgroups based on whether the proteins contain a single cupin domain, a duplicated (bicupin), or a multicupin (>2 cupin domains) structure. Allergens belonging to the cupin superfamily comprise globulin seed storage proteins like 7S globulin trimer (vicilin) and 11S globulin hexamer (legumin) [24].

Pathogenesis related (PR) proteins

PR proteins comprise a group of non-related protein families that have the function in plant defence [24]. The expression occurs as a result to pathogen infections, diverse compounds (e.g., phytohormones), ultraviolet radiation, wounds, or disadvantageous growth conditions. Fourteen groups of PR proteins are described where groups 1, 2, 3, 4, 5, 8, 10, and 14 contain allergens. PR-1 proteins (e.g., Cuc m 3 from muskmelon) possess antifungal properties where PR-2 proteins (e.g., latex Hev b 2) are involved in pathogenic defence and different physiological plant functions. PR-5 proteins comprise thaumatin-like proteins (TLPs) and can be found in apple (Mal d 2), cherry (Pru av. 2), peach (Pru p 2), kiwi (Act d 2), etc. [24]. Of big importance are PR-10 proteins to which the one of the best-known plant allergens, major birch pollen allergen Bet v 1, belongs. Allergens belonging to PR-10 proteins are Bet v 1 homologs and are about 16–18 kDa. Bet v 1 homologue proteins belong to numerous species as *Rosaceae* (peach, apricot), *Apiaceae* (carrot, celery), *Leguminosae* (peanut, soybean) families or tree nuts (hazelnut, chestnut) [44,45].

Birch pollen-related food allergies

Birch trees belong to the order *Fagales* and family *Betulaceae* and are widespread everywhere in Europe and their flowering period is between April and May [46]. Birch pollen is a known elicitor of allergies around northern and central Europe and a major indicator of allergic rhinitis as well as asthma [44,45]. In an Austrian study 16.3% of 501 participants demonstrated IgE reactivity to Bet v 1 [49]. Another study from Germany showed 14.1% sensitization to Bet v 1 among 17 641 children and adolescents [50] . The prevalence of Bet v 1 sensitization of the general population in Europe ranges from approximately 8% to 16% [47]. Data revealed that patients with birch pollen allergy possibly develop immediate reactions to fruits and vegetables besides seasonal respiratory symptoms. The occurring symptoms are displayed as itching or

swelling of the lips, tongue, and throat also known as oral allergy syndrome (OAS) or so-called pollen food syndrome (PFS) [51]. From time to time, more severe IgE mediated systemic reactions as asthma, urticaria or anaphylactic shock can arise. Birch pollen associated plant-derived food allergies occur as a result of IgE cross-reactivity between structurally related food proteins and Bet v 1. Bet v 1 specific IgE antibodies cross-react with homologous proteins from different stone-fruits, such as apple (Mal d 1), pear (Pyr c 1) and cherry (Pru av 1). A summary of Bet v 1 homologous proteins is represented in table 1. IgE cross reactivity was additionally observed in hazelnut (Cor a 1), peanut (Ara h 8), celery (Api g 1), carrot (Dau c 1), soybean (Gly m 4), kiwi (Act d 8) and jackfruit. The degree of birch pollen–related food cross-reactivity depends on structural and sequence similarities between allergens as well as the epitope repertoire identified by specific IgE antibodies [38]. The presence of specific IgE to Bet v 1-homolog food allergens fails to predict clinical symptoms after contact with the allergen source will occur. Studies showed that specific IgE against apple and/or hazelnut allergens was identified in 47% birch pollen-sensitive patient sera who did not express pollen food syndrome (PFS) [48,52]. Similarly, 75% of birch pollen-allergic patients showed IgE-mediated cross-reactions to the Bet v 1-homolog soy protein Gly m 4 *in vitro*, but only 10% of the patients established clinical symptoms after consumption of soy products [51].

Table 1: *Examples of Bet v 1 homologues allergens and their corresponding plant families.*

Plant protein family	Food	Bet v 1 Homolog Proteins
Rosaceae	Almond	Pru du 1
	Apple	Mal d 1
	Apricot	Pru ar 1
	Cherry	Pru av 1
	Peach	Pru p 1
	Pear	Pyr c 1
	Plum	Pru d 1
Apiaceae	Carrot	Dau c 1
	Celery root/tuber	Api g 1
Betulaceae	Hazelnut	Cor a 1
Actinidiaceae	Kiwifruit	Act d 8
Fabaceae	Peanut	Ara h 8
	Soybean	Gly m 4

1.3.1 Bet v 1 Superfamil

The Bet v 1 superfamily consists of eight subfamilies. Most of Bet v 1 superfamily members consist of roughly 154-160 amino acid length. [41]. The major latex proteins, ripening-related protein family, norcoclaurine synthases, and the cytokinin-binding proteins from legumes are all members of this superfamily, which have low levels of sequence similarity between them. Bet v 1, birch pollen allergen, is a major member of this superfamily. About 90% birch pollen allergic patients are sensitized to Bet v 1 [46]. This family is characterized by small gene families with isoforms that differ in their expression. Birch possesses thirteen gene loci, seven of which encoded proteins that were only expressed in pollen [53]. The PR-10 protein expression can be divided into two types: developmentally regulated and stress-induced.

Developmentally regulated PR-10 proteins are highly expressed in pollen, fruits, seeds, or storage organs and are recognized as allergens that can cross-react with Bet v 1. Pathogenic infections, plant hormones, salt stress, and injuring generate stress-induced PR-10 protein expression [54]. Individuals who are allergic to pollen frequently develop allergies to Bet v 1 homologous proteins in fruits (e.g., apples, pears, cherries, kiwis, jackfruit), vegetables (carrots, celery, and coriander), and nuts. The allergic symptoms are commonly mild and restricted to the oral cavity leading to OAS [24].

1.4 Almond allergy

1.4.1 Almond (*Prunus dulcis*)

Tree nuts are crucial foods in human diet and are consumed all around the world. Almond (*Prunus dulcis*) belongs to the *Rosaceae* family and are one of the most important nuts among tree nuts. These nuts are rich in mono- and polyunsaturated fatty acids, phytosterols and contain low glycemic index. This makes them beneficial in reduction of some risk factors for cardiovascular disease and diabetes [55]. Almonds possess anti-inflammatory and antioxidant properties because of their polyphenol compounds (e.g. flavonoids) making them valuable for hepato- and neuroprotection as well as cholesterol-lowering effects [55–58].

1.4.2 Prevalence of almond allergy

Almond together with hazelnut, cashew and walnut is an important trigger of tree nuts allergies. Studies revealed that tree nuts as cashew, walnut, almond, Brazil nut, pistachio, hazelnut, and macadamia generate most of allergic reactions [59]. Epidemiologically, almond allergy has the fourth highest prevalence among tree nut allergies (summarized in [60]). Between 9% and 15% of the US population sensitized to tree nuts report allergy to almonds [61]. Another study from Australia reported almond allergy prevalence of 0.3% in children and 0.2% in adolescents [62]. In United Kingdom it has been reported that almonds account 22% to 33% of the cases in

sensitized individuals where in Mexico City, it has been indicated that a rate of 43% of sensitization to almonds occurs in older children between the age of 6 and 17 [63,64]. Furthermore, in a Korean study a lower degree of sensitivity to almonds in females with 9.8% in comparison to males at 13.5% was reported [65].

1.4.3 Almond allergens

So far, ten distinct almond allergens have been suggested: Pru du 1, Pru du 2, Pru du 2S albumin, Pru du 3, Pru du 4, Pru du 5, Pru du 6 (amandin), Pru du γ-conglutin, Pru du 8 and Pru du 10 [58]. However, so far, only Pru du 3-6, Pru du 8 and Pru du 10 have been confirmed and included in the WHO-IUIS list of allergens (see Table 2).

Table 2: *WHO/IUIS associated almond allergens and their biochemical names, possible isoallergens, Uniprot annotations and molecular weight.*

Allergen	Biochemical Name	WHO-IUIS included	Isoallergen	UniProt	Molecular weight (kDa)
Pru du 3	non-specific Lipid Transfer Protein 1 nsLTP1	(2009)	Pru du 3.0101	C0L0I5	9
Pru du 4	Profilin	(2006)	Pru du 4.0101 Pru du 4.0102	Q8GSL5	14
Pru du 5	60S acidic ribosomal protein P2	(2007)	Pru du 5.0101	Q8H2B9	10
Pru du 6	Amandin, 11S globulin legumin-like protein	(2010)	Pru du 6.0101 Pru du 6.0201	E3SH28 E3SH29	360
Pru du 8	Antimicrobial seed storage protein	(2018)	Pru du 8.0101	A0A516F3L2	31
Pru du 10	Mandelonitrile lyase 2	(2019)	Pru du 10.0101	Q945K2	60

Pru du 3, a non-specific lipid transfer protein (nsLTP) also known as PR-14 are resistance to abrupt pH changes, heating, and pepsin digestion [58,59]. These proteins are usually assembled on the outer epidermal layer of the nut and are known to comprise a hydrophobic core to reduce lipid transference (e.g., steroids, fatty acids, phospholipids, and glycolipids) between membranes. Three distinct isoallergens Pru du 3.01, 3.02 and 3.03 were identified comprising a similar molecular weight of 9 kDa [58,66]. Almond profilins, Pru du 4, encoded by *Pru du 4.01* and *Pru du 4.02* genes are categorized as panallergens. They can establish high-affinity complexes with monomeric actin, leading to its polymerization into filaments. Studies showed that allergens Pru p 4.01 and Pru av 4 from peach and sweet cherry exhibit highest similarity (99 and 98%, respectively) with almond profilins. They are categorized as minor allergens since 45% (8/18) almond allergic patients were sensitized to Pru du 4 [67]. Pru du 5 (60S Acidic Ribosomal Protein P2) is considered as a major almond allergen encoded by the *P. dulcis* 60S acidic ribosomal protein gene (*AL60SRP*). Data indicated up to 50% of anti r60sRP IgE in patient sera sensitized to almonds [68]. An almond 11S seed storage globulin, Pru du 6 (amandin) is a hexameric allergen of a molecular weight of 360 kDa. It represents up to 65% of total almond protein content and is highly stabile to thermal processing and still recognised by IgE of almond allergic patients. (able to induce allergic reaction).

1.1.1.1 Bet v 1-homologue Pru du 1

As already described, PR-10 proteins are upregulated in plants inducing intracellular defense mechanisms and responses to pathogens (e.g., fungi, viruses, and bacteria), chemicals and other environmental factors. Major allergen form birch pollen Bet v 1 is the most prominent member of the family and is also one of the most cross-reactive plant allergens known as panallergens . Almond allergic patients from Central Europe usually presents with mild symptoms (mainly oral allergy syndrome) indicating birch-pollen-associated almond allergy. Seven genes were identified in almond leaves coding for different Bet v 1-homologues (*Pru du 1.01, Pru du 1.02,*

Pru du 1.03, Pru du 1.04, Pru du 1.05, Pru du 1.06A and Pru du 1.06B) [69]. In the publication of Chen et al., genomic DNA was isolated from leaves which raised the question whether a Bet v 1 homologue is also expressed in almond kernels [69]. The putative proteins hold peptide sequences of 160 amino acids where only *Pru du 1.04* would contain 159 amino acids and have predicted molecular weights between 17.1 and 17.5 kDa. Prior to this work, the identification of the respective isoforms on protein level which is expressed in almond kernels together with immunological data to justify their classification as allergens were missing.

1.4.4 Cross-reactivity of almond allergens

Almond sensitization is very common in northern European countries in patients sensitized to birch pollen [70]. Multiple sensitizations to various pollens, fruits, nuts, and other vegetables can appear as a result of cross-reactivity between sensitizing allergen Bet v 1 and Bet v 1 related allergens [71]. On the other hand, almond allergies can occur even in individuals without previous relevant pollen sensitization. Such is usually induced by the Pru du 3 almond allergens from the nsLTP family. Studies have demonstrated IgE cross-reactivity between Pru du 3 and hazelnut Cor a 8 as well as between Pru du 3 and walnut Jug r 3 [72,73]. Additionally, IgE cross-reactivity has been detected between almond profilin Pru du 4 and grass pollen profiling Phl p 12 [67]. Favorably, the symptoms are mild and are mainly limited to the oral cavity where many profilin-sensitized patients do not display symptoms [74].

1.5 Aim of the thesis

Almond together with hazelnut, cashew and walnut is an important trigger of tree nuts allergies in children and adults. Epidemiologically, almond allergies possess the fourth highest prevalence among tree nut allergies and can be further subdivided into primary and secondary almond allergies. Data showed that the primary almond sensitization with severe symptoms is induced by sensitization to Pru du 6. On the other hand, secondary almond allergies (birch-pollen associated almond allergy) are prevalent in birch-endemic regions followed by milder symptoms. It has been hypothesized, that the secondary almond allergy has been induced by IgE cross-reactivity of a potential Bet v 1 homologous protein present in almond kernels. Although seven genes were identified in almond leaves coding for different Bet v 1-homologues expression of a corresponding protein in almond kernels and its IgE binding were not confirmed. Thus, the aim of the thesis was to identify Bet v 1 homologous proteins in almond nuts and produce the protein recombinantly for its biochemical and immunological characterization.

2 Materials and Methods

2.1 Materials

Chemicals/Reagents	Manufacturers
Ammonium peroxodisulfate (APS)	Gibco BRL, MD, USA
Agar Bromophenol blue Bovine serum albumin (BSA) Coomassie Brilliant Blue R-250 (CBB R-250) Dithiothreitol (DTT) Glycine ($C_2H_5NO_2$) Imidazole Kanamycin Milk powder N, N, N, N-tetramethylethylenediamine (TEMED) Sodium dodecyl sulphate (SDS)	Carl Roth GmbH +Co. KG, Karlsruhe, Germany
Acetic acid (HAc) D (+)-Glucose monohydrate Isopropanol (C_3H_8O) Magnesium chloride ($MgCl_2$) Methanol (CH_3OH) Peptone Potassium chloride (KCl) Potassium dihydrogen phosphate (KH_2PO_4) 2-Propanol Sodium azide (NaN_3) Sodium chloride (NaCl) Sodium hydroxide (NaOH) Sodium dihydrogen phosphate (NaH_2PO_4) Sodium dihydrogen phosphate monohydrate (NaH2PO4 x H2O) Yeast extract	Merck, KGaA, Darmstadt, Germany
Hydrochloric acid (HCl) Glycv erol ≥99.5% Polyoxyethylene (20) sorbitan monolaurate (Tween 20)	Sigma-Aldrich, St. Louis, MO, USA

Ponceau S	
TRIS pure	Biomol GmbH, Hamburg, Germany
Ethanol absolute	VWR, Vienna, Austria
Cutsmart Buffer (10x) EcoRI NcoI	New England BioLabs® Inc., Ipswich, MA, USA
DPBS MEM media	Gibco
Agarose	Fermentas, St. Leon- Roth, Germany
Complete Protease Inhibitor Cocktail	Roche Diagnostics GmbH, Mannheim, Germany
Acrylamide/Bis solution, 37.5:1 (Reagent C)	Serva Electrophoresis GmbH, Heidelberg, Germany
TRIS pure	Biomol GmbH, Hamburg, Germany
Ethanol absolute	VWR, Vienna, Austria

2.2 Methods

2.2.1 Identification of an almond Bet v -1 homologue in almond kernels

2.2.1.1 Almond protein extraction

Protein extract was prepared by using almond kernels (*Prunus dulcis*) purchased from a local supermarket. The kernels were slowly grounded by a blender and the proteins got extracted by stirring with 5 volumes of PBS containing protease inhibitor tablets (1 tablet/50 ml) at 4 °C for 2 h. Subsequently, the homogenate was transferred into 2 ml Eppendorf tubes and centrifuged for 1 h at 4 °C (21 000 x g). The supernatants were transferred into fresh Eppendorf tubes, stored at -20 °C and used for further experiments.

10x Phosphate Buffered Saline (PBS)

100 mM Na_2HPO_4 x 12 H_2O

1.36 M NaCl

27 mM KCl

18 mM KH_2PO_4

1x PBS = 1:10 dilution

2.2.1.2 Identification of a Bet v -1 homologue in almond kernels by Nano-LC ESI Orbitrap MS/MS

Protein identification was performed by the VetCore Facility for Research (Veterinary University of Vienna, Vienna, Austria) using Nano-LC ESI Orbitrap MS/MS as previously described by Kabasser et al. [75]. Briefly, proteins were digested o/n at 37 °C using Trypsin/LysC Mix (Promega, Madison, WI). Obtained peptides were extracted by three changes of 5% trifluoro acidic acid (TFA) in 50% aqueous acetonitrile. Afterwards, the peptides were ultrasonicated for 10 min per change and dried down in a vacuum concentrator (Eppendorf, Hamburg, Germany). Later, the peptides were dissolved in 0.1% TFA. During mass spectrometry analysis, LC was linked to a high-resolution Q Exactive HF Orbitrap mass spectrometer. Two MS spectra were collected. MS1 spectra in a range 350-20000 m/z for 50 ms and MS2 spectra in the range 200-2000 m/z for 50 ms.

2.2.1.3 Amino acid sequence alignment between Pru du 1.06A and other Bet v 1 homologous proteins

Amino acid sequence alignment between Pru du 1.06A and other Bet v 1 homologous proteins was determined by Clustal Omega. In table 3, all PR-10 allergens used for the alignment were summarized with respective UniProt number.

Table 3. *Summary of Pru du 1.06A, Bet v 1 and other homologous proteins with corresponding UniProt number.*

Species	Allergen name	UniProt number
Betula verrucosa (European white birch)	Bet v 1.0101	P15494
Prunus dulcis (Almond)	Pru du 1.0101	B6CQS9
Prunus persica (Peach)	Pru p 1.0101	Q2I6V8
Malus domestica (Apple)	Mal d 1.0101	P43211
Prunus avium (Sweet cherry)	Pru av 1.0101	O24248
Arachis hypogaea (Peanut)	Ara h 8.0101	Q6VT83
Glycine max (Soybean)	Gly m 4.0101	P26987
Corylus avellana (Hazelnut)	Cor a 1.0101	Q08407
Juglans regia (English walnut)	Jug r 5.0101	A0A1J0RET5

2.2.2 Recombinant expression of Pru du 1.06A in *E.coli*

2.2.2.1 DNA construct

Codon optimization, gene insertion and synthesis of the Pru du 1.06A (UniProt accession number: B6CQS9) DNA sequence into the pET28b(+) vector (Novagen) was carried out by BioCat GmbH. The Pru du 1.06A was fused to a hexa histidine affinity tag (6xHis tag) and further expressed in *E.coli* strain NiCo21 [DE3] (New England Biolabs, Ipswich, MA, USA). As depicted in Figure 3, the pET28b(+) vector contained an aminoglycoside phosphotransferase at 560–1375 bp conferred resistance to kanamycin. Between 5069-5027 bp the Pru du 1.06A-HisC was inserted containing a 18bp long 6xHis tag. The transcription was induced by the T7 RNA polymerase from bacteriophage T7 starting at the T7 promoter sequence. The transcription was terminated after reaching the T7 terminator sequence.

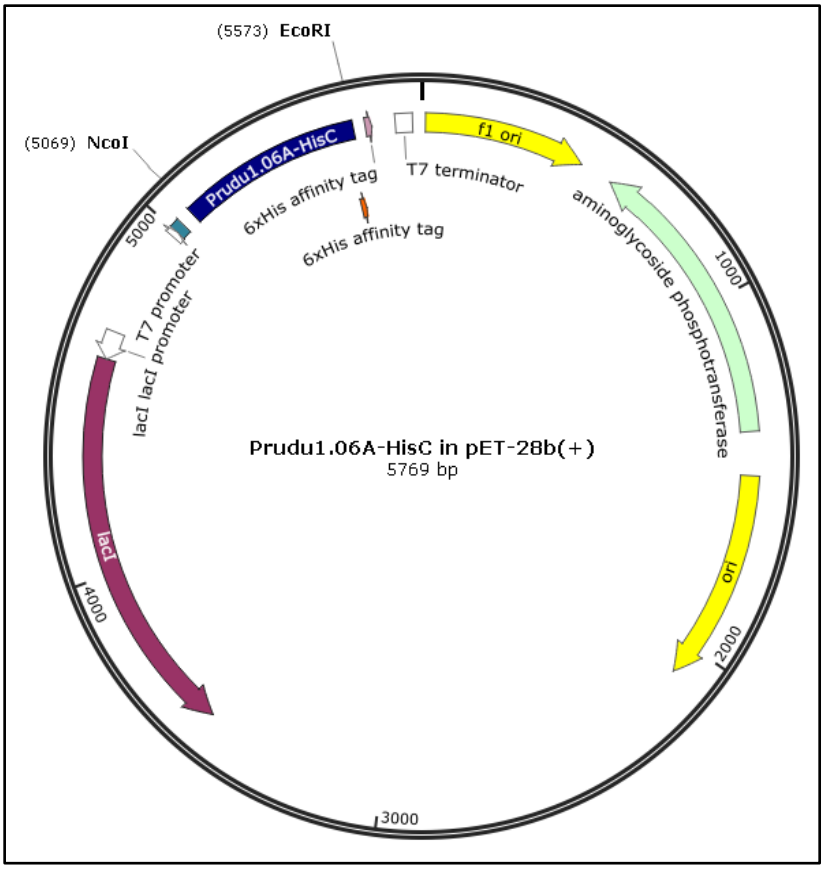

Figure 3: *Pru du 1.06A-HisC pET-28b (+) plasmid map. Genes, promotors, terminator, and relevant restriction sites were shown.*

2.2.2.2 DNA plasmid amplification by E.coli DH5α

In order to obtain high yield of plasmid DNA, chemically competent *E.coli* DH5α cells were transformed as followed. Lyophilized vector DNA provided by the manufacturer was dissolved in nuclease free ddH_2O to a final concentration of 100 ng/µl. Further steps were performed under a laminar flow hood to avoid any possible contaminations. Prior transformation, *E.coli* DH5α cell aliquots were thrown on ice for 10 min. Afterwards, 1 ng of pET28b(+) Pru du 1.06A and empty pET28b(+) vector DNA were added to respective aliquots of *E.coli* DH5α cells followed by flicking the tubes 5 times to ensure mixing the cells with DNA. Subsequently, cells were incubated on ice for 30 min. Uptake of plasmid DNA was induced by heat shock transformation (42 °C for 45 sec) followed by incubation on ice for another 5 min. To induce cell growth as well as to allow the expression of the selection marker, 300 µl of Super Optimal broth with catabolite repression (SOC) medium were added to the samples and cells were incubated at 37 °C for 1 h in a shaking incubator (New Brunswick™ Excella® EL4, Eppendorf AG, Hamburg, Germany). Already prepared pre-warmed Luria Bertani medium (LB medium) agar plates containing kanamycin (25 µg/ml) were used for plating of 100 µl of transformed cells. As a control, cells containing the empty pET28b(+) vector were used, treated and plated in the same way as described above. All plates were incubated o/n at 37 °C. Next day, selected colonies were picked by pipette tips from the agar plates and inoculated into tubes already filled with 5 ml of liquid LB medium supplemented with kanamycin (25 µg/ml). The cultures were incubated o/n at 37 °C under constant shaking. On the following day, 500 µl of culture was mixed with 20% sterile glycerol and stored at -80 °C for reuse.

SOC medium	**LB low-salt medium**
2% Peptone	1% Peptone
0.5% Yeast extract	1.5% Agar (only for agar plates)
10 mM NaCl	0.5% Yeast extract
2.5 mM KCl	0.5% NaCl
10 mM $MgSO_4$	pH 7.5 adjusted with NaOH
10 mM $MgCl_2$	Autoclaved (20 min, 120 °C)
20 mM Glucose monohydrate	
pH 7.0 adjusted with NaOH	
Filtered sterile	

2.2.2.3 Vector DNA extraction

The following day, plasmid DNA was isolated and extracted by PureYield™ Plasmid Miniprep System (Promega Corporation, Fitchburg, WI, USA). All steps were performed according to manufacturers' instructions. After cell lysis, only plasmid DNA remained in a provided DNA binding column eluted by adding 50 µl of nuclease free ddH_2O followed by centrifugation (16 000 x g, RT for 1 min).

2.2.2.4 Determination of DNA concentration

The most commonly used method for nucleic acid concentration estimation is performed by measuring the absorption of ultraviolet light by purines and pyrimidines at 260 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA. If the ratio is significantly lower, it may indicate contamination by proteins that absorb strongly at or near 280 nm [76].

The concentration of isolated DNA samples was performed on a NanoDrop 2000 Spectrophotometer (DeNovix, Wilmington, USA). As a blank value, the absorption of ddH_2O was measured. One microlitre of each DNA sample was pipetted on the pedestal of the device

and absorption was measured at wavelengths 260 nm and 280 nm. The pedestal was cleaned with ddH_2O after usage.

2.2.2.5 Verification of plasmid DNA

After DNA isolation (section 2.2.2.3), plasmid containing the gene coding for rPru du 1.06A together with the empty pET28b(+) vector were verified by restriction digestion analysis. Restriction enzymes EcoRI and NcoI were used. After mixing the components listed below, the sample was incubated at 37 °C. After 2 h, the digestion was terminated by heat inactivation (65 °C; 20 min).

Digestion of pET28b(+) Pru du 1.06A

12 µl DNA (600 ng/µl)
1.5 µl EcoRI
1.5 µl NcoI
2.0 µl Cutsmart buffer (10x)
3.0 µl ddH_2O
---
20 µl

2.2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis is a form of electrophoresis used to separate nucleic acid (DNA or RNA) based to their size within an electric field. Negatively charged DNA/RNA fragments migrate towards a positively charged end of the agarose gel when an electric current is applied. The agarose gel is a three-dimensional matrix containing pores through which DNA can pass. Smaller molecules separate better at higher gel concentrations whereas larger molecules separate better by lower gel concentrations. However, higher gel concentrations require longer run times. At the end, the separated DNA may be viewed under a UV light using a fluorescent dye [77].

After digestion of pET28b(+) Pru du 1.06A as described in section 3.2.2.5, DNA fragments were analyzes by 1.2% agarose gel electrophoresis. Agarose was dissolved in 1x TBE buffer and further cooked in a microwave till a homogenous liquid was obtained. GelRed was added to the solution and poured into a casting frame with a comb. Afterwards, the gel was left on RT to polymerize for ~ 45 min. Ten µl of digested DNA sample and non-digested DNA plasmid were loaded into the gel slots. The first slot was loaded with 2 µl of 1 kb DNA ladder (New England Biolabs, Ipswich, MA, USA). Subsequently, the box was filled with 1x TBE buffer and the electrophoresis was performed for 2 h at 80 V. DNA bands and gel images were visualized and recorded by ChemiDoc Imaging System (BioRad).

Agarose gel	**TRIS/Borat/EDTA (TBE) buffer (5x)**
TBE	445 mM TRIS pure
Agarose	445 mM Boric acid
Gel Red	10 mM Na_2-EDTA
	dissolved in ddH_2O
	1x TBE = 1:5 dilution

2.2.2.7 Transformation of chemically competent E.coli strain NiCo21

The second DNA transformation was performed in chemically competent *E. coli* strain NiCo21 [DE3] (New England Biolabs, Ipswich, MA, USA). Prior transformation, 25 µl *E.coli* NiCo21 cell aliquots were thawn on ice for 10 min. Isolated DNA (section 2.2.2.3) as well as empty pET28b(+) vector were added to aliquots of *E. coli* NiCo21 cells followed by flicking the tubes 5 times to ensure mixing the cells with DNA. Subsequently, cells were incubated on ice for 30 min. Uptake of plasmid DNA was induced by heat shock transformation (42 °C for 45 sec) followed by incubation on ice for another 5 min. To induce cell growth as well as to allow the expression of the selection marker, 300 µl of SOC medium were added to the samples and cells were incubated at 37 °C for 1 h in a shaking incubator. Already prepared pre-warmed LB agar

plates containing kanamycin (25 µg/ml) were used for plating of 50 µl of transformed cells. All plates were incubated o/n at 37 °C. Next day, selected colonies were picked by pipette tips from the agar plates and inoculated into tubes already filled with 5 ml of liquid LB medium supplemented with Kanamycin (25 µg/ml). The cultures were incubated o/n at 37 °C under constant shaking. On the following day, 500 µl of culture was mixed with 20% sterile glycerol and stored at -80 °C for reuse.

2.2.2.8 Expression of rPru du 1.06A

From the o/n bacterial cultures, selected clones (9 clones expressing rPru du 1.06A and 3 control clones/empty DNA vector) were inoculated in 5 ml ZYM-5052 autoinduction culture medium at 30 °C as previously described [78]. The best expressing clone was estimated by 15% SDS PAGE analysis (section 2.2.3.2) and further used for large scale protein expression in 1 L autoinduction medium.

2.2.2.9 Cell lysis and protein extraction

After protein expression, the bacterial o/n cultures were transferred into beakers and further centrifuged (3 000 x g ; at 4 °C for 10 min). The supernatant was discarded and the proteins were isolated by adding 5 ml of lysis buffer (50 mM sodium phosphate, pH 7.7) containing protease inhibitor. To obtain better cell wall disruption, the beakers containing homogenized pellet were further frozen by dipping into liquid nitrogen. After thawing, the same procedure was repeated for two times. Further cell disruption was performed by high-pressure microfluidics (Microfluidics LV1; Microfluidics Corp., Westwood, MA, USA). Firstly, the device was washed three times with ddH_2O followed by three times washing with lysis buffer. Then, cell suspension was injected into the system at 5 ml and pressed through a lysis chamber

at high pressure. This procedure was repeated three times. Afterward, the cell debris was removed by centrifugation (3 000 x g; at 4 °C for 30 min) and further analyzed by SDS-PAGE.

2.2.3 Purification of Pru du 1.06A by fast protein liquid chromatography

FPLC is a routine technique used to identify and purify individual components of a protein mixture with high reproducibility and resolution. The protein mixture gets dissolved in a solvent that is introduced in small volumes (microliters) through a chromatography column by a mobile phase (aqueous buffer solution). The protein components move through the column at different velocities and the separation is achieved by interference of molecules with the stationary phase (solid components) of the column. FPLC is a form of high-performance chromatography using pumps in order to control the speed at which the mobile phase passes the stationary phase [79].

2.2.3.1 Affinity chromatography

Affinity chromatography is an efficient method used to isolate proteins by their affinities for specific molecules such as ligands immobilised on the stationary phase of the column. Non-targeted molecules are removed by applying a washing buffer disrupting their weaker interactions with the stationary phase. Targeted proteins are eluted by applying an elution buffer to release the target protein for collection and further analysis. The elution buffer may be applied by using a step change or gradient [80,81]. Metal ion chelates (such as nickel chelate) are important non-biological binding agents used in immobilised metal ion affinity chromatography (IMAC). The method has been used to isolate histidine-tagged recombinant proteins that have the binding affinity for nickel, cobalt, zink, copper and iron ions [82]. On the other hand, chitin affinity chromatography is a one-step purification method (Figure 4). It allows isolation of proteins of high purity by using intein cleavage in order to produce a protein of native or near-native sequence without the use of proteases to remove the affinity tag [83].

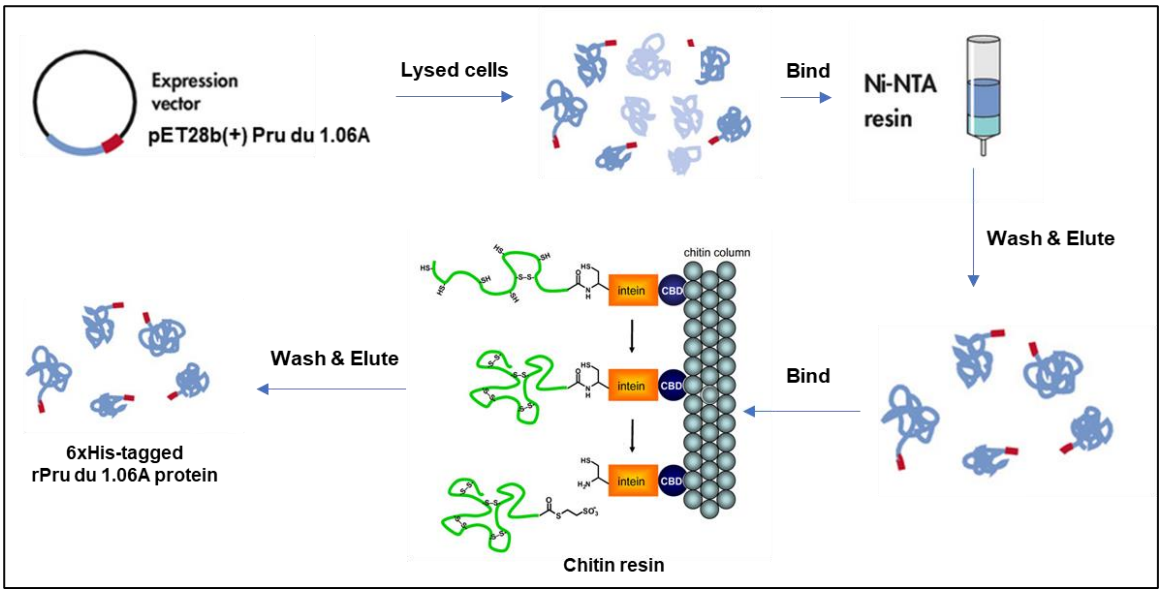

Figure 4: *Illustration of rPru du 1.06A purification. Figure taken and modified from [84,85].*

The protein sample (section 2.2.2.9) was further purified by nickel chelate affinity chromatography and chitin affinity matrix (New England Biolabs, Ipswich, MA, USA). A 2 ml self-packed Ni-NTA Agarose column (nickel-charged affinity resin) was used on a ÄKTA HPLC system (GE Healthcare, Uppsala, Sweden) to purify rPru du 1.06A containing a 6xHis tag. Before loading, the column was washed with 5 column volumes (CVs) of ddH_2O and equilibrated with the same amount of buffer A. Prior loading, the sample was filtered through a 0.2 µm syringe filter (Filtropur S, Nümbrecht, Germany) to remove remaining pellet residues. Subsequently, 60 ml of sample was applied with a flowrate of 0.5 ml/min on the mentioned column. Column-bound proteins were eluted by a step gradient (8%, 16%, 30% and 100% of buffer B) with a flow rate of 1 ml/min. Fractions of 1 ml were collected and further analysed by 15% SDS-PAGE and Coomassie staining. After purification, the column was washed with 5 CVs of ddH_2O and 20% ethanol, respectively. In order to remove imidazole, the purified sample was firstly transferred into 3.5 kDa MWCO Spectra/Por® dialysis membranes (Spectrum Laboratories Inc., Rancho Dominquez, CA) and dialyzed o/n at 4 °C against 50 mM sodium phosphate buffer, pH 7.7 with constant stirring. Next day, the dialyzed protein sample

was additionally purified by chitin affinity matrix using gravity flow to run the column. Two millilitres chitin resin microparticles were added to 10 ml 50 mM sodium phosphate buffer, pH 7.7 followed by centrifugation (3 000 x g at RT for 5 min). The supernatant was discarded, and the same procedure was repeated one more time. The protein sample was loaded on the prepared column and rotated on a wheel for 3 h at 4 °C followed by centrifugation (8 000 x g at RT for 10 min) and analysis on 15% SDS-PAGE.

Buffer A	**Buffer B**
25 mM TRIS/HCl	25 mM TRIS/HCl
0.3 M NaCl	0.3 M NaCl
pH 8.0	0.2 mM Imidazole
	pH 8.0

2.2.3.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining

SDS-PAGE is one of the most widely used laboratory methods to separate proteins according to their molecular weight. Proteins get linearized, denatured, and negatively charged by adding SDS detergent that binds to the polypeptide chains inducing even distribution of the charge per unit mass. Separation of proteins occurs as proteins move with different velocity through the polyacrylamide gel. Polyacrylamide gels are formed by the reaction of bis-acrylamide (N,N'-methylenebisacrylamide) and acrylamide resulting in a highly cross-linked gel matrix. The gel´s pore structure allows smaller proteins to pass faster compared to larger ones. The movement of negatively charged proteins towards a positive electrode is induced by an electric field. A set of molecular weight markers (proteins of predetermined weight) get additionally loaded onto the gel to estimate the unknown molecular weight of proteins [86]. *Separated protein bands can be visualised by Coomassie brilliant blue R-250 (CBB R-250) dye. The dye*

interferes with aromatic and basic amino acid residues by ionic interactions under acidic conditions [87].

For protein analysis, 15% analytical polyacrylamide gels of 1.0 mm thickness were prepared using a gel casting system. The resolving gels were made by mixing Reagent C, lower buffer and ddH_2O. By adding ammonium persulphate solution (APS) and tetramethylethylenediamine (TEMED) into the mixture, the polymerization process was induced. The solution was quickly poured between plates of an assembled casting frame. Subsequently, by adding isopropanol on the surface oxygen was removed, achieving a plain surface. Stacking gel was prepared by mixing reagent C, upper buffer, ddH_2O, APS and TEMED during polymerization of the running gel. After removing isopropanol, the stacking gel was added, and a comb (ten to fifteen slots) was inserted to form wells. After polymerization of stacking gel, the plate was removed from the casting frame, the comb was removed from the gel and the plates were assembled into an electrode assembly, which was put into an electrophoresis chamber. The chamber was filled up to a running module with 1x electrophoresis buffer. Already prepared almond extract and purified allergen were thrown at RT and 2 µg were loaded per slot. Before loading the samples onto the gel, they were mixed by adding 4x sample buffer (SB) into Eppendorf tubes followed by heating at 95 °C for 5 min. Using reducing SB containing dithiothreitol (DTT) the intra- and inter-molecular disulphide bonds were reduced whereby using non-reducing SB, disulphide bonds remained intact. SDS-PAGE was performed using the Mini-PROTEAN Tetra Handcast System (Bio-Rad). Samples and a pre-stained protein ladder, which estimate the protein size, were loaded matching to a defined scheme and run at 120-160 V for about 45 min. Immediately after electrophoresis, the gels were removed from the electrode assembly and put into a petri dish filled with Coomassie Brilliant Blue dye (CBB) for 1 h. The protein bands were visible by removing Coomassie dye and replacing it by destaining solution.

Lower buffer	**Upper buffer**	**10x Electrophoresis buffer**
1.5 M TRIS/HCl	0.5 M TRIS/HCl	250 mM TRIS pure
0.4% SDS	0.4% SDS	192 mM Glycine
dissolved in ddH_2O	dissolved in ddH_2O	1% SDS
pH 8.8	pH 6.8	dissolved in ddH_2O
		1x Electrophoresis buffer: 1:10 dilution

Resolving gel (15%)	**Stacking gel (4.5%)**	**4x Sample buffer (reducing)**
2.5 ml Reagent C	300 µl Reagent C	200 mM TRIS/HCl
1.25 ml Lower Buffer	500 µl Upper Buffer	300 mM DTT
1.25 ml ddH_2O	1.20 ml ddH_2O	4% SDS
2.5 µl TEMED	1 µl TEMED	40% Glycerol
25 µl APS (10%)	20 µl APS (10%)	0.025% Bromophenol blue
		dissolved in ddH_2O
		pH 6.8

Coomassie Brilliant Blue staining solution	**Coomassie Brilliant Blue destaining solution**
0.125% CBB R-250	20% Methanol
50% Methanol	15% Acetic acid
10% Acetic acid	ddH_2O added
ddH_2O added	

2.2.3.3 Determination of protein concentration

NanoDrop technology is an innovative sample retention system that utilises the surface tension to measure microvolume samples between two optical pedestals without the use of capillaries or cuvettes. The microvolume spectrophotometer is ideal for circumstances whereupon sample is limited. The sample is positioned on top of the detection surface creating a liquid column. A

flash lamp provides a light source and a linear CCD array utilised by a spectrometer is used for analysis of light passing through the sample. The NanoDrop determines the optimal path length automatically (1 mm to 0.05 mm) providing the most broad range of protein concentration measurements without dilutions [88].

The bicinchoninic acid (BCA) protein assay is used for protein quantification by colorimetric detection. In this two-component assay, a reduction of Cu^{2+} to Cu^{+} (biuret reaction) occurs in an alkaline medium. Further, cuprous cations get detected by bicinchoninic acid. Single amino acids and dipeptides are unaffected, but tripeptides and polypeptides will generate a blue-to-violet complex. The complex may be measured at a wavelength between 550 and 570 nm and the absorption correlates linearly with amount of proteins [89].

The obtained rPru du 1.06A was concentrated by Amicon® Ultra-15 centrifugal units (Merck Millipore LTD., Tullagreen, Ireland) according to the manufacturer's instructions with a cut-off of 3 kDa. Subsequently, the protein concentration was measured by NanoDrop 2000 Spectrophotometer (DeNovix, Wilmington, USA) at 280 nm. To obtain a more precise protein concentration, the procedure was repeated by using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Different concentrations of sample as well as standard serial dilutions (0, 25, 50, 100, 150, 200 and 250 µg/ml) of bovine serum albumin (BSA) were prepared according to manufacturer's instructions. Samples and standards were applied in duplicates (25 µl/well) into a 96-well microplate (Greiner Bio-One GmbH, Germany). For the colour reaction, 200 µl of working reagent (50:1 (v/v) of reagent A to reagent B) were added to each well. Finally, the plate was incubated at 37 °C for 30 min and the absorption was measured at 562 nm on a Tecan SPARK microplate reader (Tecan Group, Männedorf, Switzerland).

2.2.3.4 Mass determination and protein identification by mass spectrometry

Mass spectrometry (MS) is a sensitive analytical method used for identification, detection and quantification of molecules based on their mass-to-charge (m/z) ratio. The molecules get further ionised by an ion source and fragmented into positively charged ions. The ions encounter a magnetic and/or electric field from mass analyser, which diverts the paths of the ions based on their m/z towards the ion detector. The mass spectrometers are connected to computer-based software platforms that can measure ion oscillation frequencies and obtain mass spectra by image current detection. The ions can further be identified by established databases that can predict the character of the molecule based on the m/z value [90].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry contains the soft ionisation source MALDI and the TOF mass analyser. It is appropriate for mass analysis of peptides, lipids, saccharides, or other organic macromolecules. Before analysis, samples get mixed and co-crystalized with a matrix. Furthermore, the mixture gets spotted and dried onto a metal target plate for further analysis. Subsequently, the plate gets loaded on the MALDI-TOF instrument. After the ionisation process, the sample and matrix acquire a single positive charge and get further separated depending on the m/z ratio by TOF analyser (Figure 5) [91].

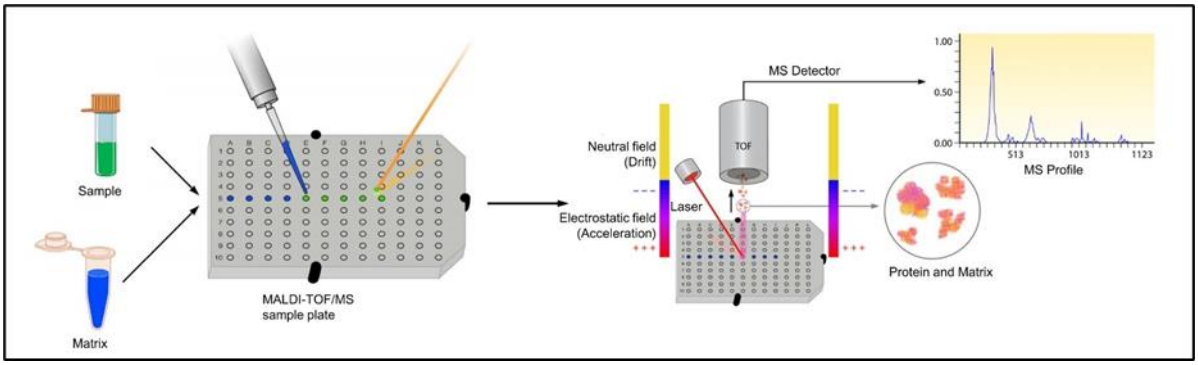

Figure 5: *Process of MALDI-TOF mass spectrometry. Figure was taken from Clark A. E. et al.[92].*

2.2.3.4.1 Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS)

Using Matrix Assisted Laser Desorption Ionization – Time of Flight mass Spectrometry (MALDI TOF-MS) the molecular weight (Mw) of rPru du 1.06A was determined. The sample was concentrated and desalted by ZipTip® Pipette Tips (Merck Millipore). Subsequently, 0.5 µl of desalted protein was mixed with an equal amount of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) and spotted onto a MALDI-target metal plate. Further analysis and measurements were conducted on a MALDI-TOF mass spectrometer (Microflex, Buker, Daltonics, Bremen, Germany) in linear mode.

2.2.3.5 Circular Dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy for fast determination of the secondary structure, folding and binding properties of proteins. It measures the difference in absorbance of right-handed and left-handed circularly polarised light when passing through a substance. Different secondary structures can be analysed between 260 and 180 nm. The α-helical structures of proteins are characterised by two minima at 222 nm and 208 nm (negative band) and one maximum (positive band) at 193 nm. Proteins with β-sheet structure possess a minimum at 218 nm and maximum at 195 [93,94].

For secondary structure determination of rPru du 1.06A, CD spectroscopy was performed in the range of 190-260 nm wavelengths on a Jasco J-810 spectropolarimeter (Jasco International Co., Tokyo, Japan) at RT. The sample (0.2 mg/ml in 50 mM sodium phosphate buffer) was transferred to a 1 mm pathlength quartz cuvette. To access the effect of heating on the proteins´ secondary structure, further measurements at RT in intervals of 10 °C up to 95 °C (25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C and 95 °C) were performed. The spectra represent the

average of three accumulations recorded at 50 nm/min with a 2 sec time constant, 0.5 nm resolution and sensitivity ±100 mdeg.

2.2.3.6 Dynamic light scattering (DLS)

DLS analyses is a technique used to detect aggregates in solutions, to determine protein size and "monitor" ligand binding. A monochromatic light source (laser) is shot through a sample. The method is based on Brownian motion of dispersed particles where particles are constantly colliding with solvent molecules. These clashes cause a certain amount of energy to be transferred inducing particle movement. Smaller molecules move faster than the large ones. If particles are present in the sample, laser light gets scattered in all directions and can be further detected at a certain angle. The intensity of the scattered light fluctuates over time "due to" constantly changing distances between particles. Those fluctuations get recorded and contain information about the time scale of the movement of the scatterers [95].

The hydrodynamic radius distribution of the sample was measured by DLS. Prior measurement, the sample was centrifuged for 10 min at max. speed (21 000 x g). Afterwards, the sample was transferred into a quartz cell and placed in the tube holder inside the DSL analyser DynaPro NanoStar (Wyatt Technology, Santa Barbara, Calif).

2.2.3.7 In silico analysis

In silico experiments are performed by computer simulation. Using specific computational programs such as UCSF Chimera molecular structures of proteins can be visualized. These include density maps, trajectories, and sequence alignment.

The 3D structure of rPru du 1.06A and rBet v 1.0101 was respresented by UCSF Chimera. The PDB for rBet v 1 was obtained directly fromfrom RCSB PDB Protein Data Bank (https://www.rcsb.org/) [96]. On the other hand, for 3D representation of rPru du 1.06A the PDB was acquired from using homology-modelling server SWISS-MODEL (https://swissmodel.expasy.org/) [97].

2.2.4 Immunological characterization

2.2.4.1 Patient´s sera

The immunological characterization of purified rPru du 1.06A was performed by IgE ELISA, immunoblotting and mediator release assay. The techniques were performed by using 17 sera from subjects with almond allergy (Table 6), 6 sera from subjects without clinically manifested almond allergy but sensitized to almonds, and 3 control sera from non-allergic donors' named as normal human serum (NHS). Almond allergy was diagnosed based on case history and positive skin prick test (≥ 3 mm wheal diameter) to almond or positive almond ImmunoCAP analysis (sIgE ≥ 0.35 kU/L), or a positive open provocation test result. The study obtained ethical approval from the Ethics Committee of the Medical University of Vienna and written consent was given by all patients.

Immunoblotting (western blotting) is a fast and sensitive assay for detection of specific antigen-antibody interaction. Electrophoretic separated proteins are further transferred (also known as "blotted") by irreversible binding to a membrane (nitrocellulose or polyvinylidene difluoride (PVDF) membrane). The membrane is coated with a primary antibody that binds to target proteins. Further, a fluorophore, radioisotope or enzyme tagged secondary antibody is applied to detect the primary antibody. Depending on the type of secondary antibody, a substrate is added for the final detection by catalysis of colour or light reaction [98].

Recombinant Pru du 1 and rBet v 1 were subjected to reducing 15% SDS-PAGE and blotted to a 0.2 µm nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Whatman paper, membrane and gel were equilibrated for 5 min in 1x transfer buffer in a clean petri dish. The western blot sandwich was assembled as followed: two pieces Whatman paper, nitrocellulose membrane, gel, two pieces Whatman paper. Blotting was performed for 45 min at 15 V using a Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell (Bio-Rad). The transfer was checked by staining the membrane with Ponceau S for 10 min at RT. The membrane was air dried, cut into several stripes and transferred into blot-boxes. Afterwards, the membrane stripes were shortly washed with TBS containing 0.05% Tween 20 (TBS-T) and then blocked with TBS-T containing 3% non-fat dry milk powder for 2 h at RT to prevent unspecific binding. After three washing steps with TBS-T (10 min each), the individual stripes were incubated o/n at 4 °C with selected pooled sera of almond allergic patients (diluted 1:20) in TBS-T 1% milk powder. Alternatively, the monoclonal anti-Bet v 1-antibodies BV16 (diluted 1:1 000; Absolute Antibody Ltd, Redcar, UK) and BIP1 (diluted 1:10) were used for o/n incubation at 4 °C. Afterwards, unbound antibodies were removed by washing the membrane stripes with TBS-T as done before. The secondary antibody, a 1:5 000 in TBS-T (1% BSA) HRP conjugated goat anti-human IgE (KPL, Gaithersburg, MD, USA) or a 1:20 000 diluted HRP conjugated anti-mouse IgG antibody (KPL, Gaithersburg, MD, USA) were applied to the stripes. After an incubation time of 2 h, at RT, unbound antibodies were removed by washing 3 times with TBS-T. Bound antibodies were detected by incubating the membranes with Clarity max TM Western ECL Substrate (Biorad, Hercules, CA, USA) according to the manufacturers' instructions.

For IgE inhibition immunoblotting, the serum pool was pre-incubated (2 h, at RT) with 50 µg/ml of rPru du 1, rBet v 1 or nPru du 6 before adding to the membranes. The detection of bound antibodies was performed as described above. rBet v 1 and nPru du 6 were kindly

provided by Stefanie Schmalz and Stefan Kabasser (Institute of Pathophysiology und Allergy research, Medical University of Vienna, Austria).

10x TRIS-buffered Saline (TBS)	**10x Transfer buffer**	**1x Transferbuffer (2 L)**
0.05 M TRIS/HCl	0.25 M TRIS pure	200 ml Transfer buffer (10x)
1.5 M NaCl	1.92 M Glycine	400 ml Methanol
0.5% NaN_3	20% Methanol	ddH_2O added up to 2 L
dissolved in ddH_2O	dissolved in ddH_2O	
pH 7.4		
1x TBS = 1:10 dilution		

Ponceau S staining solution
0.1% Ponceau S
5.0% Acetic acid

2.2.4.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique applied for quantification and detection of soluble molecules such as peptides, proteins, antibodies, and hormones. Today, there are four different ELISA formats: direct, indirect, sandwich and competitive ELISA (Figure 6). In indirect ELISA, the solid surface of the multi-well plate is coated with an antigen (e.g., an allergen). Subsequently, a test serum is added. If specific IgE antibodies are present in the serum, they will bind to the allergen. To detect the specific binding, an enzyme labelled secondary antibody (e.g., alkaline phosphatase) against human-IgE is added. In order to visualize the interaction, a substrate is added inducing a catalytic and enzymatic reaction leading to a yellow colour changed product. The colour can be quantitated in a spectrophotometer and is a measure of the level of antigen-antibody-complexes at 405 [99,100].

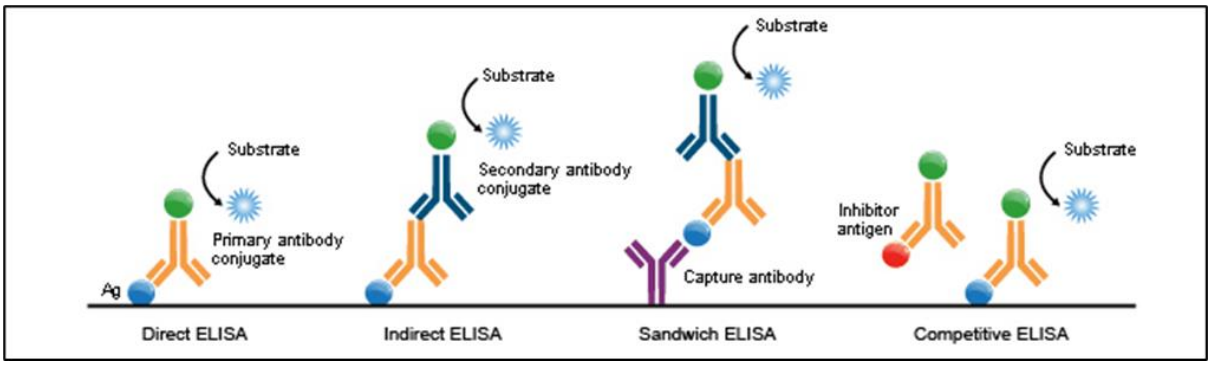

Figure 6: *Four different ELISA types. Figure taken from [101].*

2.2.4.2.1 Indirect IgE ELISA

To analyse IgE-binding activity to rPru du 1.06A, indirect IgE ELISA was performed using Nunc MaxiSorp flat-bottom 96 well immunoplates (Thermo Fisher Scientific, Roskilde, Denmark). Each well on microtiter plates were coated with 100 µl of protein, diluted in coating buffer to a final concentration of 2 µg/ml of purified allergen and 10 µg/ml of almond extract. The plates were incubated o/n at 4 °C to allow adherence of the protein to the solid plate. Next day, sera from almond allergic patients and NHS were diluted in a ratio between 1:3 to 1:10 using dilution buffer. In order to remove unbound molecules, the plate was washed 3 times with TBS-T. Non-specific binding of antibodies was avoided by blocking of coated proteins with blocking buffer at RT (200 µl/well) for 2 h. Afterwards, the plates were washed three times with TBS-T. Diluted patients' sera and NHS sera were added to respective wells in duplicates (100 µL/well) and the plates were incubated o/n at 4 °C. On the following day, sera were removed and the plates were washed as described above. In order to detect IgE binding, each well was incubated with 100 µl of a 1:1000 diluted secondary antibody (alkaline phosphate-conjugated Mouse Anti-Human IgE) for 2 h at RT. Wells were washed three times with TBS-T to remove unbound antibodies. As a final step, an alkaline phosphate conjugated substrate (SIGMAFAST™ p-Nitrophenyl phosphate tablets, dissolved in ddH_2O) was added (100 µL/well) and absorption of light at 405 nm was measured on a Tecan SPARK microplate reader (Tecan Group, Männedorf, Switzerland). Optical density (OD) values were counted as positive

if the mean of tested sera was higher than the OD value of negative control plus three times the standard deviation.

2.2.4.2.2 Dose-dependent inhibition IgE ELISA

In allergy research, inhibition IgE-ELISA is used for cross-reactivity determination between allergens. Proteins sharing less than 50% sequence identity are rarely cross-reactive [102]. Allergen cross-reactivity occurs when IgE antibodies, originally raised against a specific allergen, bind to an identical or similar surface area of another related allergen. As previously described, ELISA plates get coated with allergens or protein extracts. Before patient´s sera get added onto the ELISA plate, they get pre-incubated with other protein extracts or allergens acting like inhibitors in a dose-dependent way. The human IgE antibody absorbs the added inhibitor allergen, and the antibody cannot bind to the previously coated allergen. Decreased IgE binding to original target allergen can be determined by light absorption measurement as described above.

Cross reactivity between rPru du 1.06A and rBet v 1.0101 was assessed in a dose-dependent manner by inhibition IgE ELISA. Additionally, following Bet v 1-homologue allergens were tested: peanut (Ara h 8), soyabean (Gly m 4), apple (Mal d 1), peach (Pru p 1.0101) and hazelnut (Cor a 1). Microtiter plates were coated with aforementioned allergens. Representative sera from four almond allergic patients were diluted in a ratio between 1:20 to 1:50 and pre-incubated for 2 h at RT with rBet v 1.01 (0.0001-10 µg/ml) or rPru du 1 (0.001-100 µg/ml). Two NHS sera served as negative controls. Further steps were carried out as described for indirect IgE ELISA (section 2.2.4.2.1). Absorption was measured at 405 nm and the degree of inhibition was calculated as the percentage reduction of IgE binding compared to controls, where no inhibitor protein has been added.

Coating buffer	**Blocking buffer**	**Dilution buffer**
25 mM $NaHCO_3$	3% BSA	0.5% BSA
pH 9.6 adjusted with NaOH	dissolved in TBS-T	dissolved in TBS-T

2.2.4.3 Rat basophil leukemia (RBL) cell-based immunoassay

Humanized RBL luciferase reporter system is used for detection of allergen specific IgE. IgE antibodies get produced during type 1 hypersensitivity and bind to high-affinity receptors (FcεRI) on the surface of mast cells and basophils. Consequently, sensitized mast cells and basophils undergo degranulation with release of a range of mediators (e.g., histamine, cytokines, and others) inducing an effective allergic reaction. This assay relies on the use of donor serum/plasma. Reporter cell lines are incubated o/n with high dilutions of sera and on the next day, the respective cells get stimulated and tested with the suspected allergen. In the presence of specific IgE, FcεRI crosslinking by the allergens will result in activating a signal transduction cascade. As a result, the nuclear factor of activated T cells (NFAT) undergoes translocation and binds to specific promoters. This induces activation of the reporter gene and production of luciferase leading to possible detection even after 3 h after activation (Figure 7)

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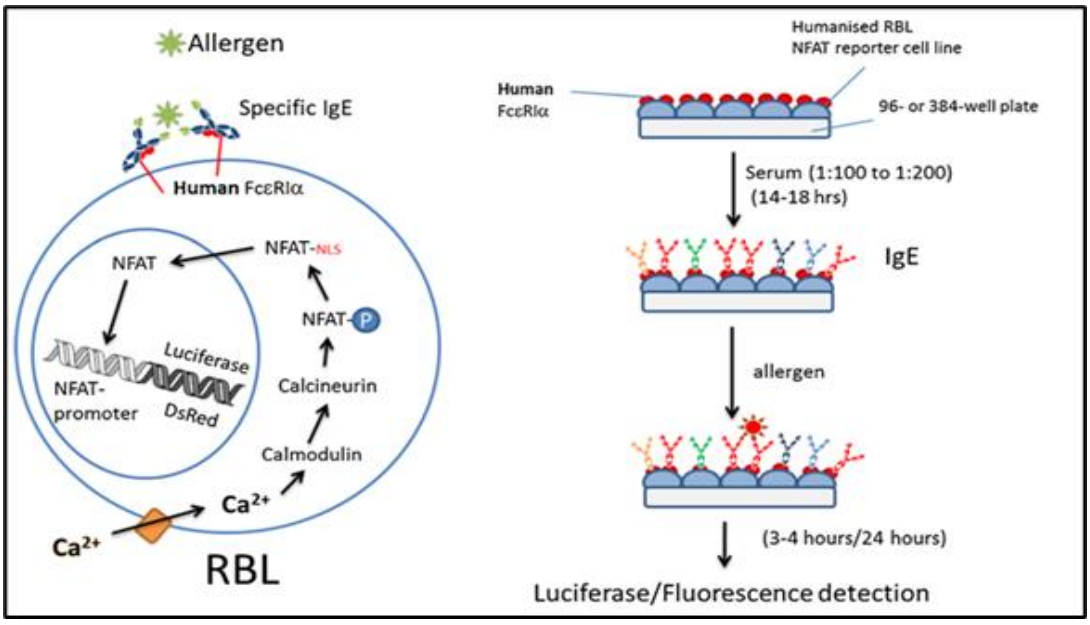

Figure 7: *Principle of the humanized RBL reporter system. Figure taken from [103].*

To evaluate the biological potency of rPru du 1.06A the mediator release assay was performed using RBL mast cells, RS-ATL8 expressing α, β and γ-chains of the human high-affinity IgE receptor (FcεRI). RBL assay was carried out as described by Nakamura et al [99]. Cells were cultured in RBL assay medium. After reaching the stationary growth phase, the cells were harvested by a cell scraper in 10 ml Dulbecco's phosphate-buffered saline (DPBS). Subsequently, the cells were counted by a Bürker-Türk hemocytometer and the cell number was adjusted to 5×10^4 cells/well. Afterwards, RS-ATL8 cells were seeded into a sterile 96 well microtiter plates (Opti-Plate, Perkin Elmer) and further incubated for 3h at 37 °C in a 5% CO_2 incubator. Later, 50 µl of diluted sera (final dilution 1:10-1:50) in RBL assay medium was added to the supernatant. After overnight incubation, the cells were washed once with sterile DPBS and then stimulated by the addition of rPru du 1.06A (0.1-10 000 ng/ml) or rBet v 1.0101 (0.01-1000 ng/ml) allergens for 3 h at 37 °C in a 5% in CO_2 incubator. After stimulation, 50 µl of luciferase substrate solution containing cell lysis reagent (ONE-Glo, Promega Corp., Tokyo, Japan) was added to the cells, and chemiluminescence was measured using a Tecan SPARK microplate reader (Tecan Group, Männedorf, Switzerland). As positive control, anti-human IgE

(1:100) was used. Luciferase expression levels were represented as a fold increase of light units compared with the background expression, after subtraction of a blank control (without cells).

RBL assay medium

Minimal Essential Medium (MEM)

10% heat-inactivated fetal calf serum (FCS)

2 mM glutamax

1% penicilin-streptomycin

1 mg/ml geneticin

0.2 mg/ml hygromycin B

3 Results

3.1 Detection of Pru du 1.06A in almond kernels

Almond proteins were extracted in PBS containing protease inhibitors to prevent protein degradation by proteases. Prepared almond protein extract was blotted and analysed using two different anti Bet v 1 monoclonal antibodies (BV16 and BIP1) to detect potential Bet v 1 homologous almond protein. Results indicated unspecific binding by anti-BIP1 antibody where bands at ~ 70-95 kDa were detected whereas anti-BV16 antibody did not detect any bands (data not showed). This suggested lower abundance of the Bet v 1-related protein in prepared almond extract. Subsequently, the almond extract was analysed by tandem mass spectrometry to identify peptides of potential eight Bet v 1-homologous proteins previously identified by genomic analysis of putative allergen genes in almond [69]. As depicted in Figure 8, the isoform Pru du 1.06A was identified based on three matching peptides (marked in red).

```
Pru du 1.01 :  MGVFTYESEFTSEIPPPRLFKAFVLDADNLVPKIAPQAIKHSEILEGDGGPGTIKKITFG 60
Pru du 1.02 :  MGVFTYETEFTSVIPPEKLFKAFILDADNLIPKIAPTAVKGTEILEGDGGVGTIKKVTFG 60
Pru du 1.03 :  MGVFTYETEFTSVIPPARLFKAFILDADTLIPKIAPSAVKGAEIVEGDGGVGTIKKITFG 60
Pru du 1.04 :  MGVFTYETEINSVIPPARLFNAFVLDADNLIPKIAPQAVKSTEILEGDGGVGTIKKINFG 60
Pru du 1.05 :  MGVFTYETEFTSVVPPARLFKALVLDADNLVPKIAPQAIKSAEIVEGDGGVGTIKKTSFG 60
Pru du 1.06A:  MGIFTYTDESTSVIPPPRLFKALVLEADTLIPKIAPQSVKSAEIVEGDGGVGTIKKISFG 60
Pru du 1.06B:  MGVFTYSDESTSVIPPPRLFKALVLEADTLIPKIAPQSVKSAEIVEGDGGVGTIKKISFG 60
               **:***  * .* :** :**:*::*:**.*:***** ::* :**:***** ***** .**

Pru du 1.01 :  EGSQYGYVKHKIDSIDKENHSYSYTLTEGDALGDNLEKISYETKLVASPSGGSIIKSTSH 120
Pru du 1.02 :  EGSQYGYVKHRIDGIDKDNLTYSYTLIEGDVLSDVIEKIAYDIKLLASPNGGSIVKTTSH 120
Pru du 1.03 :  EGSQYGFVKHRVDGIDKDNFSYSYTLIEGDALSDVIEKIAYETKLVASPNGGSIIKTTSH 120
Pru du 1.04 :  EGSTYSYVKHRIDGIDKENFVYKYSVIEGDAISETIEKISYETKLVASGS-GSVVKSTSH 119
Pru du 1.05 :  EGSEYGYVKHQVDALDKDNFVYNYSLIEGDALSDKIEKISYEIKLVASADGGSVIKNTSS 120
Pru du 1.06A:  EGSHYSYVKHQIDGLDKDNFVYNYSLVEGDALSDKVEKISYEIKLVASADGGSVIKSTSN 120
Pru du 1.06B:  EGSHYSYVKHRIDGLDKDNFVYNYSLVEGDALSDKVEKITYEIKLVASADGGSIIKSTSN 120
               *** *.:***::*.:**:*  *.*:: ***.:.: :***:*: **:** . **::*.**

Pru du 1.01 :  YHTKGDVEIKEEHVKAGKEKASNLFKLIETYLKGHPDAYN 160
Pru du 1.02 :  YHTKGDVEIKEEQVKAGKEKAAGLFKLVEGYLLANPDAYN 160
Pru du 1.03 :  YHAKGDVVIKEEQVKAGKAKASGLFKLVEDYLTANPDAYN 160
Pru du 1.04 :  YHTKGDFEIKEEHVKAGKEKASHLFKLIENYLLEHHDAYN 159
Pru du 1.05 :  YHTKGDVEIKEENVKAGKEKAHALFKIIETYLVANPDAYN 160
Pru du 1.06A:  YHTKGDVEIKEEDVKAGKEKATGLFKLIENYLVANPDAYN 160
Pru du 1.06B:  YHTKGDVEIKEEDVKAGKEKATGLFKLIENYLVANPDAYN 160
               **:***. ****.***** **  ***::* **  : ****
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Figure 8: *Amino acid sequence alignment of different Pru du 1 isoforms identified in almond kernels [69]. Matching peptides of Pru du 1.06A identified by tandem mass spectrometry of almond protein extract are underlined in red.*

3.1.1 Amino acid sequence comparison of Pru du1.06A with Bet v 1 and other allergenic Bet v 1 homologous proteins

In Figure 9, amino acid sequence alignment of Bet v 1, Pru du 1.06A and seven other allergenic Bet v 1 homologous proteins is shown. All listed proteins contained a conserved P-loop region (AA 47-55) underlined and indicated in bold. Based on the percentage of amino acid sequence identity, data showed a higher degree of identity between botanically related Pru du 1.06A to Pru p 1 (by 73.1%), Pru a v 1 (72.5%) and Mal d 1 (67.3%). To birch pollen allergen Bet v 1 Pru du 1.06A showed 55.3 % amino acid sequence identity. Amino acid sequence identities are summarized in Table 4.

Figure 9: *Amino acid sequence comparisons. Amino acid sequence alignment of Pru du 1.06A with major birch allergen Bet v 1.0101 and other PR-10 related proteins. The P-loop region (AA 47-55) is underlined and indicated in bold.*

Table 4: *Amino acid sequence identities (AA-id, %) of Pru du 1.06A with major birch allergen Bet v 1.0101 and PR-10 related proteins from peach (Pru p 1), apple (Mal d 1), cherry Pru av 1), peanut (Ara h 8), soybean (Gly m 4), walnut (Jug r 5) and hazelnut (Cor a 1).*

	Bet v 1.0101	Pru p 1.0101	Mal d 1.0101	Pru av 1.0101	Ara h 8.0101	Gly m 4.0101	Jug r 5.0101	Cor a 1.0101
Pru du 1.06A	55.3%	73.1%	67.3%	72.5%	48.1%	51.9%	70.6%	52.2%
Bet v 1.0101		58.1%	55.0%	58.1%	45.0%	46.9%	65.6%	72.2%
Pru p 1.0101			81.2%	98.1%	49.4%	54.4%	74.4%	57.5%
Mal d 1.0101				83.1%	46.9%	52.5%	72.5%	54.4%
Pru av 1 1.0101					50.0%	54.4%	73.8%	56.9%
Ara h 8 8.0101						70.4%	52.5%	63.8%
Gly m 4 4.0101							55.0%	46.9%
Jug r 5.0101								63.8%

3.1.2 Expression of recombinant Pru du 1.06A in *E.coli*

3.1.2.1 Transformation of E.coli DH5alpha and DNA verification

Due to low abundancy of Pru du 1.06A in almond extract, a synthetic *Pru du 1.06A* gene containing a 6x His-tag with a codon optimized for expression in *E.coli* was ordered. As already described in section 2.2.2, plasmid pET28b(+) containing *Pru du 1.06A* gene was used for recombinant expression. First transformation was performed in chemically competent *E.coli* DH5alpha cells to obtain higher DNA yield. Plasmid and empty vector (negative control) were plated on two different agar plates containing kanamycin as selection marker. Next day, white and round colonies were formed and further used for DNA extraction (section 2.2.2.3). Total amount of DNA derived from 5 ml LB/kanamycin medium was estimated and summarized in Table 5.

Table 5: *DNA quantification by NanoDrop 2000 Spectrophotometer.*

Name	Concentration
pET28b (+) / empty vector	49.47 ng/µl
pET28b (+) Pru du 1.06A	20.16 ng/µl

To determine the proper insertion of Pru du 1.06A into DNA construct, an enzymatic digestion with restriction enzymes NcoI and EcoRI was performed. NcoI cuts at restriction enzyme recognition site C↓CATGG where EcoRI cuts the G↓AATTC. After termination of the digestion reaction, digested and undigested DNA were loaded on a 1.2% agarose gel. As showed in Figure 10, digested plasmid yielded two DNA fragments where degested vector is visiable at 5267 pb and the insert of interest was visible at 502 bp. Empty plasmid was visible only as a vector band of 5267 pb.

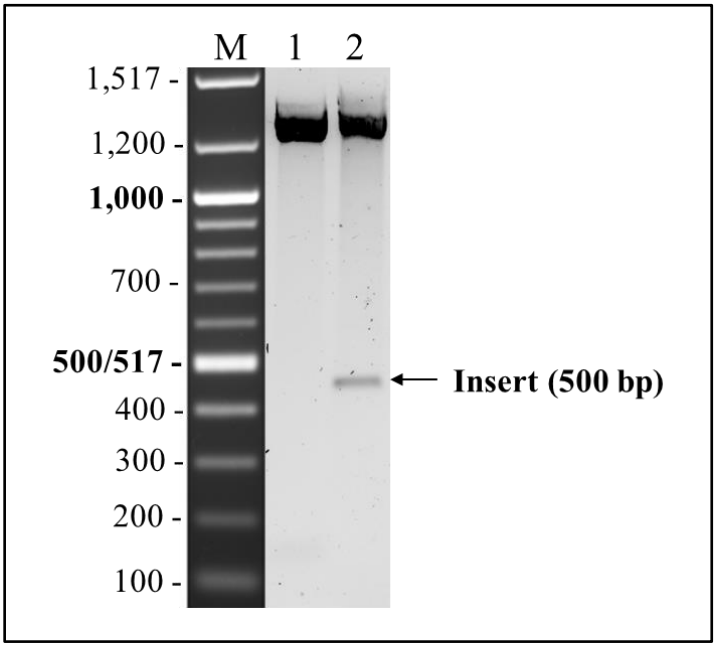
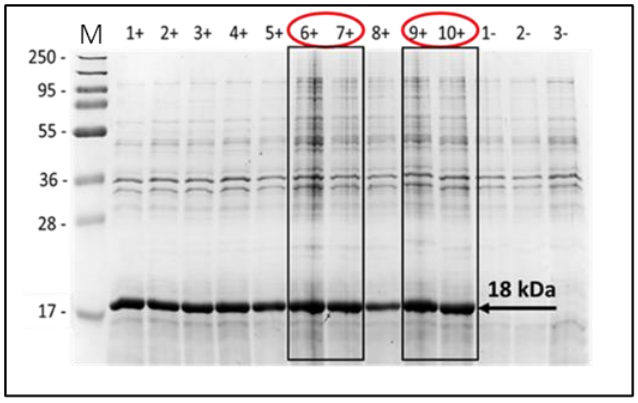


Figure 10: *Restriction analysis of E.coli DH5alpha expression vector by agarose gel electrophoresis (1.2%). Digested pET28b(+) Pru du 1.06A was represented in lane 2 where digested empty vector DNA in lane 1. DNA ladder is represented in lane M.*

3.1.2.2 Transformation of E.coli NiCo 21 and large scale expression

To induce Pru du 1.06A expression, a second transformation was performed into chemically competent *E.coli* NiCo 21 cells. On next day, ten clones (1-10) and three clones containing empty vector (negative control) were picked and further inoculated into tubes filled with 5 ml LB medium supplemented with kanamycin to select best expressing colonies. After overnight expression, cultures were centrifugated and the pellets were lysated using 4X SDS sample buffer. Subsequently, the samples were analysed on a 15% SDS PAGE following Commassie staining (Figure 11A). All colonies contained a notable protein band at ~ 18 kDa corresponding to the theoretical molecular mass of Pru du 1.06A. Other visible bands between 28–95 kDa indicated possible *E.coli* proteins. To test the solubility of the recombinant protein, clones marked in red (6, 7, 9 and 10) were further lysed by lysis buffer and high-pressure microfluidics. Commassie staining of the samples analysed by SDS-PAGE showed that all four clones contained a band at ~ 18 kDa. As depicted in Figure 11B, clone 7 was further used for purification, physicochemical and immunological characterization.

A)



B)

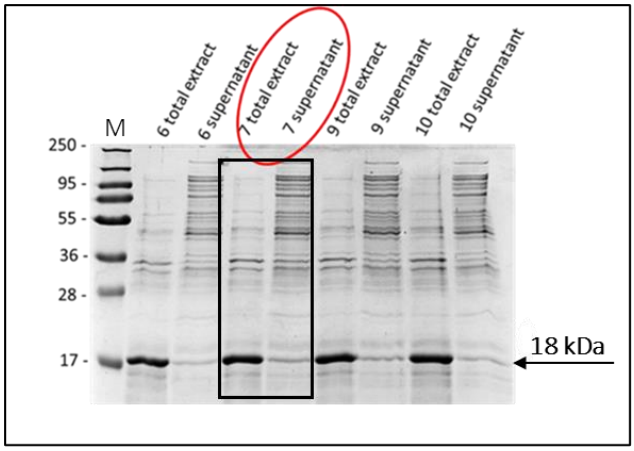


Figure 11: *Coomassie stained 15% SDS-PAGE gels under reducing conditions. A) Best expressing clones (1-10) and negative controls (1-3) are listed. Lanes marked with M showed the molecular weight protein marker. Lanes marked with numbers corresponded to best expressing clones. Clones marked in red (6+, 7+, 9+ and 10+) were further used for cell lysis. B) Clone +7 was selected for large scale expression.*

3.1.3 Purification of rPru du 1.06A by nickel chelate affinity chromatography

As described in section 2.2.3, recombinant Pru du 1.06 was purified by two purification steps. Firstly, the 6xHis tag of Pru du 1.06A was immobilized with high-affinity to a Ni-NTA agarose column containing nickel ions. The tight association between the His tag and the resin enabled contaminants to be washed away easily. Total protein extract was loaded onto the column by 100% of buffer A where elution was performed by a step gradient (8%, 16%, 30% and 100%) of buffer B. As depicted in Figure 12, two major peaks were obtained by elution with 8% and 16% of buffer B. Collected fractions were subsequently pooled and named as pool 1 (fractions

4-12) and pool 2 (fractions 13-21). In order to determine the purity of pooled samples, a 15% SDS-PAGE analysis was carried out under reducing and non-reducing conditions (Figure 13A). Both pools showed the desired band at 18 kDa where higher purity was obtained in pool 2. Nevertheless, both pools showed remaining contamination and impurities between 28–95 kDa. for higher purity of the protein, the second purification step was achieved by chitin affinity matrix using gravity flow. Both pools were again analysed by 15% SDS PAGE where improved purity was obtained in both pools (Figure 13B).

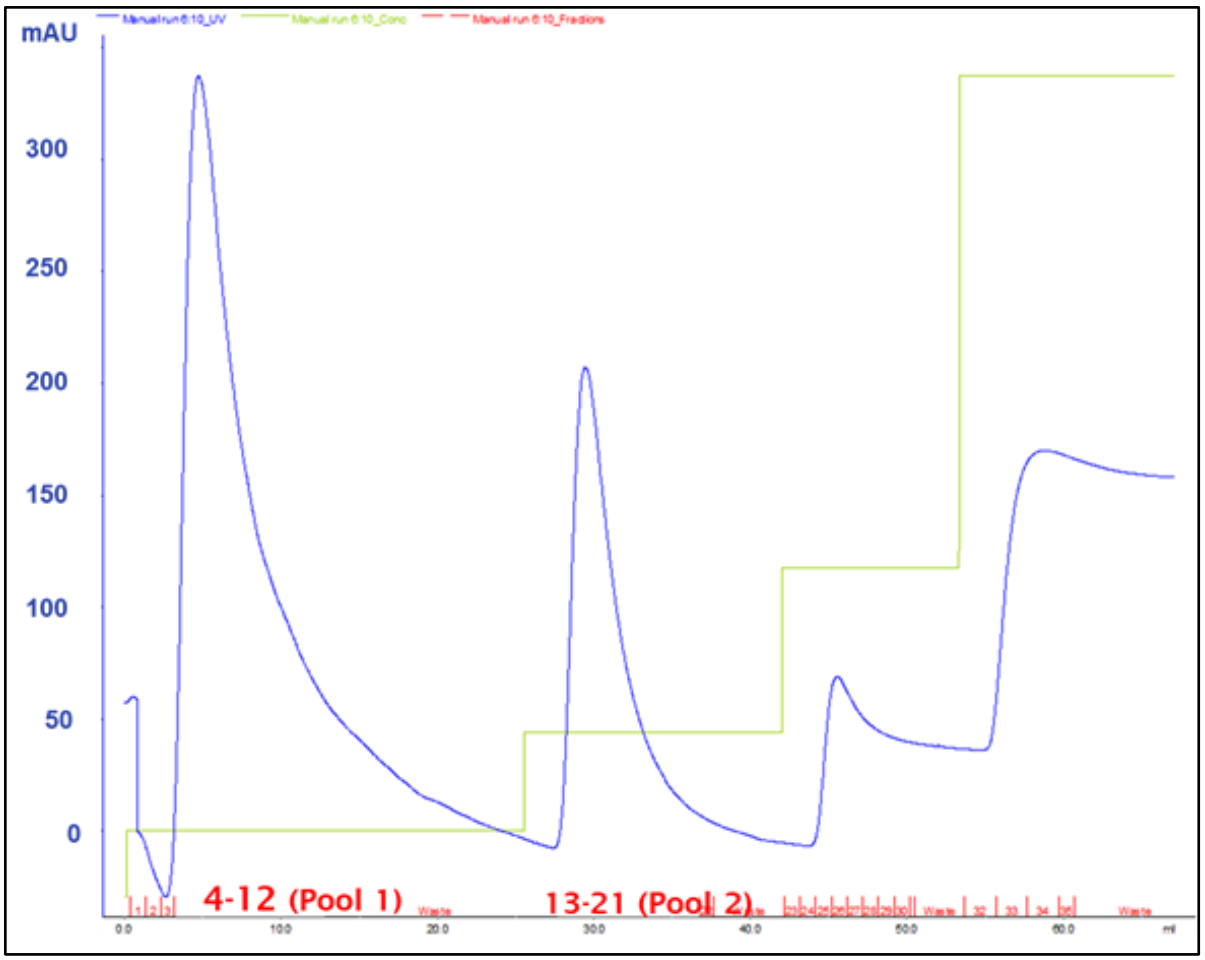


Figure 12: *Purification of rPru du 1.06A by nickel chelate affinity chromatography. The blue curve respresented the absorption of 280 nm where the green line corresponds to buffer B step gradient. The fractions marked with red rectangles were pooled.*

A) B)

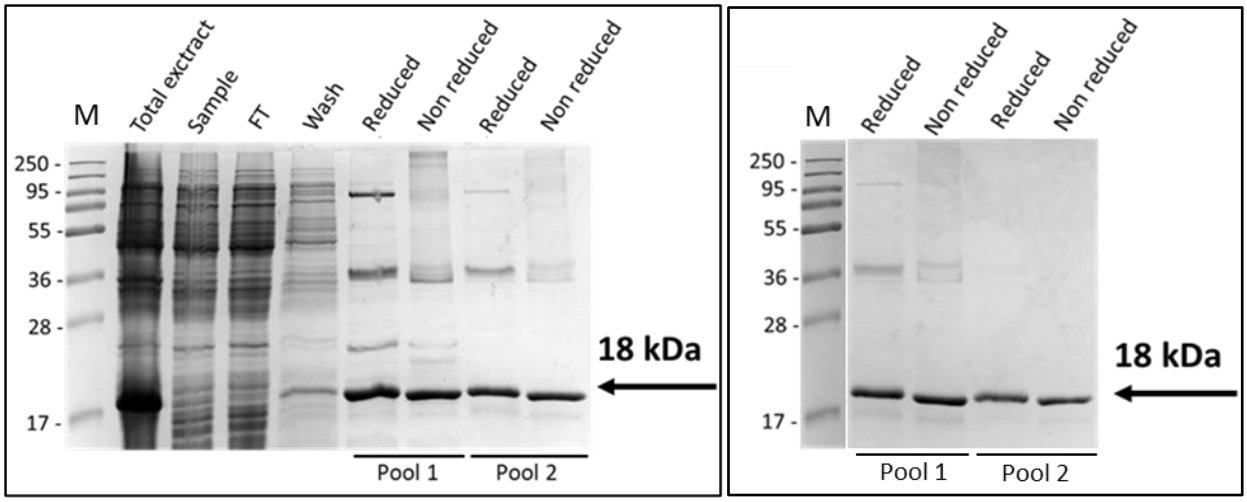


Figure 13: *Coomassie stained 15% SDS-PAGE gels under reducing and non-reducing conditions. A) Fractions obtained by nickel chelate affinity chromatography lanes 6 and 7 represents pool 1 where lanes 8 and 9 represents pool 2. B) Analysis of pool 1 and pool 2 after an additional step of chitin affinity chromatography.*

3.1.4 Physicochemical characterization of rPru du 1.06A

Purified rPru du 1.06A was analysed by MALDI-TOF MS. The sample was fixed on a MALDI target plate and measured as already described in section 2.2.3.4. The analysis revealed a protein peak at a molecular weight of 18090.82 Da equivalent to the theoretical calculated mass of 18087.26 Da (Figure 14).

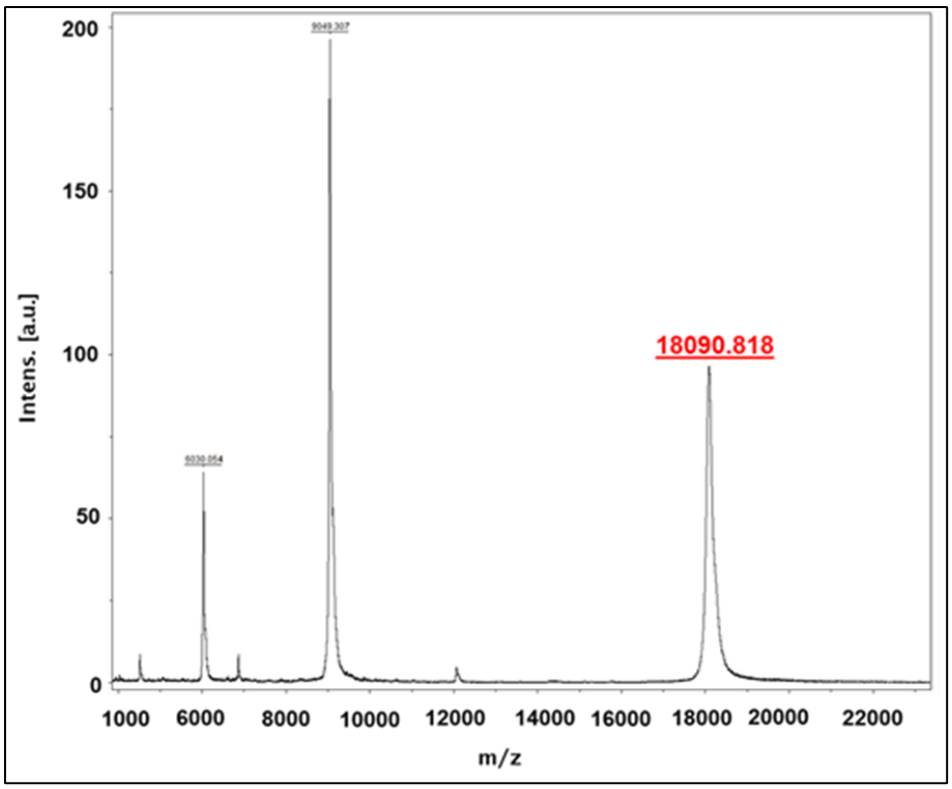


Figure 14: *MALDI-TOF mass spectra of purified rPru du 1.06A.*

The secondary structure of rPru du 1.06A was determined by CD in a range of 190-250 nm. As depicted in Figure 15, CD spectrum detected α-helical content (having two minimas at 222 nm and 208 nm and one maximum at 193 nm) and β-strand structure (containing a minimum at 218 nm and maximum at 195 nm). This indicated that rPru du 1.06A was nearly identical to rBet v 1 secondary structure. Thermostability assay of rPru du 1.06A revealed loss of structural integrity at 55 °C where upon heating up to 95 °C and cooling down to the start temperature at 25 °C, the protein structure stayed intact [104].

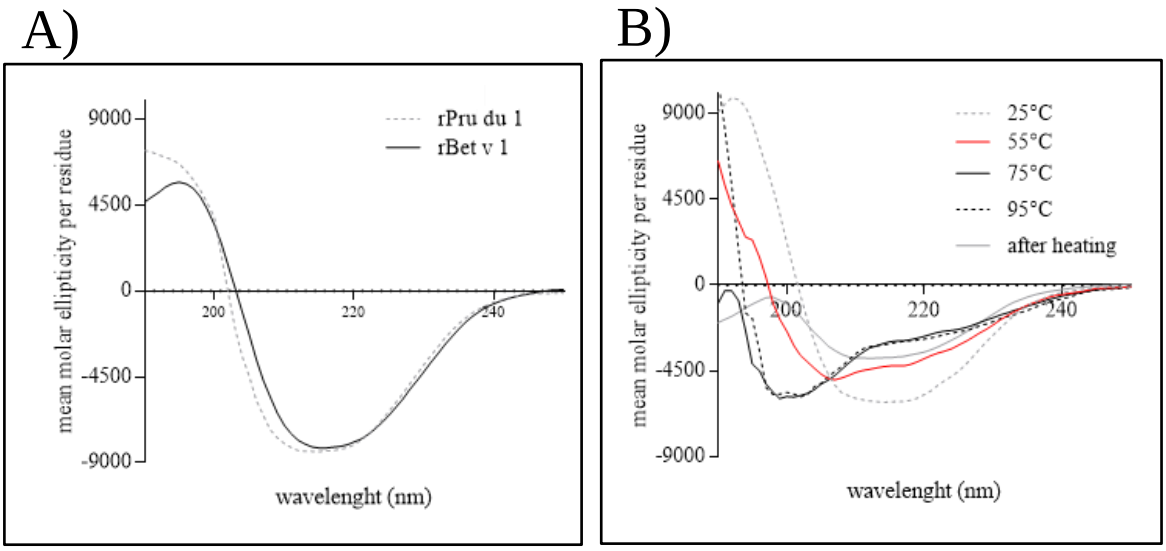
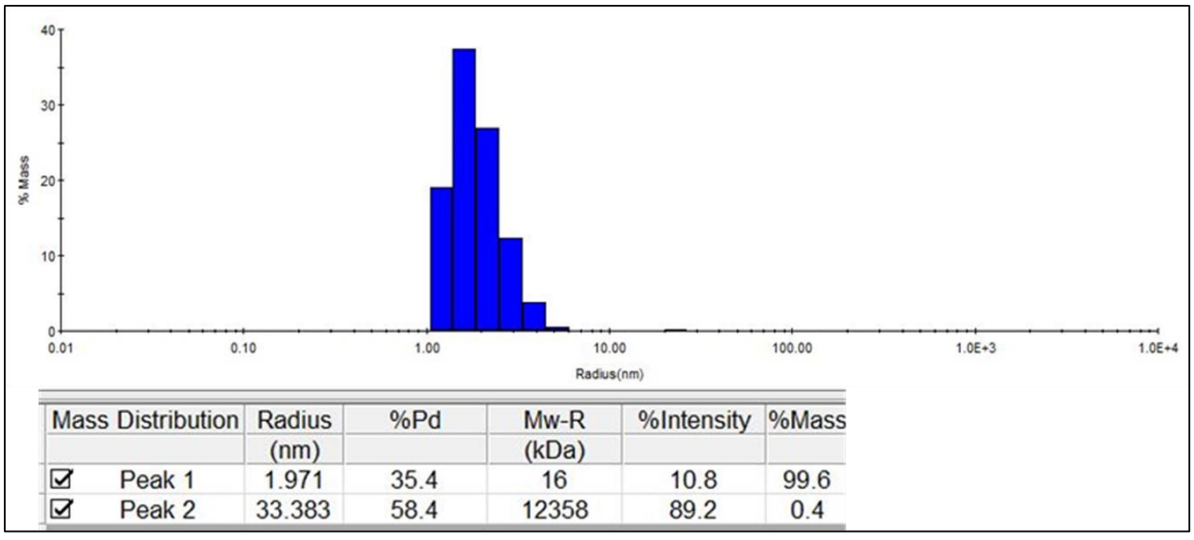


Figure 15: *CD spectra of rPru du 1.06A and r Bet v 1 . A) Measurement was performed at room temperature (25 °C) between 190-260 nm. B) Folding of protein was measured by heating up the sample up to 95 °C followed by immediate cooling at 25 °C.* Parts of the results of this thesis and Figure 15 have been published in (Kabasser et al.) [104] .

The oligomerization of the protein was determined by dynamic light scattering. As depicted in Figure 16, measured sample contained 99.6% monomers and 0.4% of oligomers which indicate mis-folded protein aggregates.



Mass Distribution	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
☑ Peak 1	1.971	35.4	16	10.8	99.6
☑ Peak 2	33.383	58.4	12358	89.2	0.4

Figure 16: *Dynamic light scattering (DLS) of purified rPru du 1.06A.*

In order to obtain better overview of the structural similarities between rPru du 1.06A and rBet v 1, 3D structure was visualized by UCSF Chimera. The PDB used for rPru du 1.06A was

obtained from strawberry pathogenesis-related 10 (PR-10) Fra a 1.02 protein, Q64W mutant by indicating sequence homology by 77.4%. As already determined by CD spectroscopy, both allergens indicated typical α-helical content and β-strand structure. The P-loop region was marked in red (Figure 17).

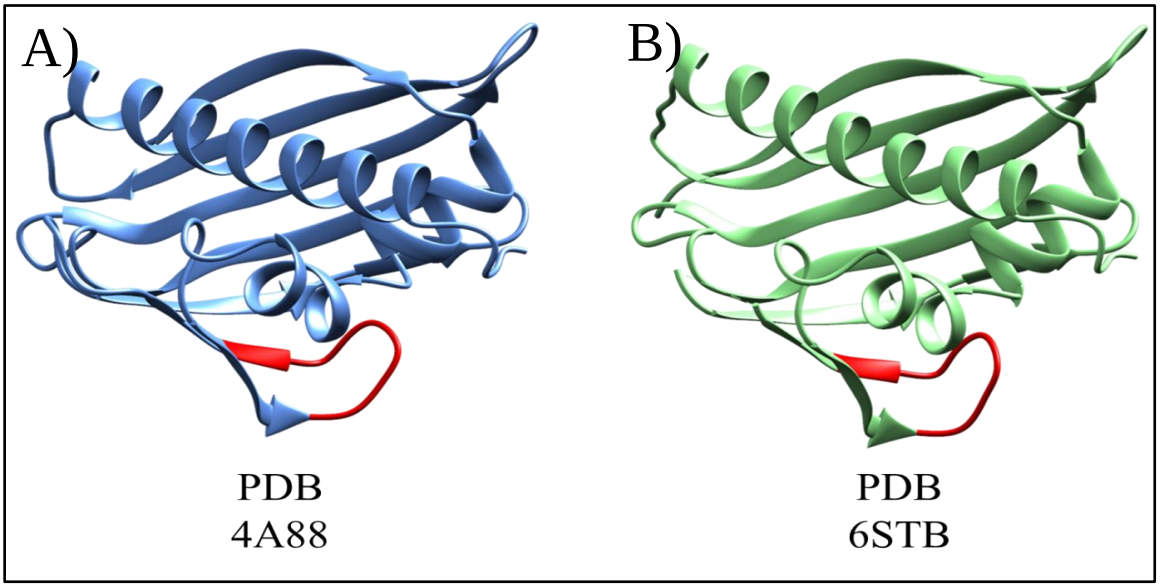


Figure 17: *Cartoon representation of the three-dimensional structrues of Bet v 1 (A) and Pru du 1.06A (B) with corresponding PDB. P-loop of both proteins was marked in red.*

3.1.5 Immunological characterization

3.1.5.1 Immunoblotting and IgE ELISA

As depicted in Figure 18A, rPru du 1.06A was detected at a molecular weight of 18 kDa by immunoblotting using polyclonal and monoclonal antibodies. In Figure 18A, lane 1, detection of the protein by an anti-His antibody represented a positive control and in lane 2, the recognition of rPru du 1.06A by a rabbit polyclonal anti-Bet v 1 antibody is visible. More specific recognition was obtained in lanes 3 and 4 by Bet v 1-specific monoclonal antibodies BV16 and BIP1. In lane 5 to 8 the corresponding secondary antibodies were used as negative control. In Figure 18B, immunoblot results of IgE cross inhibition analysis between rPru du

1.06A and rBet v 1 are presented. A pool of sera from three almond allergic subjects was used. Uninhibited serum pool recognized rPru du 1 and rBet v 1 at 18 kDa, respectively. On the other hand, when the serum pool was inhibited with rBet v 1, complete IgE binding inhibition to rPru du 1.06A was observed (Figure 18B, lane 2). Lane 3 indicated complete self-inhibition. In contrary, when serum pool was inhibited with rPru du 1.06A only partial inhibition of Bet v 1 was observed. In lane 2, complete self-inhibition of rBet v 1 was achieved. Additionally, a Bet v 1 unrelated almond allergen Pru du 6 (legumin) did not inhibited IgE binding of the serum pool to Pru du 1 and Bet v 1 (lane 4). As negative controls, NHS and corresponding secondary antibodies were used in (lane 5 and 6). These results indicated IgE cross-reactivity between rPru du 1.06A and rBet v 1.

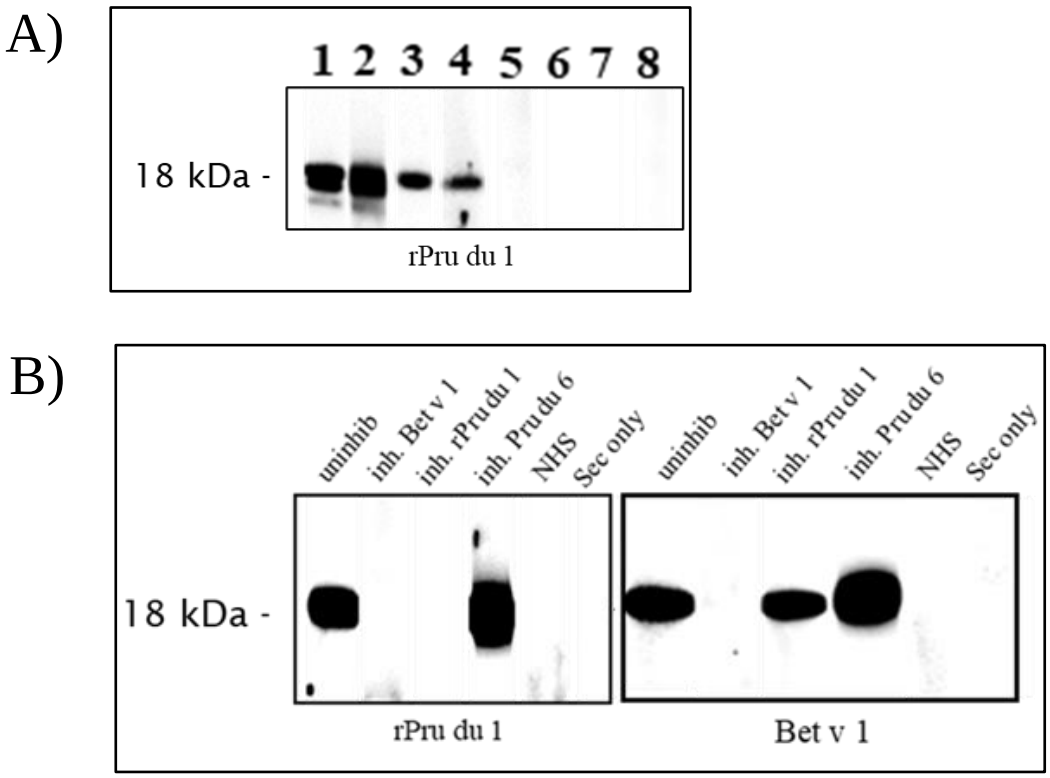


Figure 18: *Detection of rPru du 1.06A by immunoblotting. A) Lane 1: anti-His antibody, lane 2: polyclonal Bet v 1-specific antibodies, lane 3: monoclonal human PV16; and lane 4: mouse monoclonal BIP1 antibody. Lanes 5 to 8 served as negative control. B) Cross-inhibition of IgE binding to rPru du 1 and Bet v 1 performed with a serum pool. Serum pool was preincubated either with rBet v 1, rPru du 1.06A or Pru du 6. As negative control, NHS and secondary antibody were used.*

3.1.5.2 IgE reactivity of rPru du 1.06A and cross-reactivity with rBet v 1

In order to determine the concentration of sIgE to almond extract, Pru du 1.06A and Bet v 1, in almond allergic and almond-tolerant subjects, qELISA was performed. sIgE concentration (kU_A/L) was calculated by a factor of 1U=2.4 ng/mL and summarized in Table 6.

Table 6. *List of almond allergic patient sera (n=17) indicating information about symptoms to almond, allergies to food and inhalant allergies and sIgE levels to almond extract and allergens (rPru du 1 and rBet v 1),. OAS: oral allergy syndrome. URT: urticaria. AE: angioedema.*

Patient no.	Symptoms to almond	Other food allergies	Inhalant allergies	Allerges specific sIgE (kU/L)		
				Almond	rPru du 1	rBet v 1
1	OAS	hazelnut	alder, birch, hazel	0.0	18.4	44.9
2	OAS, URT, AE	eggs, milk	birch, hazel	0.0	94.9	157.7
3	OAS	apple, carrot, hazelnut	alder, birch, mugworth	0.0	21.7	74.5
4	OAS	apple, carrot, cherry, hazelnut, nectarine, walnut	alder, birch	0.0	4.2	28.4
5	OAS	apple, carrot, cherry, hazelnut, nectarine, peach pear, walnut	alder, birch, grass, oak	0.0	52.2	513.6
6	OAS	apple, carrot, cherry, peach, peanut, soy	birch	0.0	36.3	99.2
7	URT,	cashew, hazelnut, walnut	birch	0.0	5.9	46.0
8	URT, AE	apple, orange	birch, hazel	0.0	12.1	24.9
9	OAS, AE	none	birch	0.0	0.0	0.7
10	OAS, URT	seafood	none	0.0	0.0	0.6
11	OAS	apple, celery, cherry, potato	alder, birch, hazel	1.3	0.8	1.8
12	OAS	hazelnut, peanut	birch	0.0	40.8	62.5
13	OAS	cashew, hazelnut, peanut, pine nut, walnut	birch	49.0	0.0	480.4
14	URT	apple, apricot, celery, hazelnut, peanut, soy	birch	0.0	8.5	21.0
15	URT, AE	cashew, macadamia, seeds	birch, grass	5.0	5.2	692.9
16	AE	hazelnut, pea, peanut, walnut	none	22.6	3.0	6.8
17	URT, AE	apple, hazelnut, peanut, strawberry, walnut	birch	0.2	1.1	3.6

A control group of six subjects sensitized birch pollen allergen Bet v 1 and to almond but without allergic symptoms to almond were used as negative controls.

The majority of tested subjects (11 out of 17) experienced oral allergy syndrome (OAS). Twentynine percent of OAS positive subjects showed reactivity to almond extract while 82% of subjects reacted mainly to rPru du 1.06A (Figure 19A). By implementing Spearman correlation, data showed weak correlation between IgE reactivity to rPru du 1.06A and almond extract (Spearman correlation coefficient: -0.53). Otherwise, a strong correlation in IgE activity was observed between rBet v 1 and rPru du 1.06A (Spearman correlation coefficient: 0.60) [104] .

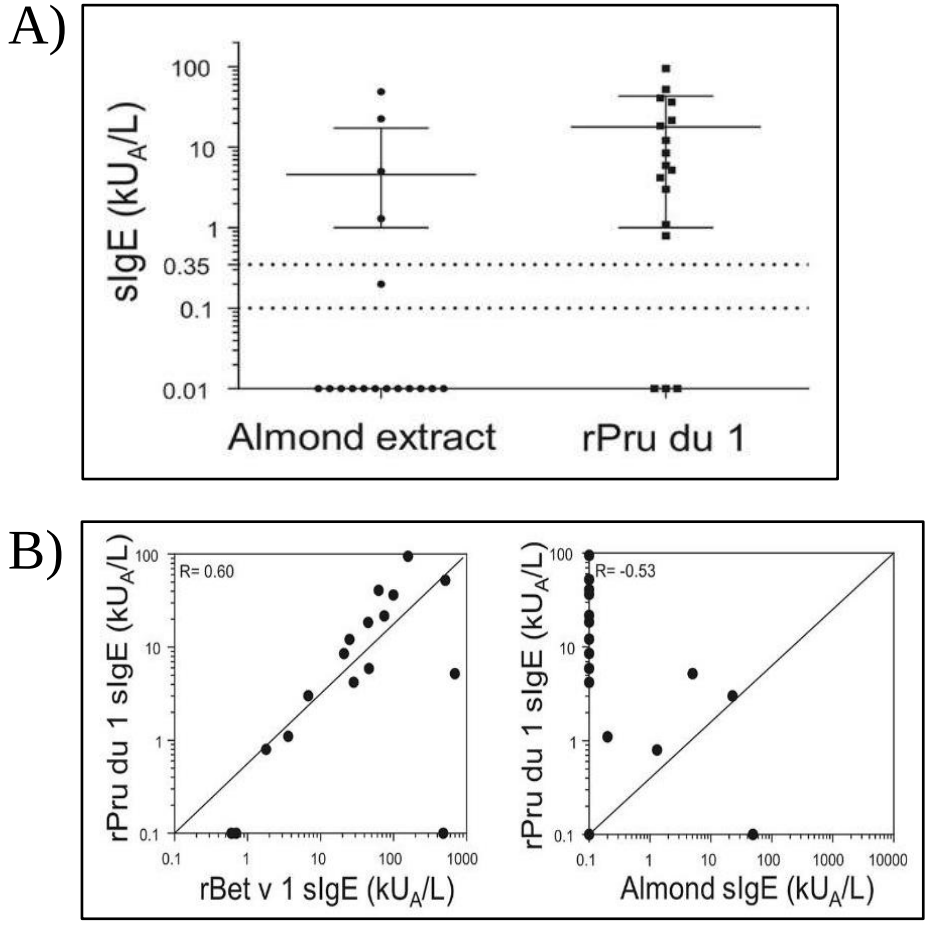
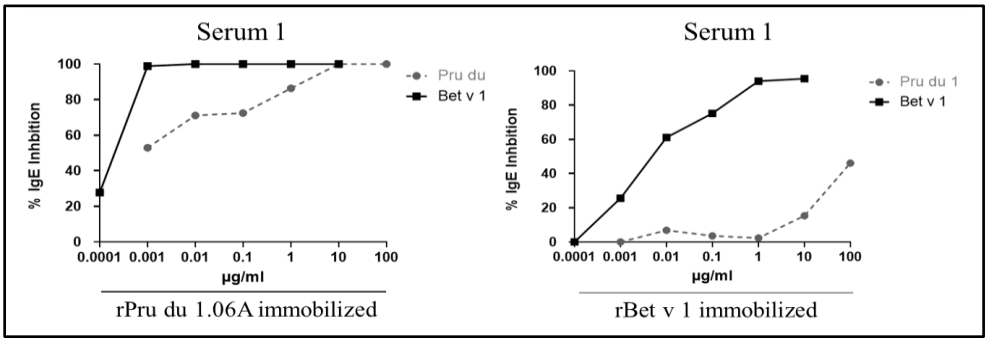
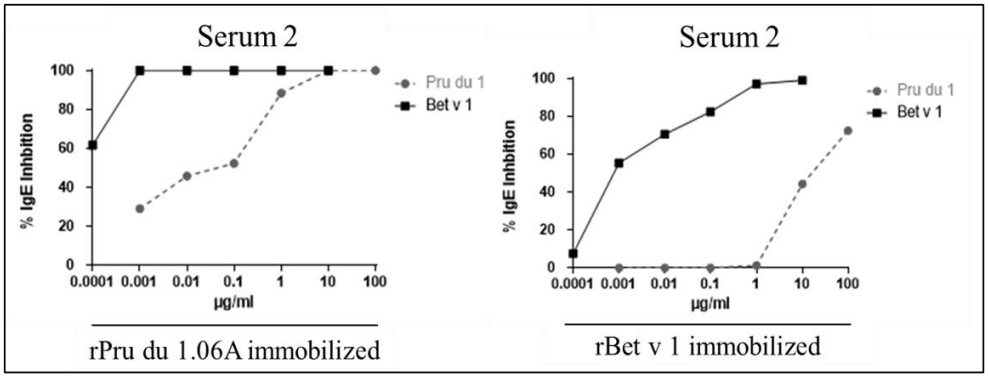
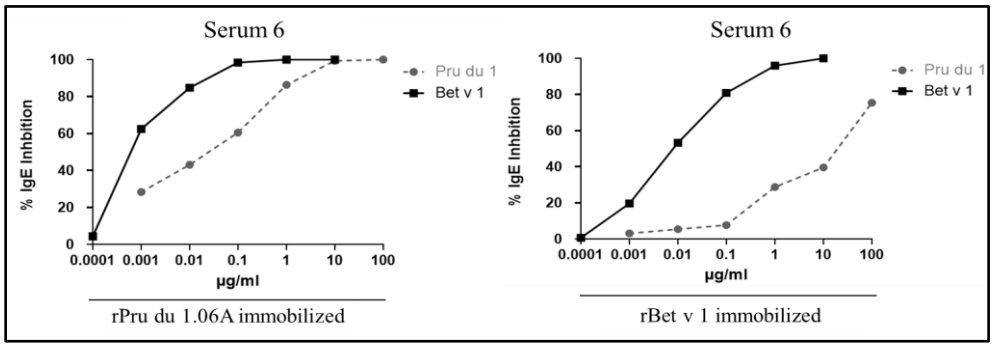


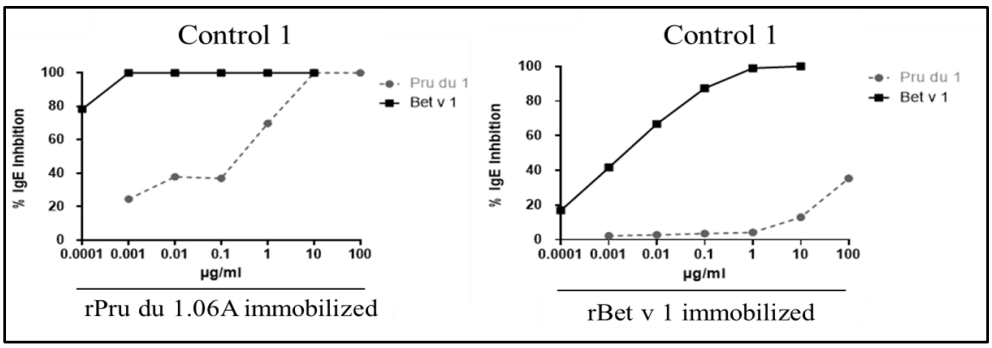
Figure 19: *Immunological characterization of rPru du 1.06A. A) IgE reactivity to almond extract and rPru du 1.06A of 17 almond allergic subjects. B) Comparison of sIgE reactivity to rBet v 1 and almond extract in almond allergic subjects by Spearman correlation. Parts of the results of this thesis and Figure 19 have been published in (Kabasser et al.[104].)*

IgE cross-reactivity between rPru du 1.06A, rBet v 1 as well as Bet v 1-homologue allergens was evaluated by dose-dependent inhibition IgE ELISA (Figure 20). Using Serum 1, sIgE reactivity to rPru du 1.06A was inhibited by Bet v 1 by 100% at a concentration of 0.001 µg/ml, while a concentration of 10 µg/ml of rPru du 1.06A was necessary for self-inhibition. On the other hand, rBet v 1 self-inhibition of 100% was reached at a concentration of 1µg/ml, while rPru du 1.06A inhibited sIgE to rBet v 1 only by 40% at a concentration of 100 µg/ml. Similar inhibition patterns were obtained in Serum 2 and 6. In both sera, sIgE reactivity to rBet v 1 was inhibited by 80% at a concentration of 100 µg/ml of rPru du 1.06A. Similar results were obtained with the two control sera from patients sensitized to Pru du 1 but without almond allergy. IgE specific to Pru du 1 from control sera 1 and 2 was inhibited by rBet t v 1 by 100% at a concentration of 0.001 µg/ml and rPru du 1.06A self-inhibition of 100% was obtained at a concentration of 10 µg/ml. In contrary, rPru du 1.06A inhibited sIgE to rBet v 1 by only 40% at the highest concentration of 100 µg/ml. rBet v 1 self-inhibition by 100% was visible at 10µg/ml. In all four almond allergic subjects, rBet v 1 already at concentrations of 0.0001 µg/ml and 0.001 µg/ml was able to inhibit IgE reactivity to immobilized rPru du 1.06A by 60 % where at concentrations of 0.001 µg/ml and 0.1 µg/ml 100% inhibition was achieved. On the other hand, the results showed that rPru du 1.06A inhibited IgE reactivity to immobilized rBet v 1 by 60% at a concentration of 100 µg/ml.


Serum 1
% IgE Inhbition
100
80
60
40
20
0
0.0001 0.001 0.01 0.1 1 10 100
µg/ml
Pru du
Bet v 1
rPru du 1.06A immobilized
Serum 1
% IgE Inhbition
Pru du 1
Bet v 1
rBet v 1 immobilized


Serum 2
% IgE Inhbition
100
80
60
40
20
0
0.0001 0.001 0.01 0.1 1 10 100
µg/ml
Pru du 1
Bet v 1
rPru du 1.06A immobilized
Serum 2
% IgE Inhbition
Pru du 1
Bet v 1
rBet v 1 immobilized


Serum 6
% IgE Inhbition
100
80
60
40
20
0
0.0001 0.001 0.01 0.1 1 10 100
µg/ml
Pru du 1
Bet v 1
rPru du 1.06A immobilized
Serum 6
% IgE Inhbition
Pru du 1
Bet v 1
rBet v 1 immobilized


Control 1
% IgE Inhbition
100
80
60
40
20
0
0.0001 0.001 0.01 0.1 1 10 100
µg/ml
Pru du 1
Bet v 1
rPru du 1.06A immobilized
Control 1
% IgE Inhbition
Pru du 1
Bet v 1
rBet v 1 immobilized

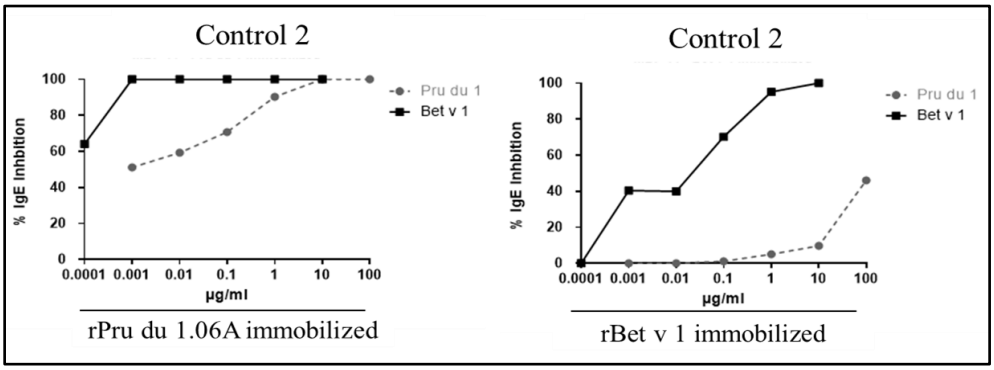

Control 2
% IgE Inhbition
100
80
60
40
20
0
0.0001 0.001 0.01 0.1 1 10 100
µg/ml
Pru du 1
Bet v 1
rPru du 1.06A immobilized
Control 2
% IgE Inhbition
Pru du 1
Bet v 1
rBet v 1 immobilized

Figure 20: *IgE cross-reactivity between rPru du 1 and rBet v 1. IgE cross-inhibition ELISA was performed by using rPru du 1.06A and rBet v 1 on solid phase. IgE reactivity of sera from subjects with almond allergy (n=3) as well as control subjects (n=3) was determined upon dose-dependent inhibition with rBet v 1 and rPru du 1.06A.*

Furthermore, rBet v 1-homologue allergens such as rAra h 8 inhibited IgE reactivity at a concentration of 10 µg/ml to rPru du 1.06A by an average of 81%,rMal d 1 by 74%,rCor a 1 by 47% and rGly m 4 and rPru p 1 showed the weakest sIgE inhibition of approx. 36% (Figure 21).

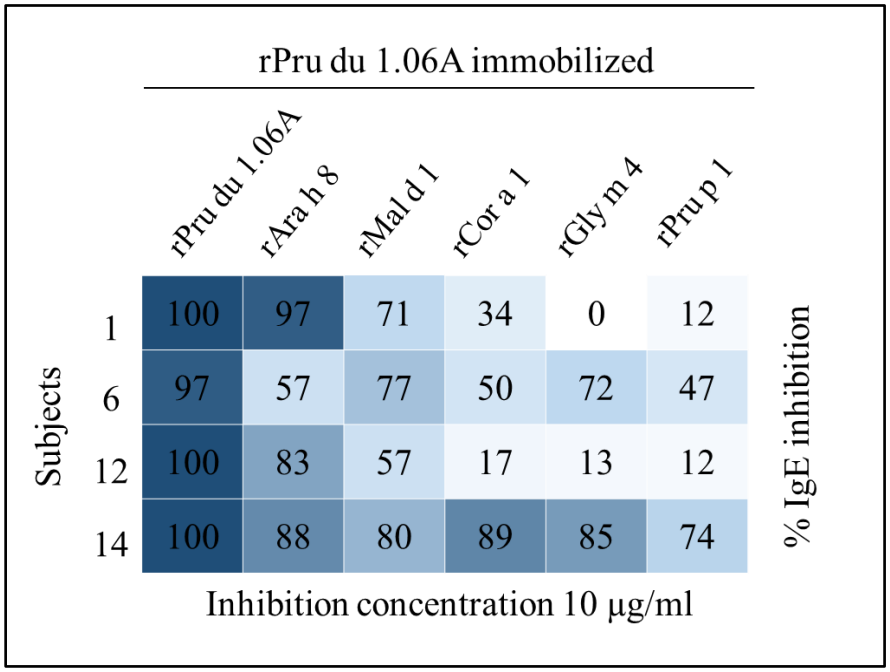


Figure 21: *IgE cross-reactivity between rPru du 1 and other allergenic Bet v 1-related proteins. IgE inhibition to Pru du 1 was performed by adding 10 µg/ml of different PR-10 related proteins to sera from four almond allergic patients.*

3.1.5.3 Basophil activation capacity of rPru du 1.06A

To evaluate the biological potency of rPru du 1.06A to activate effector cells, *in vitro* basophil activation assay with RS-ATL8 rat basophil leukemia (RBL) cells was performed using four sera from almond allergic patients (serum 2, 3, 6, and 12). As shown in Figure 22, using serum 6, up to 1 µg/ml of rPru du 1.06A was required for maximum activation of cells as compared to Bet v 1. On the other hand, serum 2 and 3 required up to 1000 µg/ml of rPru du 1.06A for

maximum activation. In comparison to rPru du 1.06A, lower concentrations of rBet v 1 (0.1 µg/ml) were required for full cell activation.

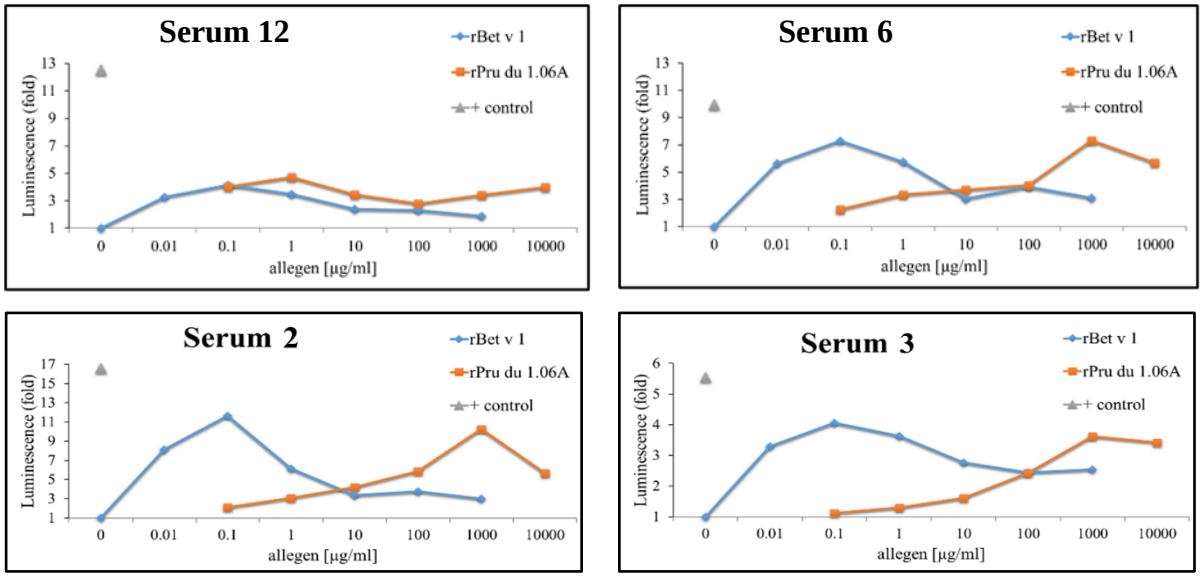


Figure 22. *Allergenic potency of rPru du 1.06A compared to rBet v 1. Allergenic activity of rPru du 1.06A and rBet v 1 was determined by basophil activation assay with RS-ATL8 rat basophil leukemia (RBL) cells sensitized with sera from 4 subjects.*

4 Discussion and Conclusion

Birch trees as well as other trees of Fagales order (alder and hazel) play a role as allergen sources mostly in temperate climate regions such as Northern and Central Europe, North America and East Asia [24]. In these areas where birch trees are abundant, there is a connection between birch-pollinosis and almond allergy which typically present a milder form of almond allergy (oral allergy syndrome) and often with low levels of IgE specific to almond extract. According to Uotila et al., who studied sensitizations to nuts in a birch-endemic area, 71% of subjects with birch sensitization were co-sensitized to almond [105]. Furthermore, of 1099 patients sensitized to almond, 569 (52%) reported symptoms, of whom 378 (66%) showed only OAS. Primary sensitization to the major birch pollen allergen Bet v 1 is the main elicitor of secondary plant food allergies due to high IgE cross-reactivity between Bet v 1 and homologous food allergens [106]. Pror to this study, a pollen-related almond allergen has not been identified. Thus, the aim of this study was to identify a Bet v 1-homologue in almond, produce its recombinant counterpart and analyse its biochemical as well as immunological properties.

As known from previous study, there are seven identified genes in almond leaves coding for different Bet v 1-homologues (Pru du 1.01, Pru du 1.02, Pru du 1.03, Pru du 1.04, Pru du 1.05, Pru du 1.06A and Pru du 1.06B) [69]. In the publication of Chen et al., genomic DNA was isolated from leaves, which raised the question whether a Bet v 1 homologue is also expressed in almond kernels. Thus, the first step of our study was to detect a possible Bet v 1 homologue in almond kernel protein extract using two different specific anti-Bet v 1 antibodies (BIP-1 and BV16) [106,107]. However, in immunoblot, we observed no antibody binding to any proteins with any similar molecular weight to Bet v 1. Despite using conditions optimized for Bet v 1-related protein extraction, the almond Bet v 1-homologue was apparently underrepresented in the extract. In general, Bet v 1 homologues have a low degree of stability, which complicates

the extraction process from natural sources. Underrepresentation of Bet v 1 homologues is commonly observed in tree nut extracts, as for example in peanut, hazelnut and walnut, which highlights the importance of using recombinant Bet v 1-related allergens for in vitro studies and diagnosis [108–110].

We therefore analyzed the almond extract with a more sensitive methodology, namely Nano-LC ESI Orbitrap MS/MS. By using this approach we detected only one isoform Pru du 1.06A on a protein level. The remaining six isoforms are probably not expressed in the kernels or may be expressed in almond leaves or roots. We concluded that Pru du 1.06A is represented only in minor quantities in almond protein extract.

It is already known that Bet v 1 homolgous allergens are sensitive to enzyme activity as well as high temperatures [112]. Storage conditions may also play a role in degradation of the allergens [113]. These might be some of the underlying reasons for the very low quantities of Pru du 1.06A in the extract.

Different Bet v 1 homologues are recombinantly produced since they are not easily detected in natural food or [113–115]. These allergens are further used for molecular allergy diagnosis of patients with pollen-associated food allergies. Today, Bet v 1 homologues from walnut (rJug r 5) and hazelnut (rCor a 1) are used and additionally spiked to the respective extracts [24]. We hence proceeded with recombinant expression of Pru du 1.06A, in order to determine its further physicochemical and immunological characteristics by SDS-PAGE, MALDI-TOF MS analysis and CD spectroscopy. After recombinant expression and purification of rPru du 1.06A, the exact molecular weight of 18 kDa of Pru du 1.06A was confirmed by MALDI-TOF corresponding to the theoretical molecular mass. The secondary structure of Pru du 1.06A was determined by CD spectroscopy. The protein was correctly folded possessing typical for Bet v 1-homologous proteins α-helical content and β-strand structures. Based on the amino acid

sequence identities and secondary structure of Pru du 1.06A, we observed high similarities to Bet v 1.

To characterize IgE reactivity of rPru du 1.06A, sera from patients from Central Europe with mild symptoms (mainly oral allergy syndrome) indicating birch-pollen-associated almond allergy were tested. In this cohort, the majority of patients reacted positive with rPru du 1.06A, whereas sensitization to almond protein extract was nearly absent. Based on IgE cross-inhibitions between Bet v 1 and Pru du 1, our data demonstrated that pre-incubation of sera with Bet v 1 completely (100%) inhibited IgE binding to rPru du 1.06A. On the other hand, rPru du 1.06A showed weaker IgE inhibition capacity than Bet v 1. This indicates primary sensitization to Bet v 1 and IgE cross-reactivity to some of the shared epitopes on Pru du 1.06A. Bet v 1 and Bet v 1 homologus proteins posses a P-loop region, which is highly conserved among all pathogenesis-realted protein family members [106,107] This region may be responsible for cross-reactivity of Bet v 1 with its homologues. The contribution of the P loop region to IgE binding was previously shown in studies which, by mutating single amino acids in this region, showed consequent reduction in IgE binding [117]. In addition to P-loop region other part of Bet v 1 are also able to bind IgE [106,107]

Almond together with peach, apple, cherry belongs to the plant family *Rosaceae*. Indeed, the majority of the recruited subjects were allergic to apples, hazelnut, walnuts, cherries, peach and celery. The sequence alignment between Bet v 1, Pru du 1.06A, Pru p 1.0101, Mald 1.0101, Ara h 8.0101, Gly m 4.0101, Cor a 1.0101, and Jug r 5.0101 revealed high amino acid sequence identities between Bet v 1 homologs from botanically related plants such as peach, apple and cherry. This is in line with results of dose-dependent IgE inhibition assay showing high IgE cross-reactivity between rPru du 1.06A and Mal d 1. Based on this, we can speculate that patients with sensitization to Pru du 1.06A may show IgE cross-reactivity to homologues proteins from other food allergens belonging to *Rosaceae* family. Interestingly, while apple

allergen Mal d 1 inhibited IgE binding to Pru du 1 by 80%, even higher inhibition of over 90% was achieved by peanut allergen Ara h 8. This suggest that also other Bet v 1 homologous from botanically unrelated sources share important IgE epitopes with Pru du 1.

In addition to IgE binding, we investigated the capacity of Pru du 1.06A to induce IgE cross-linking on the effector cells. Our experiments on RBL cells provided evidence that Pru du 1.06A can indeed trigger effector cells activation in a dose-dependent manner, conforming the biological activity of rPru du 1. However, biological activity was lower compared with that of Bet v 1, which was observed similarly for other birch pollen related allergens, such as Ara h 8 from peanut , or Jug r 5 from walnut [108,113]. The highest allergenic activity was observed at a Pru du 1.06A concentration of 1000 µg/ml. In contrast, rBet v 1 induced cell activation already at a concentration of 0.1 µg/ml. These results provide compelling evidence that in our patient cohort of the birch pollen associated almond allergy individuals, Bet v1 as a primary sensitizer was a more potent allergen.

Precise diagnosis of almond allergy is complicated by the poor correlation between sensitization and clinical reactivity [104,117,118]. Molecular allergy diagnosis was already proven to be useful to elucidate different sensitization profiles and more accurately reflect clinical reactivity, avoiding the need for a challenge in patients with food allergies [24,119]. Allergens, such as Ara h 2, 12 Jug r 1, 13 Cor a 14, 14 and Ana o 3, 15 were described as superior in predicting allergic reactions to peanut, walnut, hazelnut, and cashew, compared to the extracts [104]. Furthermore, considering that is difficult to purify allergens from natural protein extracts, the use of recombinant almond allergens play an important role in the application in diagnostics of almond allergens. Our data indicate that birch pollen associated almond allergy is characterized by presence of IgE specific to Pru du 1. However, in birch-endemic areas, birch pollen sensitization may occur also in patients who suffer a severe almond allergy which is unrelated to pollen and characterized by IgE specific to seed storage proteins such as almond legumin Pru

du 6 [75]. To determine the nature of almond allergy is crucial in reducing unnecessary diet restrictions and yet keeping severely allergic patients safe. In comparison to detection of specific IgE to almond protein extract, detection of Pru du 1 and Pru d u 6 specific IgE would allow more detailed patients advising and reduce the number of unnecessary food challenges or unnecessary avoidance of almonds.

In summary, the expression of a Bet v 1 homologue in almond kernels was confirmed by mass spectrometry and it has received the designation Pru du 1.0101 (http://www.allergen.org/). It was expressed as a recombinant protein in *E. coli* and physico-chemical characterization confirmed the identity, purity, and correct structural conformation of the purified protein. The recombinant protein bound IgE from majority of almond allergic patients sensitized to birch pollen allergen Bet v 1. IgE cross-inhibition results clearly confirm that birch-pollinosis associated almond allergy is due to the high cross-reactivity of Bet v 1-specific IgE antibodies and its related allergen in almond.

In conclusion, this study provides the first experimental conformation that the link between birch-pollinosis and mild secondary almond allergy is based on the presence of a cross-reactive Bet v 1 homologue in almond. These results may further help in allergy diagnostic approaches to discriminate between primary allergy to almond and birch-pollen associated almond allergy.

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6 Appendix

6.1 List of illustration

6.2 List of tables