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Humoral and cellular characterization of minor allergens in birch pollen

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Content

Abstract

Allergic diseases have reached epidemic dimensions in urban areas and reduce the patients´ quality of life. Pollen from wind pollinated plants is among the most potent allergen sources. Pollen from the European white birch *Betula verrucosa* contain the well characterized major allergen Bet v 1 and several minor allergens which are recognized by less than 50% of birch pollen-allergic patients.

Diagnosis and therapy of Type I allergy depends on the quality of the employed allergen extracts. Disadvantages such as batch to batch variability or standardization of the concentration of different components in allergen extracts may be overcome by the use of recombinant allergens.

The major aims of this thesis were i) the expression, purification and characterization of the minor birch pollen allergens Bet v 3, Bet v 4, Bet v 6, Bet v 7 and ii) the isolation and characterization of T-cell clones (TCC) specific for proteins in birch pollen.

All minor allergens were expressed in *E.coli* with a His-tag and purified by Ni- affinity chromatography. The LPS content was reduced to negligible levels. Correct folding and allergenicity of the allergens was tested by IgE ELISA experiments conducted with sera from birch pollen-allergic patients. The ability of the allergens to activate birch pollen-specific T-cells was assessed in proliferation assays using peripheral blood mononuclear cells (PBMC), allergen-specific T-cell lines (TCL) and TCC derived from birch pollen-allergic patients. All recombinant minor allergens were able to bind IgE antibodies and induced proliferation in PBMC from birch pollen-allergic patients.

Allergen specific TCC were expanded from birch pollen-specific TCL and characterized in proliferation assays. Various subset-specific markers as well as molecules up regulated by specific activation were analyzed by flow cytometry. Cytokine levels in the supernatants of allergen-activated TCC were determined by cytokine bead arrays. Our results demonstrate that the majority of the TCC isolated from TCL expanded with birch pollen extract were specific for Bet v 1 and belonged to the T_H2-like subset. TCC non-reactive with Bet v 1 belonged to the T_H0-subset. None of the clones reacted with the recombinant minor allergens Bet v 3-7. One TCC was found to be Bet v 2-specific.

In summary, endotoxin free batches of recombinant Bet v 3, Bet v 4, Bet v 6 and Bet v 7 were produced and their IgE reactivity was confirmed. For the first time, the T-cell activating capacity of these minor allergens was shown.

In the future, the produced and characterized allergens may help to examine the differences between Bet v 1 and minor allergens. Furthermore, the stored TCC are valuable in the search for new proteins with immunological value in birch pollen.

Introduction

The immune system

The immune system (IS) is the biological defense mechanism against pathogens and tumor cells. The general immune response can be divided in the early innate and late adaptive phase.[7]

The innate immune system

The innate immune system is the first line of defense and includes epithelial barriers, phagocytes, natural killer cells (NK-cells), complement proteins and cytokines. Its main function is the host defense against infection and it further contributes to the removal of environmental particles, microbial products and allergens.[7] Innate immune cells express germ line-encoded pattern recognition receptors that recognize pathogen associated molecular patterns (PAMPS) which are shared by many microbes and are often essential for their survival.[8] Toll like receptors (TLRs) are the most important group of pattern recognition receptors.

Leukocytes of the innate immune system kill ingested microbes by phagocytosis and can promote inflammation and tissue remodeling at the site of infection. The innate immune system also influences the adaptive immune response through co-stimulatory molecules and cytokines[7] Interaction of allergens with the innate immune system usually leads to immunologic tolerance. In patients with allergic predispositions, this interaction triggers chronic inflammation and the loss of immunologic tolerance.[8]

The adaptive immune system

Whilst the innate IS exerts a general response against structures bearing PAMPs, the cells of the adaptive IS are responsible for the specific immune response against pathogens. This second line of defense, consisting mainly of B- and T-lymphocytes, is confronted with a vast number of different antigens. Therefore it depends on professional antigen presenting cells (APCs) which activate and influence T- lymphocytes. These cells then orchestrate the optimal immune response. Antigens are presented to T-cells by major histocompatibility complex (MHC) molecules, expressed on the surface of nucleated cells. Class I MHC molecules are expressed by all cells, MHC II molecules are expressed mainly by antigen presenting cells (macrophages, dendritic cells...).[7] Through genetic recombination and mutation the IS is able to produce a vast variety of effector cells with receptors that specifically recognize antigens.[9] It can further develop a specific memory which allows a fast immune response to subsequent exposure of antigen.[7]

B-lymphocytes

B-lymphocytes play a major role in the antibody-mediated humoral immune response. B-cells develop in the bone marrow and mature B-cells are found primarily in secondary lymphoid tissues, in lymphoid follicles and in bone marrow. Their main function is the production of antibodies but they can also function as antigen presenting cells.

B-cells recognize antigens in their native form with their B-cell receptor, a membrane bound antibody. Antibodies are a type of glycoprotein also called immunoglobulin (Ig). They are composed of two identical heavy and light chains. Variable regions of the heavy and light chain on the N-terminus form the antigen binding site. There are five immunoglobulin isotypes in mammals: IgM, IgA, IgD, IgG and IgE.

In contrast to T-cells, B-lymphocytes don't need antigen processing prior to their activation. Activation of naive B-cells by antigen recognition supplemented by T_H cell stimulation leads to differentiation into antibody secreting plasma cells. This can also induce irreversible class switching from IgM to the other Ig-isotypes, depending on the cytokine milieu provided by the T_H cell. Especially the switch to IgE promoted by IL-4 plays a major role in allergic reactions.[7]

T-lymphocytes

T-lymphocytes play a central role in cell to cell mediated immunity and contribute to the humoral immune response by interacting with B-cells. They mature in the thymus and and populate secondary lymphoid tissues.

T-cell receptor complex

T-cells recognize processed peptide fragments of foreign proteins in the context of MHC class I and II molecules (MHC restriction) with their T-cell receptor (TCR). In most T-lymphocytes, the TCR is a heterodimer composed of two disulfide linked polypeptide chains called α and β (αβ TCR). These are homologous to the light and heavy chains of immunoglobulin (Ig) molecules. Each chain consists of a variable (V) and constant (C) region. The variable regions recognized processed protein antigens as well as polymorphic residues on the MHC molecule of the antigen presenting cell.[7]

The γδ TCR is very similar to the common αβ TCR in terms of structure and interaction with proteins of the TCR complex. This receptor is expressed on less than 5% of all T-cells. These cells are not MHC restricted and recognize different forms of antigens including lipids and peptides presented by MHC like molecules.

The TCR heterodimers are non-covalently associated with transmembrane proteins called CD3 and ζ. These proteins transduce T-cell activating signals into the cell after successful peptide recognition by the TCR. CD4 and CD8 are coreceptors expressed on the surface of

mature T-cells. CD4 is expressed on MHC II restricted T-helper cells and CD8 is found on MHC I restricted cytotoxic T-cells.

T-lymphocytes consist of functionally distinct populations. They can be distinguished by the expression of several surface proteins (cluster of differentiation, CD- Molecules).

The major T-cell subsets and their characteristic surface markers, transcriptional regulators and effector molecules are listed in Table 1.

T-cell subset	Surface phenotype	Transcription factors	Secreted effector molecules	Short description
T helper-cells	αβ TCR, CD3, CD4,	Depending on the T_H subtype	Depending on the T_H subtype	Different subtypes orchestrate immune response via direct cell contact and cytokines.
Cytotoxic T-cells	αβ TCR, CD3, CD8	EOMES, T-bet, BLIMP1	Perforin, granzyme, IFNγ	Kill infected and transformed cells.
Regulatory T-cells	αβ TCR, CD3, CD4, CD25, CTLA4, GITR	FOXP3, STAT5, FOXO1, FOXO3	IL-10, TGFβ	Immunosuppression mediated by IL-10 production and contact dependent mechanisms.
Memory T-cells	CCR7, CD44, CD62L, CD3, IL-7R (CD127), IL-15R	BCL-6, BCL-6B, MBD2, BMI1	Rapid and high production of inflammatory cytokines	Mount recall responses to antigens. Provide immediate protection upon antigen challenge.
NKT cells	NK1.1, SLAMF1, SLAMF6, TGFβR, Vα24, Jα18	PLZF	IL-4, IFNγ, IL-17A	Can have pro and anti-inflammatory functions.
γδ T cell	γδ TCR, CD3		IFNγ, IL-17A, IL-17F, IL-22	Can have pro- and anti-inflammatory functions depending on the context.

Table 1: Characteristics of T-cell subtypes. [10]

Cytotoxic T- cells

The major effector function of Cytotoxic T- cells (T_c cells) is the killing of infected or dysfunctional host cells. They express the coreceptor CD8 and recognize antigens displayed by MHC class I molecules. Activation requires MHC I associated antigen recognition together with costimulators on APCs (cross presentation) or signals provided by helper T-cells. When exposed to infected cells, the response of differentiated cytotoxic T-lymphocytes involves the release of cytoplasmic granules with membrane pore forming proteins and enzymes initiating apoptosis in the target cell.[7]

T-helper cells

T-helper cells (T_H cells) are essential for the IS as they can direct the immune response via interaction with other cells and secretion of cytokines. T_H cells express the CD4 co-receptor and recognize processed antigens associated with MHC class II molecules. When activated in specific cytokine environments, naive CD4+ T-cells differentiate into different subsets with distinct effector functions. These specified cells then mobilize and orchestrate other cell types to effectively clear invading pathogens. Based on their cytokine profile, T_H cells can be subdivided into several T_H subsets summarized in Table 2.[9]

T_H cell subset	Surface phenotype	Transcription factors	Secreted effector molecules	Short description
T_H1 cells	αβ TCR, CD3, CD4, IL-12R, IFNγR, CXCR3	T-bet, STAT4, STAT1	IFNγ, IL-2	Important in host defense against intracellular pathogens. Secretion of IFNγ activates macrophages
T_H2 cells	αβ TCR, CD3, CD4, IL-4R, IL-33R, CCR4, IL-17RB, CRTH2	GATA3, STAT6,	IL-4, IL-5, IL-13, IL-10	Major factor in humoral immune response against extracellular parasites. Can potentiate allergic responses.
T_H9 cells	αβ TCR, CD3, CD4	PU.1	IL-9, IL-10	Promote allergic inflammation in host defense against extracellular parasites.
T_H17 cells	αβ TCR, CD3, CD4, IL-23R, CCR6, IL-1R, CD161	RORγt, STAT3, RORα	IL-17A, IL-17F, IL-21, IL-22, CCL20	Part of the protective immune response against extracellular bacteria and fungi, primarily at mucosal surfaces
T_H22 cells	αβ TCR, CD3, CD4, CCR10	AHR	IL-22, TNFα	Identified in inflammatory skin disease. Role in the immune response still unclear

Table 2: Characteristics of T_H-cell subtypes[10]

T_H1 differentiation usually occurs in response to intracellular microbes. T_H1 mediated responses include the activation of macrophages and neutrophils and the production of IgG antibodies by B-cells. IFNγ is the signature cytokine of this subtype. Also the expression of the transcription factors STAT-4 and T-bet are T_H1-associated.[11]

T_H2 cells play a major role in the humoral immune response. Secretion of IL-4, IL-5, IL-9, IL-13 and expression of the transcription factors STAT-6 and GATA-3 are their distinctive feature. They induce class switching in B-cells through CD40-CD40L interaction and the secretion of IL-4 and IL-13. An imbalance in favour of a Th2 mediated response is believed to be the cause of IgE.mediated allergic disorders.[12,13]

T_H9 cells are $Foxp3^-$ $IL\text{-}9^+$ $IL\text{-}10^+$ T-cells that do not suppress T-cell responses even though they produce IL-10.[14] TGF-β induces the differentiation of T_H2 cells into T_H9 cells.[15] This effect can be potentiated by IL-4 and inhibited by IFNγ.[16]

T_H17 cells contribute to the host defense against extracellular bacteria and fungi mainly at mucosal surfaces.[17] Their main transcription factor is RORγt and activation leads to tissue inflammation[18]. Approximately 1% of the $CD4^+$ T-cells in peripheral blood are T_H17 cells[7].

The T_H22 subset is characterized by the secretion of IL-22 and TNF-α. These cells don't produce IFNγ, IL-4 or IL-17[19]. Dermal dendritic cells and Langerhans cells can induce the differentiation of T_H cells into this subtype.[20] They express proteins involved in skin remodeling and infiltrate the epidermis of patients with inflammatory skin disorders[19].

T-cell cloning

To analyze birch pollen specific T-cells at the single cell level, T-cell clones (TCCs) can be established from birch pollen-specific T-cell lines by means of limiting dilution. The major advantage of this method is the ability to:

- identify the protein and epitope specificity of a single cell with proliferation assays
- determine secreted cytokine levels of a single clone
- isolate mRNA to further investigate TCR composition and TF expression
- determine the expression of surface proteins by flow cytometry

Birch pollen allergy / Type I hypersensitivity

Allergic diseases have reached epidemic dimensions in urban areas. They are associated with high economic costs and have a negative effect on the patient's quality of life.[21] Approximately 20% of the population in industrialized countries suffer from immediate hypersensitivity reactions.[22]
Pollen from wind-pollinated plants is among the most potent allergen sources. It contains several proteins which are responsible for cross-reactive allergies to fruits, nuts and vegetables. Allergen sensitization often occurs via the respiratory mucosa[23] and requires certain concentrations of pollen.[24]

Genetic predispositions play a critical role in the development of allergies (atopic predisposition). People with a history of atopy in the family have an increased risk to develop allergic symptoms.[25]

Patients suffering from type I allergies have aberrant T-cell responses to harmless antigens, dominated by T_H2 cells. As a result B-cells produce increased amounts of IgE antibodies against common environmental proteins.

Sensitization phase

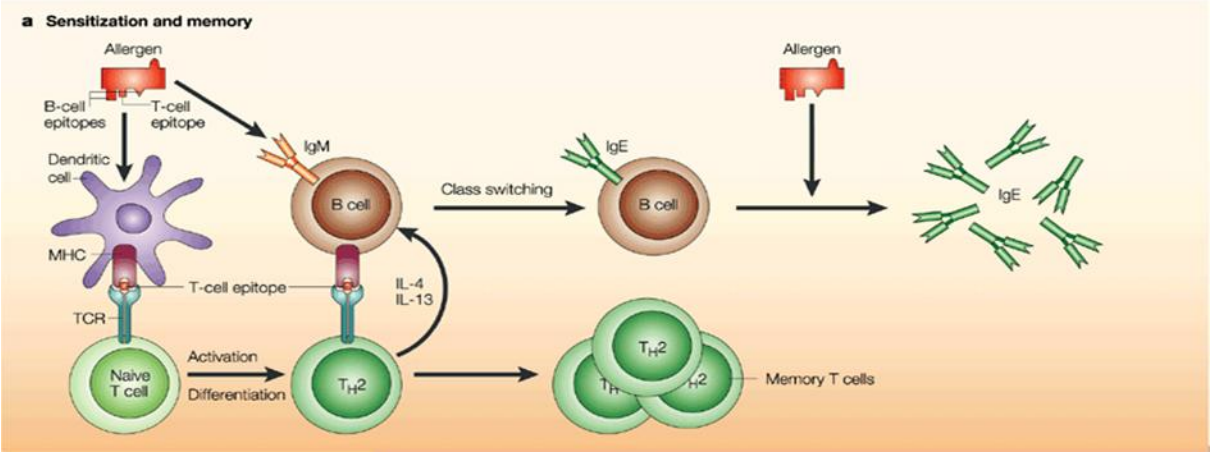


Illustration 1: Schematic representation of the sensitization phase[1]; Adapted from "The future of antigen-specific immunotherapy of allergy by Valenta R., 2002, Nature Reviews Immunology 2, 446-453;

An allergic reaction is preceded by a sensitization phase. Atopic individuals react to repeated exposure to allergens with the activation of specific T_H2 cells. APCs take up the allergen and present it to naive T-cells via their MHC II molecules, directing them towards the T_H2 phenotype as shown in Illustration 1. Mouse studies have suggested, that low-dose TLR

agonists such as LPS can influence dendritic cells in this process.[26] T_H2 cells secrete cytokines that lead to the development of characteristic allergic phenotypes such as class switching of B-cells to IgE (IL-4, IL-13), recruitment of mast cells (IL-4, IL-9, IL-13) and maturation of granulocytes (IL-3, IL-4).[27] Allergen-specific IgE antibodies bind to high affinity IgE receptors (FcεRI) on the surface of mast cells, basophilic granulocytes and eosinophilic granulocytes which are located in several tissues such as the nasal mucosa. FcεRI bound IgE antibodies are stable for several months to years.

The presence of allergen-specific IgE antibodies must not lead to allergic symptoms but most sensitized patients react to subsequent exposure of an allergen with immediate clinical allergic symptoms.

Immediate and late reaction

In the challenge phase or immediate reaction, mast cell bound IgE antibodies bind to the allergen as depicted in Illustration 2. Allergens can have several epitopes or can form multimers, a process that lead to binding of neighbouring IgE molecules to the same allergen. This process called cross-linking leads to mast cell degranulation and the release of inflammatory mediators such as histamine and leukotriens.[27] This causes the fast occurring symptoms 1-30 minutes after allergen contact which are typical for type I allergies such as skin hives, allergic rhinitis and conjunctivitis. The release of chemokines and proinflammatory cytokines from Th2 cells recruits macrophages, eosinophiles and basophiles that release inflammatory mediators causing a late response 6-27 hours after allergen contact[1](Illustration 3).

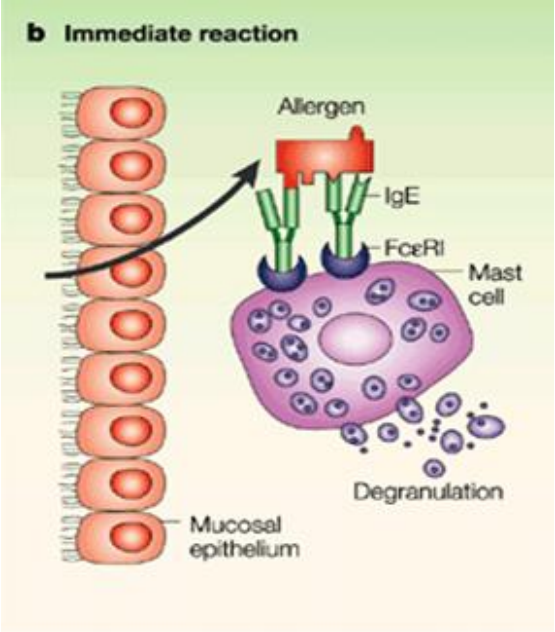


Illustration 2: Overview of the immediate reaction[1] Adapted from "The future of antigen-specific immunotherapy of allergy by Valenta R., 2002, Nature Reviews Immunology 2, 446-453;

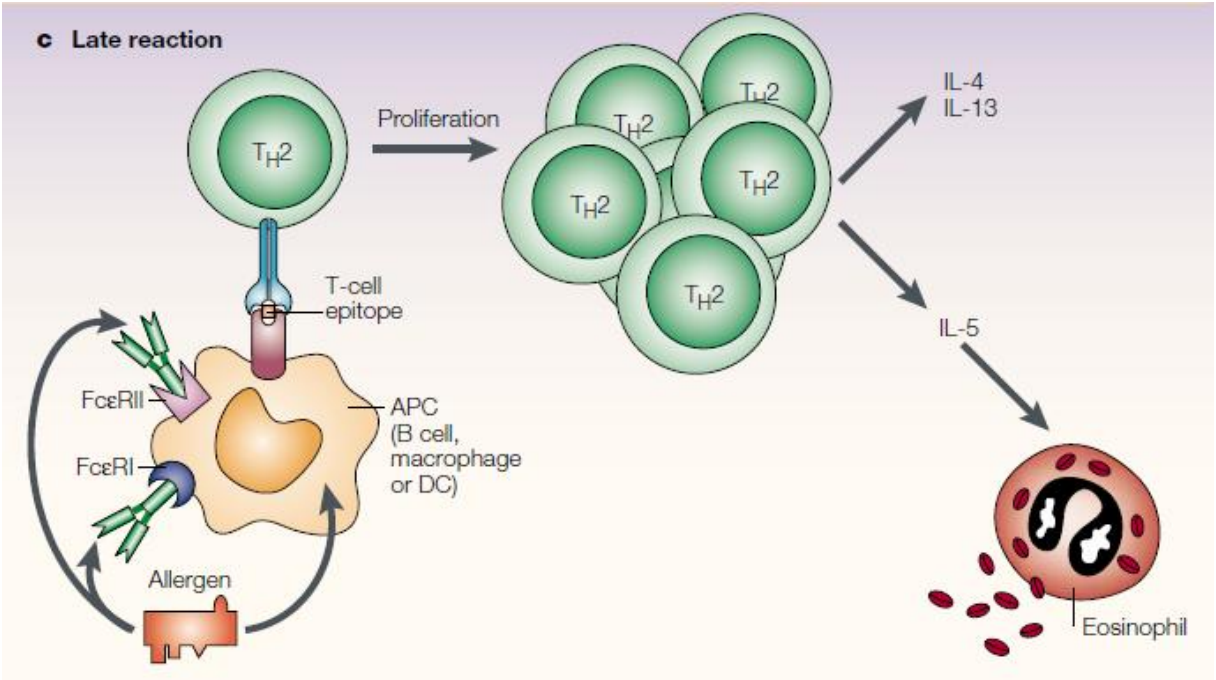


Illustration 3: Overview of the late reaction occuring 6-27h after allergen contact.[1] Adapted from "The future of antigen-specific immunotherapy of allergy by Valenta R., 2002, Nature Reviews Immunology 2, 446-453;

Birch pollen allergens

Birch trees are widely spread in Central and Northern Europe and they release large amounts of pollen during flowering season. The major allergen in the European white birch (*Betula verrucosa*) is Bet v 1.[28] Individuals who are sensitized to birch pollen are prone to develop birch pollen-related food allergy due to an IgE- and T-cell-mediated cross-reaction between Bet v 1 and structurally related food proteins.[29,30] It is one of best characterized allergens identified so far. Birch pollen contains several other proteins that are highly cross reactive to other allergens found in trees, grasses and weeds such as profilin (Bet v 2), two calcium binding proteins (Bet v 3 and Bet v 4), an isoflavone reductase homolog (Bet v 6) and a cyclophilin (Bet v 7). All identified birch pollen allergens are summarized in Table 3.

Allergen	Number of AA	Molecular weight(kDa)	Theoretical pI	Frequency of recognition
Bet v 1	160	17	5.39	>90%
Bet v 2	133	14	5.02	~15%
Bet v 3	205	23	4.6	n.a.
Bet v 4	85	9	4.76	~15%
Bet v 6	300	33	7.82	n.a.
Bet v 7	173	18	8.68	n.a.

Table 3: Summary of so far identified birch pollen allergens, amino acids (AA), theoretical isoeletric point (pI), n.a.= no studies available[3-5]

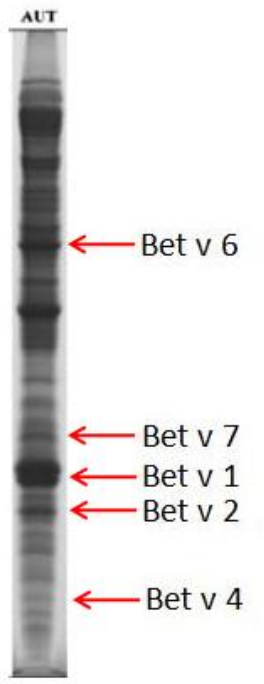


Figure 1 illustrates the different protein concentrations in an Austrian birch pollen sample. The identified minor allergens are indicated by red arrows.

The concentration of specific allergenic proteins in pollen or foods is thought to have an impact on allergenicity. Nonetheless, the quantity of allergens in birch pollen has only been estimated for Bet v 1. It accounts for 10% of the total protein content of *B. Pendula* pollen.[31] To get an idea of the allergen concentration in birch pollen, Figure 4 shows SDS-PAGE profiles of different birch pollen extracts.

Figure 1: SDS-PAGE profile of Austrian birch pollen extracts; Adapted from "Proteomic profiling of birch (Betula verrucosa) pollen extracts from different origins by Erler, A. et al., *Proteomics* 11, 1486-98 (2011). Red arrows indicate the identified minor allergens. Bet v 3 was not included in this study .[2]

Bet v 1 – the major birch pollen allergen

Bet v 1 is the major birch pollen allergen in Europe. Around 60% of birch pollen-allergic patients react exclusively to this allergen[32] and more than 90% of all tree pollen-allergic patients display IgE antibodies to Bet v 1.[3] It is part of the pathogenesis- related proteins (PR 10) family.[33] These proteins play a role in the immune system of plants but their physiological function is still unknown. Allergenic Bet v 1 homologues have been described in several foods such as hazelnut (Cor a 1)[34], peanut (Ara h 1)[35], apple (Mal d 1)[36], cherry (pru av 1)[37], pear (Pyr c 1), celery (api g 1)[38], carrot (Dau c 1)[39], soybean (Gly m 4)[40] and kiwi (Act d 8)[41].

Several isoforms of Bet v 1 are known, differing in their ability to activate T-cells and bind IgE. Bet v 1.0101 is the most common one. These isoforms can be classified into high, intermediate and low IgE binding classes shown in Table 4 .[42]

Bet v 1 isoform	Old nomenclature	IgE binding (rel %)	T-cell reactivity (rel %)	Skin prick test wheal areas (mm^2)
1.0101	Bet v 1 a	100	41	70
1.0501	Bet v 1 e	83	67	50
1.0201	Bet v 1 b	60	92	19
1.0401	Bet v 1 d	5	100	7
1.1001	Bet v 1 l	3	87	10

Table 4: (from Ferreira F. et al. 1996): Summary of Bet v 1 isoforms: IgE binding and T-cell reactiviy of the pure recombinant allergens relative to isolated Bet v 1 from birch pollen including all isoforms (natural Bet v 1). T-cell reactivity tested with 48 T-cell clones; IgE binding tested with 30 sera from birch pollen allergic patients.[42]

T-cells recognize linear processed amino acid sequences, therefore T-cell epitopes differ considerably from conformational B-cell epitopes and cross-reactivity with food proteins cannot be abolished by cooking or digestion. Bet v 1.0101 has been expressed as recombinant protein and characterized in great detail on molecular and immunological level. It contains ten prominent T-cell epitopes of which the epitope Bet v 1 $_{142\text{-}153}$ was shown to be the dominant one.[43] Hypoallergenic variants of Bet v 1.0101 have been created reducing the IgE binding capacities but maintaining the T-cell activating properties.

Minor allergens

Minor allergens are proteins recognized by <50% of sensitized patients.

Bet v 2

Bet v 2 is a 14 kDa protein of the profilin family. These molecules are actin binding proteins and are found in pollen from trees, weed and grass.[44] Approximately 15% of all birch pollen-allergic individuals are sensitized to Bet v 2.[4] Pollen profilins are cross-reacting with profilins in tomatoes, celery, carrots, peanuts, hazelnuts, bananas, apples and pineapple.[45-50]

Bet v 3

Bet v 3 is a 23,7 kDa protein that contains three typical calcium-binding motifs. It is expressed specifically in mature birch pollen and IgE binding requires protein bound Ca^{2+}.[51] The prevalence of recognition has not been determined yet.

Bet v 4

Bet v 4 is a 9 kDa protein found in birch pollen with two EF-hand calcium binding motifs.[52] Approximately 5% of birch pollen allergic patients and 10-15% of mugwort and timothy grass- pollen allergic patients have IgE antibodies recognizing Bet v 4.[53] It has sequential homologies to other calcium binding allergens found in Bermuda grass[54] and *Brassica*.[55] It also shares IgE epitopes with Cyn d 7 and Phl p 7, two EF-hand Ca^{2+} binding allergens found in grass pollen.[56] Most antibodies are directed to the C-terminal EF-hand domain but in contrast to Bet v 3, IgE recognition doesn't depend on protein bound calcium as substantial IgE binding can still be detected after calcium depletion.[57]

Bet v 6

Bet v 6 is a 35 kDa allergen expressed in birch pollen. It has a high sequence homology with isoflavone reductase homologue proteins (60-80%) such as the pear allergen Pyr c 5. It was further shown that Bet v 6 displays reductase activity.[58] Mouse antibodies directed against Bet v 6 recognize cross-reactive proteins in pear and lychee fruit.[59] Although only 10% of birch pollen-allergic subjects are sensitized to Bet v 6, [60] it is thought to represent a highly cross-reactive allergen in plant foods. The prevalence of recognition has not been determined yet.

Bet v 7

Bet v 7 is a 18 kDa member of the cyclophilin A family with a cis-trans isomerase activity.[61] Cyclophilins have been demonstrated to be stress-induced proteins[62] but the physiological function in pollen is still unknown. Proteins in alder, hazel and oak pollen extracts were detected by a polyclonal rabbit anti-Bet v 7 antibody, indicating a general immunological cross-reactivity among plant cyclophilins of group A.[63] The prevalence of recognition has not been determined yet.

To explore the mechanisms that drive Th2 polarization it is important to characterize involved allergens in great detail. In the present study, I tried to express, purify and characterize four minor allergens in birch pollen (Bet v 3, Bet v 4, Bet v 6 and Bet v 7). After purification, their IgE binding capacity, possible oligomerization and, abilitiy to activate allergen specific T-cells were examined. For further cellular characterization, T-cell clones and T-cell lines specific for proteins in birch pollen extract were isolated.

Aims of the study

The major aims of this project were to

- produce, purify and characterize recombinant Bet v 3, Bet v 4, Bet v 6 and Bet v 7

- isolate and characterize T- cell clones and T-cell lines specific for proteins in birch pollen extract from birch pollen-allergic patients

Materials and Methods

Cloning

Minor Birch pollen allergen DNA sequence

Constructs containing the sequence of minor birch pollen allergens flanked by desired restriction sites were ordered from a DNA synthesis company (GenScript – Gene synthesis service). The sequences encoding the minor allergens were obtained codon optimized for *E.coli* and cloned in the pUC57 vector.

DNA digestion

For quantitative digestion 1 µg of the DNA sample was incubated with 15 U of the desired restriction enzymes in the corresponding buffers (total volume = 50 µl) for 2-3 hours in a heat block at 37°C.

For test digestions 1 µg of the DNA sample was incubated with 5 U of the desired restriction enzymes in the corresponding buffers for 1 hour in a heatblock at 37°C.

The digested sample was then separated by agarose gelelectrophoresis.

Dephosphorylation of digested vectors

To prevent religation, digested vectors were dephosphorylized by incubating them with 5 U of Calf Intestine Alkaline Phosphatase (CIAP 1 U/µl, Fermentas) for 30 min at 37°C in a heatblock, followed by incubation at 85°C for 15 min to inactivate the enzyme.

Agarose gel electrophoresis

DNA samples were separared by agarose gel electrophoresis containing Ethidium Bromide (EtBr). For a 1 % (w/v) gel, 1 g of agarose was added to 100 ml 1 x TBE buffer. The solution was heated in a microwave till the agarose was completely dissolved and the solution was clear. After cooling to approximately 40 °C two drops of a 0,07% EtBr solution (0.7 mg/mL;

AppliChem GmbH, Darmstadt, Germany) were added and the solution was poured in a BioRad tray for polymerization.

DNA samples were mixed with Fermentas 6x loading dye before loading and the Gene Ruler™ 100 bp DNA ladder marker (Fermentas GmbH) was used as a size marker (100 bp – 2400 bp).

The gels were run at 90 V for ~ 40 min in the BioRad Sub-Cell GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories Ges.m.b.H.) using 1x TBE as running buffer.

Gel extraction of separated DNA samples using Quia Quick gel extraction kit

The weight of the later used eppendorf tube was measured and the desired DNA band was cut out of the agarose gel under UV light using a scalpel. The cut out piece was placed in the eppendorf tube and the weight of the gel piece was determined.

Then the DNA was extracted from the gel using the QIAquick Gel Extraction Kit and following the manufacturer's protocol. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

3 x gel volume of QG buffer was added to the gel (100 mg = 100 µl)

The sample was heated to 50°C in a heatblock for 10 min and vortexed every 3 minutes

1 x gel volume of Isopropanol was added and the sample was vortexed

The dissolved sample was transfered to a spin column in a 2 mL collector tube and was centrifuged for 1 min at 13 000 rpm

The flow through was discarded and 500 µl QG buffer were added

The sample was centrifuged for 1 min at 13 000 rpm and the flow through was discarded

The sample was incubated with 750 µl of PE buffer for 2 minutes at RT and then centrifuged for 1 min at 13 000 rpm and the flow through discarded

To elute the DNA, the column was placed in a new sterile eppendorf tube and 30 µl of nuclease free H_2O was added directly onto the filter

After 1 min incubation the sample was centrifuged for 1 min at 13 000 rpm

The flowthrough containing the eluted DNA was collected.

The concentration of the DNA was measured via NanoDrop and stored at -20°C

DNA Ligation

In order to add a N terminal HIS- tag to the recombinant proteins, all minor allergen genes were were cloned in the pHIS parallel 2 vector using restriction enzymes and T4 DNA ligase.

To calculate the needed amount of insert, the following formula was used:

ng insert = ng vector (~150 ng) x 10 x (bp insert / bp vector)

The digested DNA fragments were incubated with 2,5 units of T4 DNA ligase (Fermentas, 5U/µl) and the corresponding 10x T4 DNA ligase buffer and nuclease free H_2O in a total volume of 20 µl for 3 hours at RT .

The ligated constructs were then transformed in *E.coli* Top 10 cells.

Transformation in chemically competent *E.coli* strains

The constructs were transformed by a heat shock protocol first in *E.coli* TOP 10 cells (cloning strain without own plasmids) and then in *E.coli* BL21(DE3)pLysS cells (expression strain for quantitative protein expression) (Invitrogen GmbH, Lofer, Austria). All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

- 0,3 µg of the vector were added to the thawed *E.coli* cells and kept on ice for 20 min
- the cells were put into a heatblock at 42°C for exactly 45 seconds and immediately put back on ice for 2 min
- 1 ml SOC medium was added to recover the cells, thereafter they were kept for 15 min on RT
- Afterwards the cells were put into a heatblock at 37°C for 60 min and 350 rpm (not really necessary for transformation with a vector)
- 50 µl of the transformed cells were plated on a LB agar plate containing 100 µg/ml ampicillin to select for cells containing the vector
- The remaining cells were centrifuged in a table top centrifuge for 2 min with 4000 rpm
- The pellet was resuspended in 150 µl supernatant and also plated on a LB-agar plate with ampicillin
- The plates were incubated over night (o/n) at 37 °C
- On the next day the plates with the grown colonies were stored at 4 °C
- To check the transformed construct, several colonies were picked and seperate precultures were made in culture tubes containing 5 mL LB-Amp medium.
- The precultures were incubated at 37°C at 220 rpm o/n
- **Frozen stocks:** On the next day, frozen stocks were prepared by transfering 800µl of the preculture in a 2 mL cryovial and adding 70 µl of chilled DMSO to the cells

- The frozen stocks were immediately stored at -80°C
- Then plasmids were isolated from the precultures and the construct checked by a test digestion.
- Isolated plasmids containing the correct construct were then transformed by the described protocol in competent *E.coli* BL21(DE3)pLysS cells
- One single colony was picked from one plate with a sterile toothpick and put o/n in 5ml liquid LB-amp (100 µg/ml) medium at 37°C in a shaking incubator (~240 rpm) to grow a preculture for a testexpression and a frozen stock.

Plasmid preparation from *E.coli* using QUIAprep spin Miniprep Kit

Plasmids were extracted from overnight *E.coli* cultures using the QUIAprep Miniprep Kit according to the manufactureres protocol. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

Overnight culture:

Picked colonies were inoculated under sterile conditions in 15 mL culture tubes filled with 5 mL LB-Amp medium. The cultures were incubated at 37°C while shaking at 220 rpm for 16 hours.

Procedure:

- 4 mL of the overnight cultures were spinned down in two sterile 2 mL eppendorf tubes for 3 minutes at 6800 g
- The Supernatant was discarded and the pellet was dried on paper for 2 min
- The dried pellet was resuspended in 250 µl of cold P1 buffer
- 250 µl of P2 buffer were added and the tube was inverted 4-6 times
- After 4 min 350 µl of N3 were added, the tube was inverted 4-6 times
- The cells were centrifuged for 10 min at 17 900 g at RT
- The supernatant containing the plasmids was poured in the supplied spin column and centrifuged for 1 min at 17 900 g
- The flow through was discarded
- 500 µl PB were added to the column and it was centrifuged for 1 min at 17 900 g
- The flow through was discarded
- 750 µl PE were added to the column and it was centrifuged for 1 min at 17 900 g
- The column was placed in a new eppendorf tube and 50 µl of Elution buffer (EB) was applied directly onto the filter
- After 1 minute incubation, the column was centrifuged for 1 min at 17 900g
- The DNA concentration was measured via NanoDrop and the isolated plasmid was stored at -20°C

Protein expression and purification

Testexpression in E.coli BL21

The expression of minor allergens was performed in the *E.coli* BL21(DE3)pLysS expression strain (Invitrogen GmbH, Lofer, Austria). This strain encodes a T7 RNA polymerase gene downstream of a Lac operator. The genes encoding the minor allergens in the transformed vectors are located downstream of a T7 promoter and lac operator. IPTG was used to induce the expression of the T7 RNA polymerase which in turn facilitates the expression of the HIS-tagged minor allergens.

- 1,5 ml of a 5 ml o/n preculture were added to 50 ml pre-warmed LB-amp medium (--> 1:30 dilution) and kept at 37°C in a shaking incubator at 220 rpm
- Cells were grown till an OD_{600} between 0,6- 1
- The optical density was measured in a 1 ml plastic cuvette using a spectral photometer
- **0h sample**: 1 ml of the cell suspension was transformed in an eppi and centrifuged in a table top centrifuge for 2 min at 3500 g in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG). Then the pellet was resuspended in 50 µl 1x Sample buffer (SB) and stored on ice
- **IPTG induction**: Protein expression was iduced by adding 50 µl of IPTG (1M stock) to the 50 ml culture (1 mM final IPTG concentration)
- The cells were kept in the shaker at the desired temperature ranging from 18°C to 37°C at 220 rpm
- **1h- 5h samples:** Every hour a 50 µl sample was taken from the culture and centrifuged for 2 min at 3500 g. The resulting pellet was resuspended in 50 µl of 1x SB and stored on ice. The 0h-5h samples were stored at -20°C.
- **Harvesting:** After 5h the remaining culture was centrifuged in a Sorvall RC SC PLUS centrifuge (Thermo Scientific Inc.) for 20 min with 6000 g at 4°C
- The supernatant was discarded and the pellet can be stored at -20°C at this stage
- In order to break up the cells, the pellet was resuspended in 5 mL lysis buffer and vortexed till the pellet was completely resuspended
- The suspension was poured in a 50 ml falcon tube and a freeze – thaw cycle was repeated three times using liquid nitrogen (N_2) followed by a waterbath at 37°C to lyze the cells
- If the cell suspension has a gel like structure, incubation with 5 µg/ml DNase at RT can improve separating the soluble protein fraction from the insoluble
- The lysed cells were centrifuged in a Sorvall RC SC PLUS centrifuge for 30 min at 4°C with 20000 g

- **Soluble Protein:** The supernatant which was considered to be the soluble protein fraction was collected in a falcon tube and stored on ice
- **Insoluble Protein:** Recombinant produced proteins often aggregate in insoluble inclusion bodies. In order to solubilize these proteins the pellet representing the insoluble protein fraction was resuspended in Lysis buffer containing 5M Urea.
- The resulting suspension was centrifuged for 30 min at 4°C with 20000 g in a Sorvall centrifuge
- The supernatant which consists of the insoluble proteins was then collected in a falcon tube and stored on ice. The remaining pellet was discarded
- **Denaturation:** to denaturate the samples prior to the SDS PAGE, the 0h-5h samples were put in a heatblock at 95°C for 15 min. To denaturate the soluble and insoluble fractions collected after 5 h, 5µl of 4x SB were added to aliquots (15µl) of the fractions. They were also put on 95°C for 5 min

To determine the optimal expression conditions 12 µl of the collected samples were loaded on SDS-PAGE gels (15%) .

Separation of protein samples by SDS PAGE

Protein samples were separated by SDS polyacrylamide gelelectrophoresis. They were denaturated with 2-mercaptoethanol and SDS at 95°C and separated according to their molecular weight in a polyacrylamide gel by polyacrylamide gelelectrophoresis (PAGE). Gels containing 13- 17% polyacrylamide were produced (Table 5) and the separated proteins were visualized by coomassie staining.

	Stacking gel		Separating gel
	15%	17%	
Acrylamide	10 mL	11,3 mL	700 µL
Lower buffer	5 mL	5 mL	1,1 mL
ddH_2O	5 mL	3,6 mL	2,6 mL
TEMED	10 µL	10 µL	5 µL
10%APS	60 µL	60 µL	30 µL

Table 5: Separating- and stacking gel solution for 3 gels

- The reagents were mixed according to Table 1. The separating gel was poured between two sealed glas plates.
- 2 cm from the top were left for the stacking gel
- Isobutanol was added on the separation gel to ensure an even surface
- After polymerization of the gel, Isobutanol was removed, the surface was washed with ddH_20 and the stacking gel was poured on top
- After polymerization 5 µl of Fermentas PAGE Ruler^{TM} were used as a protein ladder and 12 µl protein sample was mixed with 4 µl 4 x SB and boiled at 95°C for 15 min prior to loading.
- Gels were run at 25 - 30 mAmp per gel until the dye front completely left the gel

Coomassie staining

SDS PAGE gels were incubated with the coomassie staining solution (Coomassie brilliant blue G250) under continuous shaking for ~ 30 min at RT on a slow shaker. The coomassie solution was discarded and the gels were incubated with a destaining solution containing acetic acid and methanol, until the background was completely destained and the protein bands were clearly visible.

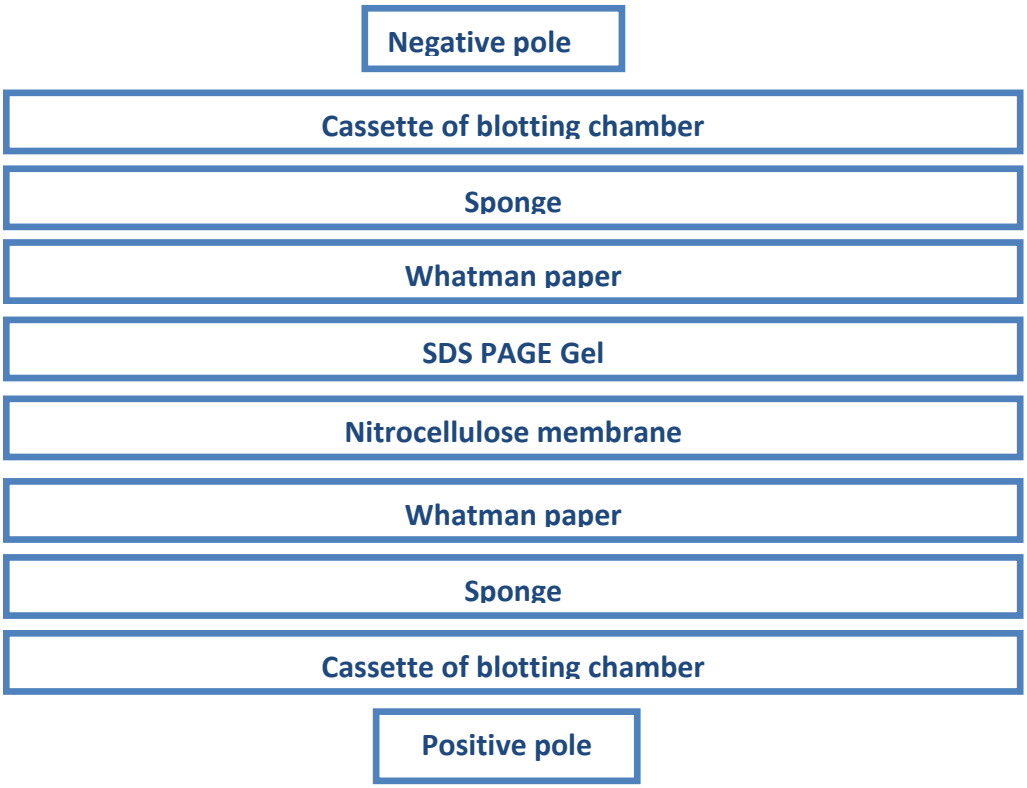
Western Blot

Western Blot was used to blot the separated proteins from a SDS PAGE gel onto a Whatman Protran Nitrocellulose Transfer membrane with 0.2 µm pore size (Whatman GmbH, Dassel, Germany) using an electric current generated in a Biosciences TE22 Tank Transfer Unit (GE Europe GmbH, Munich, Germany). Gels were blotted for 1 hour at 150 mA.

Thereafter HIS- tagged proteins were detected by a mouse Penta-His^{TM} IgG_1 antibody (QUIAGEN)

Scheme of the assembled blotting chamber:

All components were equilibrated in transfer buffer



- The membrane was dryed on paper and the bands of the protein marker were marked
- The dryed membrane can be stored at -20°C at this point
- The membrane was incubated with 20 mL Blocking buffer at RT on a shaker for 2h to block unspecific binding of antibodies
- Afterwards the membrane was washed for 3x with 15 mL Wash buffer for 10 min at RT
- Then it was incubated for 2h at RT with a mouse Penta-His™ IgG_1 antibody (QUIAGEN) diluted 1:4000 in TBS/0.1% (v/v) Tween 20 supplemented with BSA (4% w/v) (this step can also be performed o/n at 4°C)
- Then the membrane was washed for 3x with ~15 mL Wash buffer for 10 min at RT
- Incubated for 1.5h at RT with a HRP- tagged Goat anti Mouse IgG antibody (Cell
- Signaling Technology Inc, Danvers, USA) diluted 1: 10 000 with blocking buffer
- Washed 3x with ~15 mL Wash buffer for 10 min at RT
- Then the membrane was dried and placed in a clean petri dish
- A chemiluminescent reagent was used to detect the HRP linked sec. antibodies (Lumigen™ PS-3 detection reagent, GE Healthcare UK Ltd, Birminghamshire, UK)
- The cleavage of the chemiluminescent agent by the HRP creates an emmission of light which darkens the photofilm

- 4 mL Lumigen solution A were mixed with 100 µL detection reagent and the membrane was incubated for 5 min in the dark
- The membrane was fixed in a Hyperfilm detection box
- A photographic film (Amersham Hyperfilm^{TM} MP ; GE Europe GMbH, Munich, Germany) was exposed to the membrane in a darkroom and developed afterwards in a AGFA CP1000 developing unit (Agfa Graphics Germany GmbH & Co, KG, Düsseldorf, Germany)

Expression of recombinant birch pollen allergens

In order to produce minor allergens in lager quantities, the proteins were expressed in 2 L *E.coli* BL21(DE3)pLysS cultures. Optimal temperature and duration of the expression were determined by testexpressions. The cells were broken up with lysis buffer and freeze-thaw cycles. Most of the proteins were purified from the soluble fraction to maintain the natural conformation of the protein. All centrifugation steps were performed in a SORVALL RC SC PLUS centrufuge (Thermo Scientific Inc., Rockford, USA)

- o/n culture: Cells from a *E.coli* BL21 (DE3) frozen stock, containing a pHIS parallel 2-minor allergen construct were inoculated in 70 mL of prewarmed LB- amp medium
- the cells were incubated at 37°C in a shaker o/n
- on the next day 66 mL of the preculture were added to 2 L prewarmed LB- amp medium (2x 33 ml preculture in 1 L of LB Medium in a 5 L Erlenmeyer flask)
- the culture was grown at 37°C in a shaker till an OD_{600} of 1,1
- the expression of the minor allergen was induced by addition of IPTG (final concentration: 0,4 mM)
- after 4 ½ h of expression at 37°C the cells were harvested by centrifugation at 4000 g for 30 min at 4°C and the cell pellets were stored o/n at -20°C
- To lyse the cells, the pellet was resuspended in lysis buffer (1/10 of the former volume -> 200 ml) and vortexed till the pellet was completely resuspended
- The suspension was poured in several 50 ml falcon tubes and shock frozen in liquid N followed by thawing in a 37°C waterbath. This freeze-thawing cycle was repeated 2 times
- The cell suspension was incubated with DNase (5 µg/ml) at RT on a rock n roller to improve separation of the soluble and insoluble fractions
- The cell lysate was poured in centrifugation tubes and centrifuged for 30 min at 4°C with 18000 g
- The supernatant was collected, filtered through a MILLEX GV low protein filter unit (0.22 µm; Millipore Corporation) and stored at 4°C

Purification of His tagged proteins by Ni affinity chromatography

His-tagged minor allergens were purified using His-Trap FF crude 1 mL columns (GE Europe GmbH, Munich, Germany). The His-tag on the N treminus of the minor allergen binds to the immobilised nickel and any untagged proteins pass through the column. Bound proteins are eluted by an increasing imidazol gradient that competes with the His tag for the Ni agarose.

The purification of the proteins was performed using the ÄKTA^{TM} prime protein purification system (GE Europe GmbH, Munich, Germany) according to the manufacturer's instructions.

- The His-Trap FF crude 1 mL column (GE Europe GmbH, Munich, Germany) was washed with 10 mL (10 x column volume) of ddH_2O at a low flow rate (~ 1 ml/min) and equilibrated with 10 column volumes of Lysis buffer
- The ÄKTAprime system was washed with 40 mL of ddH_2O and 40 mL of Elution buffer, then equilibrated with 40mL of Lysis buffer buffer
- The filtered supernatant was loaded onto the column at a low flow rate (1-2 mL /min) with a backpressure maximum of 0.3 MPa
- the flow through was collected and stored on ice (flow through fraction)
- The column was washed with 10 column volumes of start buffer and the flow through was also collected as the WASH fraction
- Then the protein was eluted with 30 mL of an increasing imidazol gradient (100% Lysis buffer/ 0% Elution buffer → 20% Lysis buffer/ 80% Elution buffer) and fractions of 1mL were collected and stored on ice
- The ÄKTAprime system was washed with 40 mL of ddH_2O and 40 mL of 20% EtOH
- The His-Trap FF crude column was washed with 10 mL of ddH_2O and 10 mL of 100% EtOH and the stored at 4°C

Prior to dialysis, the eluted fractions were collected and analysed by means of SDS PAGE followed by coomassie staining.

Preparation of the dialysis membrane

The dialysis membrane (4 m length; Spectra/Por, Spectrum Medical Industries Inc.) was incubated for 2 hours at 60°C in 2000 mL pre-warmed dialysis preparation buffer. From time to time the solution was stirred. This step was repeated once. Afterwards, the membrane was incubated at 60°C in 2000 mL pre- warmed ddH_2O. This step was repeated till the solution was clear. The solution was slowly cooled down to 4°C. The dialysis membrane was stored in ddH_2O supplied with 1 mL/L chloroform at 4°C.

Dialysis of eluted fractions

Eluted fractions containing protein were pooled and dialysed against either PBS or a Sodium phosphate buffer to remove toxic imidazol and to minimize protein precipitation. Optimal buffer and pH of the dialysis buffer were determined empirically.

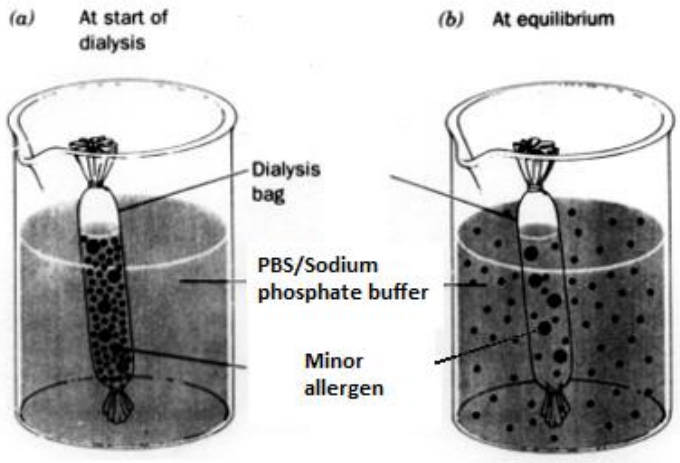


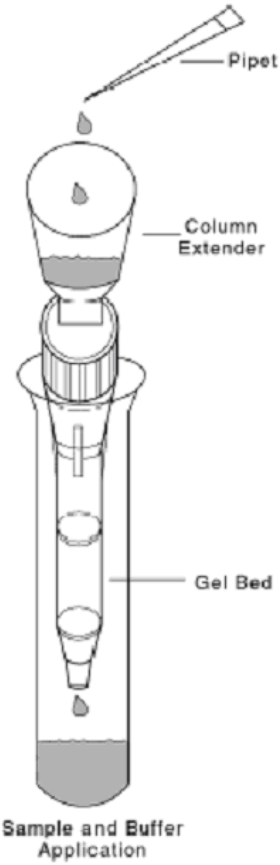
Figure 2: Scheme of the dialysis of Bet v 6 against PBS.

Pooled fractions were transfered in a semipermeable dialysis tube (cut off at 5 -7 kD, Spectra/Por, Spectrum Medical Industries Inc) and dialysed against 2 L dialysis buffer, at 4°C under stiring (schematically depicted in Figure 2). The dialysis buffer was changed 4x (1x in the morning, 1x in the evening for 2 days). 100 mM Sodium phosphate buffer with a pH of 6 turned out to be optimal for most of the minor allergens.

Endotoxin removal

To reduce LPS mediated side effects, such as activation of the TLR4 pathway in cellculture experiments, endotoxin was removed from the dialysed samples using EndoTrap® red 1 mL columns (Hyglos GmbH, Germany) according to the manufacturer's protocoll. These columns contain a resin that specifically binds LPS and lets the aquarious sample pass through.

- The protein sample was diluted 1:2 with endotoxin free water to decrease the salt concentration which could reduce the efficiency of the column
- All used equilibration and regeneration buffers (EB, RB) were filtered and degassed using a Steriltop-GP Filter Unit (0,22 µm, Millipore Corporation) connected to a vacuum pump
- All samples and buffers were kept on ice to avoid degradation of the protein
- The column was first regenerated and then equilibrated with 6 mL of regeneration/equilibration buffers
- The sample was applied and the flow through was collected
- 100 µl of the original sample were retained to determine endotoxin removal efficiency and protein loss during removal
- The column was filled with 1 mL of EB to collect the sample left in the column
- At the end the column was equilibrated with 6 mL EB
- To achieve a highly sufficient endotoxin reduction in the sample, the removal was repeated two times using the same column
- The collected samples were filtered through a MILLEX GV low protein filter unit (0,22 µm, Millipore Corporation, Billerica, USA) under sterile conditions and stored at 4°C.
- The column was filled with 1 ml RB 0,02% sodium azide, stored at 4°C and can be reused with the same protein



Bicinchoninic acid (BCA) assay

Protein concentrations were determined using the Pierce Endogen BCA (Thermo Scientific Inc., Rockford, USA) assay according to the manufacturer's protocol. Peptide bonds in the sample reduce Cu^{2+} ions of the copper sulfate in the reagents, to Cu^{1+} ions. Two Bicinchoninic acid molecules chelate with each reduced Cu^{1+} ion, forming a purple colored product that strongly absorbs at 562 nm. The concentration of the protein can be determined by comparing the absorption of the sample to a BSA standard row.

- Standard 96well plates were used for this assay
- BSA standard row:
    - 2000 µg/mL
    - 1500 µg/mL
    - 1000 µg/mL
    - 750 µg/mL
    - 500 µg/mL
    - 250 µg/mL
    - 125 µg/mL
    - 25 µg/mL
    - 0 µg/mL
- 25 µl of the standard row and the protein sample in different dilutions were added to the plate (dilutions were made with ddH_2O)
- The detection reagents were mixed in a 1 : 50 ration (Sol A : Sol B)
- 200 µl of the detection reagent were added (containing copper sulfate and bicinchoninic acid) to each well
- The plate was mixed gently and incubated for 30 min at 37°C
- The plate was cooled to RT
- The absorption at 562 nm was measured using a Spectra max Plus 384 plate reader (Molecular Devices, Sunnyvale, USA)

Determine endotoxin levels (LAL assay)

To determine the remaining endotoxin concentration in the sample, a chromogenic LAL assay was performed. Bacterial endotoxins (especially LPS) catalyze the activation of an enzyme in the Limulus Amebocyte Lysate which in turn releases p-nitroaniline (pNA) from a synthetic substrate, producing a yellow color. The pNA release is measured photometrically at 405-410 nm. The concentration of endotoxins can be calculated using an endotoxin standard.

- The vial with lyophilized *E.coli* endotoxin stock (20 EU/mL) was filled with 1 mL endotoxin free H_2O (= LAL water) and vortexed for 15 min
- The vial with the substrate was filled with 6,5 mL endotoxin free water and put in a 37°C waterbath 5 min before use
- A flat bottom 96 well plate (Corning Inc., Netherlands) was used for the assay
- Two standard rows were prepared seperately in endotoxin free 25 mL sterilin tubes
    - 1 EU/mL = 100 µl endotoxin stock + 1900 µl LAL water
    - 0,5 EU/mL = 500 µl 1 EU/mL + 500 µl LAL water
    - 0,25 EU/mL = 500 µl 1 EU/mL + 1500 µl LAL water
    - 0,1 EU/mL = 100 µl 1 EU/mL + 900 µl LAL water
- Several dilutions of the protein samples were prepared, depending on the expected endotoxin concentrations:
    - Without LPS removal = 1:100, 1:10 000
    - After 1x LPS removal = 1:100, 1:10 000
    - After 2x LPS removal = 1:100, 1:10 000
    - After 3x LPS removal = pure, 1:100, 1:1000
- The 96 well plate was heated to 37°C on a heatblock
- 50 µl of the prepared standard rows and samples were added to the wells
- 3 mL of LAL water were added to the vial containing the Amebocyte Lysate
- 50 µl of the lysate were added to the wells followed by gentle mixing, incubation time exactly (!) 10 min (start counting after adding lysate to the first well)
- After 10 min 100 µl of substrate were added in each well, followed by gentle mixing, incubation for exactly 6 min on the heatblock
- After 6 min 50 µl of the STOP reagent were added in each well
- The pNA release was measured photometrically at 405-410 nm using a Spectra max Plus 384 plate reader (Molecular Devices, Sunnyvale, USA)

Confirmation of IgE binding (ELISA)

The conservation of IgE epitopes on recombinant proteins was determined by IgE ELISA. Different allergens were coated on Nunc Maxisorb 96 well plates (Thermo Scientific Inc., Rockford, USA), followed by blocking with HSA and incubation with sera from birch pollen allergic patients. Specific IgE antibodies bind to the recombinant proteins and are detected by a secondary antibody coupled with an alkaline phosphatase.

Sera from several birch pollen allergic patients were selected with significant IgE binding to proteins with the same molecular weight as the produced allergens in an IgE immunoblot with birch pollen extract. (Bet v 3 = 23 kDa, Bet v 4 = 9 kDa, Bet v 6 = 33 kDa, Bet v 7 = 18 kDa)

Sera from non-allergic and a Bet v 1-allergic patients served as negative and positive control, respectively.

- A Nunc Maxisorb 96 well plate (Thermo Scientific Inc., Rockford, USA) was coated with different proteins
- Coating: The proteins were diluted in carbonate buffer (pH=9,6) and 50 µl were added to each well
- The plate was stored over night at 4°C
- The coating solution was removed and the plate was washed twice with PBS 0,05% Tween (200 µl/well). The solutions were removed by flicking the plate over a sink and patting the plate on a paper towel
- Blocking: To block the remaining protein binding sites the coated wells were incubated with 100 µl of PBS 0,05%Tween 1%HSA for 5h at room temperature
- Incubation with Antibody: The sera/plasma were diluted with PBS 0,05 % Tween 1 % HSA and 50 µl/well were added to the plates.
- The plates were incubated over night at 4°C
- Detection: The wells were washed 5 times with 200 µl of PBS 0,05 % Tween
- 50 µl of a Mouse anti-human IgE antibody coupled with an alkaline phosphatase (AP) (Diluted 1:3000 with PBS 0,05%Tween 1%HSA) were added to each well
- Incubated for 1h at 37°C then for 1h at 4°C
- The wells were washed for 5 times with 200µl of PBS 0,05%Tween
- Incubated with 100 µl of Paranitrophenylphosphate (1 mg/mL) per well at 37°C
- The absorption was measured photometrically at 405/550 nm using a Spectra max Plus 384 plate reader (Molecular Devices, Sunnyvale, USA) after several timepoints (2h, 3,5h, 5h and 19h)

Tissue culture

Patients with birch pollen allergy

All birch pollen allergic patients included in this study had rhinoconjunctivitis in spring and positive skin prick reactions (wheal diameter > 5mm) to birch pollen. *All patients gave written consent before enrolement in the study which was approved by the local Medical Ethical Committee of Vienna*.

PBMC isolation by Ficoll gradient

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of birch pollen allergic patients by ficoll gradient centrifugation. All centrifugation steps were performed in an Allegra^{TM} X-12R centrifuge (Beckman Coulter)

- Blood was collected in heparin tubes (9 mL LH Lithium Heparine, Vacuette) or a Blood Bag and poured in a sterile 1 L glass bottle
- The heparin tubes were washed 2 x with DPBS (Gibco®, Invitrogen GmbH, Austria) and DPBS was added to the heparinized blood till a 1:2 dilution was reached
- 50 mL Falcon tubes were filled with 15 mL Ficoll solution
- 35 mL diluted blood was gently pipetted on the top of the Ficoll solution
- The tubes were spinned 30 min at 400 g at RT without brake
- The plasma was collected in a new falcon tube, around 10 mL of plasma were left in the tube
- The white blood cell ring fraction of two gradients were combined in a new 50 mL Falcon tube using a sterile 5 mL glass pipette (aspiration of ficoll was avoided)
- The volume of the tubes was adjusted to 50 mL using DPBS
- The tubes were spinned for 10 min at 300 g at RT
- The supernatant was discarded and the pellet was loosened by tapping on the flask
- The pellets were resuspended in 5 mL DPBS and the cells from two tubes were combined in one. The empty tubes were rinsed with DPBS and added to the combined cell suspensions.The volume of the tubes was adjusted to 50 mL using DPBS
- The tubes were spinned for 8 min at 275 g at RT, the pellets resuspended in DPBS and the cells from all tubes combined in one
- The volume of the cell suspension was adjusted to 50 mL with DPBS and the cells were counted with a Bürker- Türk counting chamber (0,1 mm depth, BRAND, Germany)

Stimulation of PBMCs (Primary response)

To test a possible toxicity of recombinant allergens and to define their optimal concentration in T-cell proliferation assays primary responses were performed.

Isolated PBMCs were distributed in a 96 well round bottom culture plate (TC Microwell 96U, NUNC™, Denmark) (200 000 cells/well) and stimulated with titrated allergens, a medium control and a positive control using 2 Units of IL-2 per well. All stimulations were performed in triplicates and the allergens were diluted in AIM V (Gibco®, Invitrogen GmbH, Austria) to the desired concentration to minimize stimulation by the culture medium.

The cells were incubated for 6 days, then ^{3}H labeled thymidine (Perkin Elmer, 1 µCi/mL) was added to the PBMCs (final concentration 0,5 µCi/well). After 12-16h the cells were harvested on a fibreglas filter (Printer Filtermat A 90 x 120 mm, Perkin Elmer), 4-5 mL of szintilization liquid (Betaplate Scint, Perkin Elmer) were added and the converted radiation measured by a ß-counter (MicroBeta TriLux, Perkin Elmer)

Generation of T-cell lines

Addition of the allergen led to the enrichment of allergen specific T-cells and to the upregulation of the IL-2 receptor. Addition of IL-2 at day 4-6 led to increased survival and proliferation of T-cells. At day 8-12 blast enrichment was performed to remove all dead cells. All centrifugation steps were performed in an Allegra™ X-12R centrifuge (Beckman Coulter)

Preparation of T-cell line (Day 0)

PBMCs isolated from birch pollen allergic patients were diluted in AIM V (Gibco®, Invitrogen GmbH, Austria) and added to a 24 well cell culture plate (Corning Inc., Netherlands) (1.5 Mio PBMCs/ well). Tested allergens/proteins were diluted to the desired concentration in UCØ, one well with PBMCs only treated with UCØ as a control.

Addition of IL-2 (Day 4-6)

Between day 4 and 6, T-cell clusters were visible under the microscope. IL-2 was diluted in UCØ and added to the wells drop by drop (10 U IL-2 in 500 µl UCØ/ well) . After addition of IL-2 the cells were checked every day under the microscope. From day 8 on blastenrichment could be performed.

Blast enrichment (Day 8-12)

The stimulated PBMCs were resuspended and transfered from the 24 well plate to 25 mL sterilin tubes. Touching the bottom of the well was avoided while resuspending to prevent detachment of unwanted fibroblasts. The wells were washed 2 x with 1 mL UCØ and the cells

were then transfered in the 25 mL sterilin tubes. 7 mL ficoll solution was overlayed with the resuspended cells and the dead cells were separated by density gradient centrifugation at 390 g for 15 min without brake. The cells from the interphase (yellow ring visible above the ficoll) were transfered in a new sterilin tube together with 10 mL UCØ. The cells were centrifuged at 800 g for 10 min, the suppernatant aspirated and the pellet resuspended in 1 mL UCØ. The cells were counted with a Bürker- Türk counting chamber (0,1 mm depth, BRAND, Germany) with trypan blue and diluted to 50 000 cells/mL. 500 cells were used for T-cell cloning and the remaining cells were pooled to establish a T-cell line.

T-cell line

The remaining T-cells from the blast enrichment were pooled and transfered to a sterile 96 well round bottom culture plate (TC Microwell 96U, NUNC^{TM}, Denmark). 200 000 T-cells in 100µl UC+HUS were incubated together with 100 000 irradiated PBMCs (= Feeder cells) in 100µl UC+HUS and 2 U of IL-2 in a well.

The T-cell lines were expanded 2 x a week with 100 000 unspecific irradiated feeder cells and 2 U IL-2 per well. They were rested for 10 days prior to specific stimulation with allergens.

Specific stimulation of T-cell lines

To determine the specificity of birch pollen specific T-cell lines to different allergens secondary responses were performed.

The specific stimulations were performed in sterile 96 well round bottom culture plate (TC Microwell 96U, NUNC^{TM}, Denmark)

The expanded T-cell lines were stimulated with birch pollen extract (25 µg/mL), recombinant birch pollen allergens (5 µg/mL), and Bet v 1 a homologous proteins in peach and apple (5 µg/mL) in the presence of 200 000 irradiated, autologous PBMCs per well. IL-2 (2 U/well) and PHA (0,5%) treated cells served as positive control.

All stimulations were performed in duplicates and compared to untreated T-cells incubated with autologous PBMCs. The proliferation was assesed after 48h by ^{3}H-thymidine incorporation (0,5 µCi/well).

A mean SI of >2 was defined as specific T-cell proliferation.

Epitope mapping of Bet v 1-specific TCCs

To determine the epitopes of Bet v 1a that are recognized by T-cells, an epitope mapping was performed.

The assays were performed in sterile 96 well round bottom culture plate (TC Microwell 96U, NUNC^{TM}, Denmark).

Rested birch pollen-specific T-cell lines and clones were stimulated with a pannel of 50 overlapping, synthetic 12-mer peptides (5 µg/mL) (Mimotopes, Biotrend), representing the complete amino acid sequence of Bet v 1a[43]. The T-cell cultures were incubated with the peptides in the presence of irradiated, autologous PBMCs (100 000 cells/well).

The proliferation was assessed after 48h by ^{3}H-thymidine incorporation. A mean SI of >2 was defined as specific T-cell proliferation after incubation with the peptides.

Cloning by limiting dilution

Birch pollen specific T-cell clones (TCC) were established from birch pollen specific T-cell lines by means of limiting dilution and tested for their reactivity with birch pollen extract and recombinant allergens. Specific TCCs were characterized by flow cytometry and checked for allergen induced proliferation and cytokine production.

T-cells collected after blast enrichment were diluted in two 1:10 dilution steps with UCØ to 500 cells /5 mL. These 500 cells were incubated in a sterile 200 mL glass bottle with 175 mL UC+HUS, 300 Mio irradiated PBMCs (=Feeder cells) resuspended in 20 mL UC+HUS and 4000 U IL-2. As last step 0,5 mL PHA (100%) were added and the suspension distributed among 10 sterile 96 well round bottom cell culture plates (200 µl/well). The cells were incubated for 14 days at 37°C 5% CO_2 95% rH.

1st screening: After 14 days the cells were analysed under the microscope: (**2nd** screening after 21 days **3rd** screening after 28 days)

- Wells containing a clone that has not proliferated enough were labeled with a dot on the lid of the plate: 100µl of the supernatant were aspirated and 100 000 feeder + 2 U IL-2 were added to stimulate proliferation
- Wells containing a clone that had proliferated enough to be split 1:2, were labeled with a circle on the lid

- Clones labeled with a circle were transfered to a new sterile 96 well round bottom culture plate and split 1:2 (--> 2 wells per clone). These clones were numbered consecutively and rested for 10 days
- After 10 days, one well per expanded clone was used for specific stimulation.

Birch pollen-reactive TCCs were expanded by alternating turns of stimulation with irradiated PBMCs together with allergen or IL-2.

Specific stimulation of T-cell clones

80% of the medium from one well of each clone was aspirated and 200 µL UCØ were added. The cells were resuspended gently, transfered on a new sterile 96 well round bottom culture plate and divided among two wells. One well of each clone recieved 100 000 autologous PBMCs in UCØ = **unstimulated**. The cells in the other well were incubated with 100 000 autologous feeder in UCØ together with recombinant allergen = **stimulated.** The optimal allergen concentration was determined empirically. After 48h 100 µl of the supernatant was collected to determine the amount of produced cytokines and cell proliferation was assesed by ^{3}H Thymidine incorporation. Incubated clones with a SI (= stimulation index = stimulated/ unstimulated well) >5 were counted as allergen specific.

Cytokine measurement

Supernatants were collected 24h and 48h after stimulation. Cultures containing TCCs and PBMCs alone served as controls. Cytokine levels in supernatants were measured using the Luminex system. TCCs were assigned to Th subsets according to the released IL-4 and IFNγ levels:

Th2 = IL-4 : IFNγ greater than 5
Th1 = IL-4 : IFNγ less than 0,2
Th0 = IL-4 : IFNγ 0.2 to 5

Characterization of TCCs by flow cytometry

To further characterize the allergen specific TCCs they were rested for 7 days and stained with:

Antibody	Isotype	Dilution	Company
TCRα/β - FITC	mIgG1	1:10	BD
TIM 3 - PE	mIgG1	1:10	BioLegend
CD4 - PerCP	mIgG1	1:20	BD
CCR3 - APC	mIgG2b	1:20	BioLegend
CLA - FITC	rIgM	1:10	Pharmingen
CCR6 - PE	mIgG1	1:10	BD
CD 161 - APC	mIgG1	1:5	BD
CCR5 - FITC	rIgG2a	1:10	BioLegend
CRTh2 unlabeled	rIgG2a	1:20	Pharmingen
Goat anti rat IgG - PE		1:1000	BD

Isotype matched antibodies were used as controls. In pilot experiments some TCCs were stained 24h (activated) and 7 days (rested) after allergen stimulation. A downregulation of CD3 and Cr Th2 in the activated cells was observed. Therefore all TCCs were stained 7 days after allergen specific stimulation.

Harvesting cells: Allergen specific T-cells were pooled in micronic tubes together with 1 mL FACS buffer. The cells were centrifuged for 8 min at 700 rpm in a Allegra^{TM} X-12R centrifuge (Beckman Coulter) and the supernatant aspirated. **Blocking:** The cell pellet was resuspended in 50 µl blocking buffer (AB serum (20%) in FACS buffer) and incubated for 30 min at 4°C. **Staining:** The cells were centrifuged for 8 min at 700 g in a Allegra^{TM} X-12R centrifuge

(Beckman Coulter) and the supernatant aspirated. Then the cells were incubated with 30 µl of prepared antibody solutions after one washing step and incubated for 30 min at 4°C. Thereafter the cells were centrifuged, the supernatant aspirated, the cells resuspended in 500 µl FACS buffer and transfered in FACS tubes for analysis.

Analysis was performed on a FACStar plus (Beckton Dickinson, San Jose) with CELLquest Software version 3.3.

mRNA isolation using RNeasy kit (Quiagen)

To characterize the T-cell receptor, mRNA was islolated from allergen specific TCCs with the RNeasy mRNA isolation kit (QUIAGEN) by following the manufacturer's protocol. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

12 wells of each allergen specific TCC were reserved for mRNA isolation and expanded three times without the addition of irradiated PBMCs to avoid contamination with feeder mRNA. The cells were rested for 7 days and collected in a 25 mL sterilin tube. The cells were lysed by resuspending them in 600 µl RTL buffer (+ ß mercaptoethanol). The lysate was homogenized by passing it 5 x through a blunt 20-gauge needle. One volume of 70% EtOH was added and 700 µl of the sample loaded onto a RNeasy column in a collection tube. The column was centrifuged for 15 sec at 8000 g and the flow through discarded. This was repeated till the full lysate was loaded onto the column. Then 700 µl RW1 buffer were added to the column, it was centrifuged for 15 sec at 8000 g and the flow through was discarded. 500 µl RPE buffer were added to the column and it was centrifuged for 15 sec at 8000 g. Then 500 µl RPE buffer were loaded to the column and it was centrufuged for 2 min at 14 000 g. To elute the mRNA, the column was placed into a 1.5 mL collection tube and 30 µl nuclease free water (QUIAGEN) were added directly onto the membrane of the column. It was centrifuged for 1 min at 8000 g and put on ice. The concentration of the eluted mRNA was measured via NanoDrop^{TM} 1000 (PEQLAB, Germany) and then either direclty transcribed to cDNA or stored at -80°C.

Transcription to cDNA using the GeneAmp RNA PCR kit

The isolated mRNA from the allergen specific TCCs was transcribed to cDNA by PCR using the GeneAmp RNA PCR kit (Applied Biosystems, USA).

The following master mix was prepared on ice:

Master mix	1 tube (volume: 40 µl)
10 x PCR buffer II	4 µl
MgCl (25 mM)	8 µl
dATP, dCTP, dGTP, dTTP (10 mM)	4 µl per base
Oligo d(T)16 primer (50 µM)	2 µl
RNase inhibitor (20 U/µl)	2 µl
MuLV Reverse Transcriptase (50 U/µl)	2 µl
isolated RNA	6 µl

The PCR was performed using a peqSTAR 96 HPL Gradient thermocycler (PEQLAB, Germany) with the following settings:

Heat Lid to 110°C

Pause at 20°C

42°C for 1 hour

95°C for 5 min

Store forever at 4°C

After transcription the cDNA was stored at -20°C.

T-cell receptor family typing

The T-cell receptor family type of the α and β chain was determined by PCR to further characterize the TCR and to confirm the monoclonality of TCCs.

In this assay the isolated and transcribed cDNA of the T-cell clones was used as a template together with a constant reverse primer and family type specific forward primers in a PCR. Primer amplifying a housekeeping gene (ß-actin) were used as a positive control and reaction mixes without template served as negative controls.

The following components were mixed on ice:

Master mix	1 tube (volume: 20 µl)
Nuclease free water (QUIAGEN)	14.3 µl
10 x PCR buffer (Dynazyme)	2 µl
dNTPs (10 mM)	0.5 µl
DyNAzyme™ II DNA polymerase (2 U/µl)	0.2 µl
α/β/ßactin 3' and 5' primer (5 mM)	1 µl each
Template cDNA (diluted 1:5)	1 µl

The PCR was performed using a peqSTAR 96 HPL Gradient thermocycler (PEQLAB, Germany) with the following settings:

Heat Lid to 110°C

95°C for 1 min

Pause at 95°C → put in PCR tubes

94°C for 3 min

Start loop (35x)

95°C for 30 sec

57°C for 35 sec

72°C for 45 sec

Close loop

72°C for 7 min

Store forever at 4°C

Afterwards the samples were mixed with Fermentas 6 x loading dye and analysed on a 1% (w/v) agarose gel.

Storage of T-cell clones

Remaining allergen specific T-cell clones were stored in liquid N2.

TCC freezing medium was prepared (90% FCS, 10% DMSO, Gentamycin (2 mL/L)) and stored on ice. 2 mL cryovials were labeled and also stored on ice. Depending on their density 8-12 wells of TCCs were pooled in a 25 mL sterilin tube and centrifuged at 700 g for 8 min in a Allegra^{TM} X-12R centrifuge (Beckman Coulter). The supernatant was aspirated and the cells resuspended in chilled freezing medium. 1 mL of the cell suspension was transfered in each cryovial and stored o/n in a cryocontainer at -80°C. The next day they were stored in a liquid nitrogen dewar.

Results

Production and purification of birch pollen allergens

Cloning of DNA encoding birch pollen allergens in the pHIS parallel 2 vector

DNA sequences encoding the genes of the allergens Bet v 3 and Bet v 7, flanked by restriction sites for BamHI and Not I were ordered from a DNA synthesis company (GenScript – Gene synthesis service). For protein expression, the synthesized DNA sequences were fused with a N terminal HIS- tag by cloning it in the pHIS parallel 2 vector (sequence in the appendix) using the BamHI and Not I restriction sites.

Sequence of Bet v 3:

>gi|488604:1-618 B.verrucosa Betv III mRNA for pollen allergen

TATAGGATCCATGCCCTGTTCCACAGAAGCCATGGAAAAAGCAGGGCATGGGCATGCAAGTACACCTCGCAAGCGTAGCC
TCAGCAACTCGTCCTTCCGCCTCCGCTCAGAGAGTCTGAATACCCTCCGCCTCCGACGCATATTCGATCTATTTGACAAGAA
CAGCGATGGCATCATCACCGTCGATGAACTCAGCCGAGCCCTCAACCTTCTTGGCCTCGAAACCGACCTCTCGGAGCTCGA
ATCCACCGTCAAATCATTCACTCGAGAGGGCAACATTGGGCTTCAATTCGAAGACTTCATATCGCTGCACCAATCCCTAAAC
GACAGCTACTTTGCTTACGGCGGCGAGGATGAGGATGATAATGAGGAGGATATGAGAAAGAGCATATTGTCGCAAGAGG
AAGCAGATTCTTTCGGAGGCTTCAAGGTGTTCGACGAGGACGGGGATGGTTACATATCGGCCAGAGAACTGCAAATGGT
ACTGGGGAAGCTGGGATTCTCTGAAGGGAGCGAGATTGACAGAGTTGAGAAGATGATCGTGTCCGTTGACAGCAACCGA
GATGGCCGGGTTGATTTCTTTGAGTTCAAGGATATGATGCGTAGCGTTCTCGTGCGGAGCTCTTGAGCGGCCGCTATA

Sequence of Bet v 7:

>gi|21886602:35-556 Betula verrucosa mRNA for peptidylprolyl isomerase (cyclophilin), ppiase gene (CyP)

TATAGGATCCATGGCGTCAAACCCTAAGGTCTTCTTCGACATGGAGGTCGGTGGCCAGCCCGTTGGGCGGATGTGATGGA
GCTCTACGCCGACACCACTCCCCGCACGGCCGAGAACTTCCGGGCCCTCTGCACCGGTGAGAAGGGCAACGGCCGCTCCG
GCAAGCCCCTCCACTACAAGAAATCGTCCTTCCACAGGGTGATCCCCGGGTTCATGTGCCAGGGGGGCGACTTCACTGCC
GGAAACGGCACCGGTGGCGAGTCCATCTACGGCGCCAAGTTCGCCGATGAGAACTTCATCAAGAAGCACACCGGCCCCG
GCATCCTCTCCATGGCTAATGCCGGCCCCGGCACCAATGGATCTCAGTTCTTCATCTGTACCGCCAAGACCGAGTGGCTCG
ACGGCAAGCACGTGGTGTTCGGCCAGGTCGTGGAGGGTCTGGACATCGTGAAAGCCATCGAGAAGGTCGGGTCCAGCTC
CGGCAGGACTTCCAAGCCCGTGGTCGTCGCCGACTGTGGTCAACTCTCTTAGGCGGCCGCTATA

Restriction sites for BamHI and Not I in red

The constructs were transformed into One Shot®TOP10 Chemically competent *E.coli* cells (Invitrogen) by a heat shock protocol and plated on LB-agar plates containing ampicillin as a selection marker. Several colonies were picked and the plasmids isolated from 5 mL cultures using the QUIAprep spin Miniprep Kit (QUIAGEN). The correct size of the insert was checked by testdigestion and agarose gel electrophoresis (Figure 3).

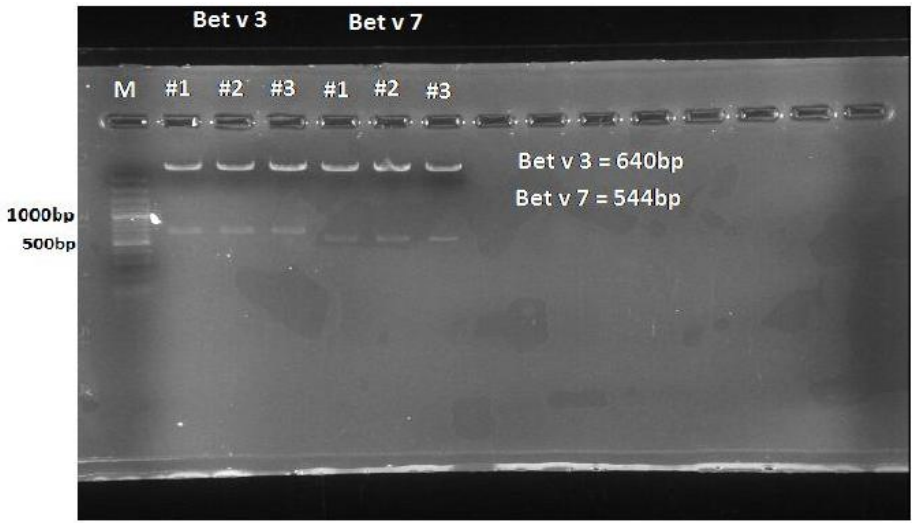


Figure 3: Test digestion of Bet v 3 and Bet v 7 constructs: Digested constructs were separated on a 1%(w/v) agarose gel containing EtBr. M: gene ruler 100 bp ladder marker; Bet v 4: 640bp; Bet v 7 : 544bp

Figure 3 shows the testdigestion of isolated plasmids from six transformed colonies. All vectors contain inserts with the correct size (Bet v 3: 640 bp, Lane 2-4; Bet v 7: 544 bp, Lane 5-7), indicating that the Bet v 3 and Bet v 7 genes were ligated successfully into the pHis parallel 2 vector.

Constructs encoding the Bet v 4 and the Bet v 6 protein fused to a N-terminal His-tag were already available.

Sequence of Bet v 4 :

>gi|809535|emb|X87153.1| B.verrucosa mRNA for BETV4 pollen allergen

CAACAGTGGAAACAAAAATGGCTGATGATCATCCACAGGACAAGGCTGAACGCGAGCGCATTTTCAAGCGCTTTGACGCC
AATGGCGATGGTAAAATCTCTGCAGCAGAGCTTGGGGAGGCCTTGAAAACACTTGGCTCCATCACACCGGATGAGGTGAA
ACATATGATGGCCGAGATTGACACCGATGGCGACGGCTTCATTTCGTTCCAAGAGTTCACGGATTTTGGTCGTGCTAATCG
TGGTTTACTAAAGGATGTTGCCAAGATATTTTAATGTCTCTGTCTCTTTCTCTTTTTTGTCATATTTTCATGTCATGATCTCCTT
GTTTAGAGTGATTTATTTTCATGGCTATGGTCCGTTTGGATTTTTCTCATTATAGAATTATTTTTGGTAGTTCGACTGTCACCT
CTTGTATTCTAATATCAATGGTGTTTGACCGTATTATTGTAACAAGAAAATGGTTCATCAGATTCGTGCATGTACATC

Sequence of Bet v 6 partial CDS:

>gi|4731375|gb|AF135127.1| Betula pendula isoflavone reductase homolog Bet v 6.0101 (BETV6) mRNA, partial cds

ATGGCTCACAAAAGCAAGATTCTGATCATCGGAGGCACCGGCTACATCGGAAAATTCATCGTGGAAGCAAGTGCAAAGTC
TGGCCATCCCACCTTTGCTTTGGTCAGAGAGAGTACGGTCTCTGATCCCGTTAAGGGAAAACTTGTCGAGAAATTCAAGGG
CTTAGGCGTCACTTTGCTCCATGGAGATCTGTATGACCATGAGAGTTTGGTAAAGGCGTTTAAGCAGGTGGACGTGGTGA
TATCGACGGTAGGCCACCTGCAGTTAGCAGATCAGGTCAAGATTATTGCTGCCATTAAAGAGGCTGGTAATATTAAGAGA
TTCTTCCCTTCGGAATTCGGAAACGACGTAGACCGTGTGCATGCTGTTGAGCCAGCAAAGACTGCATTTGCTACCAAGGCT
GAAATCCGCCGCAAGACTGAGGCTGAAGGCATCCCCTACACTTATGTGTCATCCAATTTCTTCGCTGGATATTTTCTTCCTA
CGTTGGCACAACCAGGACTCACTTCTCCTCCTAGAGAGAAAGTCGTTATCTTCGGAGATGGAAATGCCAGGGCTGTTTTTA
ACAAGGAAGACGACATAGGAACTTACACAATTAGAGCTGTGGATGACCCAAGAACACTGAATAAGATAGTCTACATCAAG
CCTGCCAAGAACATTTACTCATTCAATGAGATTGTTGCCCTTTGGGAGAAAAAGATTGGCAAAACCCTTGAGAAAATCTAT
GTTCCAGAGGAGAAACTTTTGAAGGACATCCAAGAGTCCCCAATTCCAATCAACGTGATATTAGCAATCAACCACTCAGTT
TTTGTGAAGGGAGATCATACCAACTTTGAGATTGAGGCATCCTTCGGTGTGGAGGCCTCCGAGCTATACCCAGATGTCAAA
TACACCACAGTG

Testexpression of birch pollen allergens in *E.coli* BL21 (DE3)pLysS

In order to express the minor allergens, the isolated constructs were transformed in the *E.coli* BL21(DE3)pLysS expression strain (Invitrogen GmbH, Lofer, Austria). To determine the optimal expression conditions, testexpressions were performed varying in expression time and temperature. Collected samples were applied to SDS-PAGE (15%, 15µl per slot) under reducing conditions and stained with Coomassie brilliant blue R-250. For immunoblotting, proteins were transfered onto a nitrocellulose membrane by means of tank blotting and incubated with a Penta-his IgG1 antibody (Quiagen).

Testexpression of Bet v 3 at 37°C :

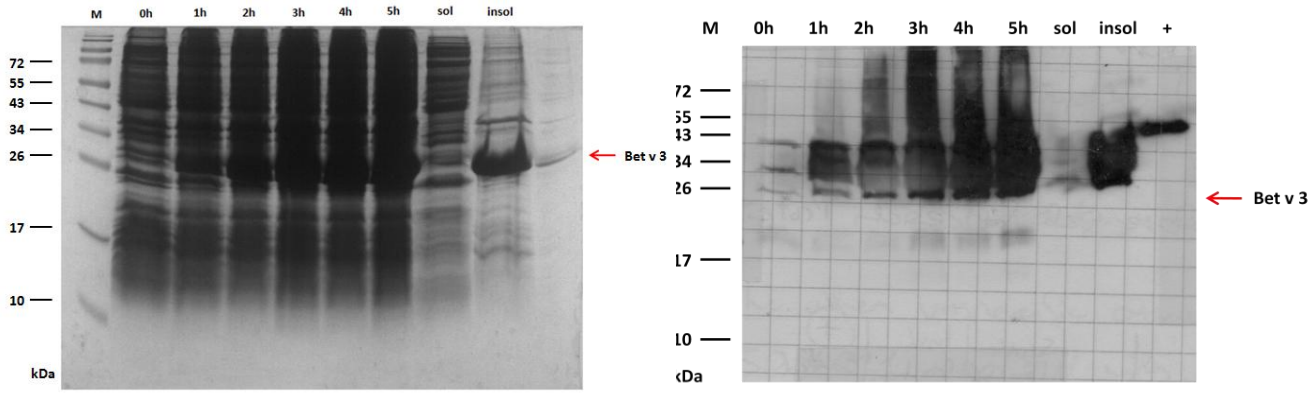


Figure 4: Left: Expression of Bet v 3 at 37°C. Fractions obtained before (0h) and after addition of IPTG (1h - 5h) were analysed by means of SDS PAGE (15%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3 with a molecular weight of 26 kDa

Right: Detection of HIS-tagged Bet v 3 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (1h - 5h). Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 2 seconds exposure to the fotofilm.

Bet v 3 (expected with a MW of 26 kDa) was found at all timepoints of IPTG induced expression (Figure 4). High amounts of Bet v 3 were found 5h after the addition of IPTG. After 5h of induced expression and cell lysis, the majority of the protein was found in the urea-treated insoluble fraction.

The results of the western blot confirmed the data shown in Figure 3. Before the addition of IPTG low amounts of Bet v 3 were detected (0h). After induction, the His- tagged Bet v 3 protein was expressed at all timepoints (1h-4h). Highest levels of Bet v 3 were found in the insoluble fraction 5h after induction.

To maintain the natural conformation of the allergen it is important to avoid denaturation steps during purification. Therefore, new testexpressions at 30°C and 18°C were performed to express the protein in a soluble form.

Testexpression of Bet v 3 at 30°C

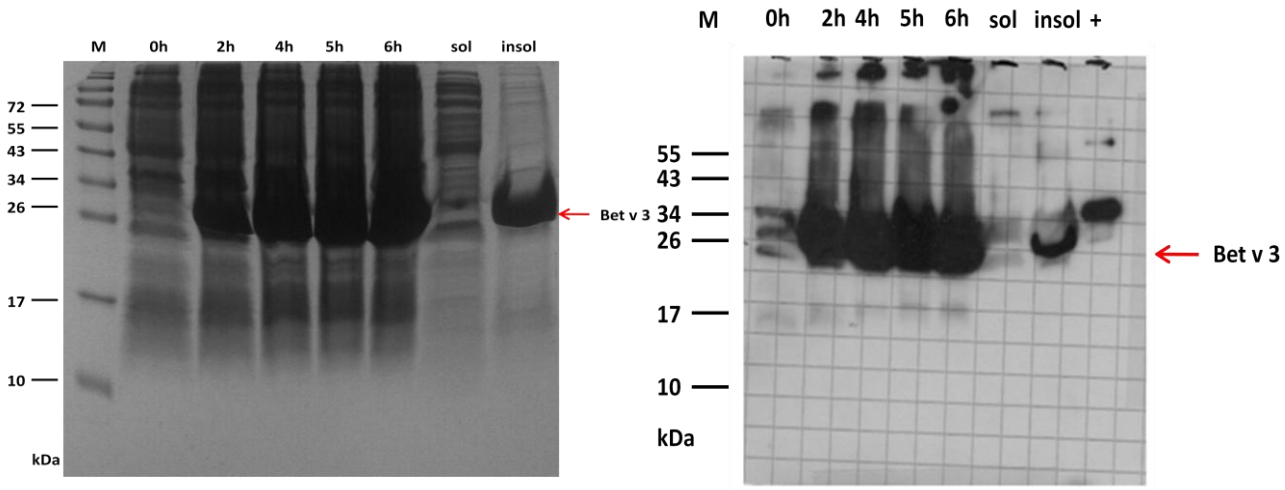


Figure 5: : Left: Expression of Bet v 3 at 30°C. Fractions obtained before (0h) and after addition of IPTG (2h - 6h) were analysed by means of SDS PAGE (15%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 6h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3 with a molecular weight of 26 kDa

Right: Detection of HIS-tagged Bet v 3 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (2h - 6h). Sol, Insol, soluble and insoluble fractions obtained after 6h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3 with a molecular weight of 26 kDa. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 1 minute exposure to the fotofilm.

The results of the testexpression at 30°C (Figure 5) were basically the same as at 37°C with the majority of Bet v 3 as insoluble protein. Therefore a new testexpression at 18°C was performed. The low temperature slows bacterial growth and protein expression which could lead to a soluble expression of the protein.

Testexpression of Bet v 3 at 18°C

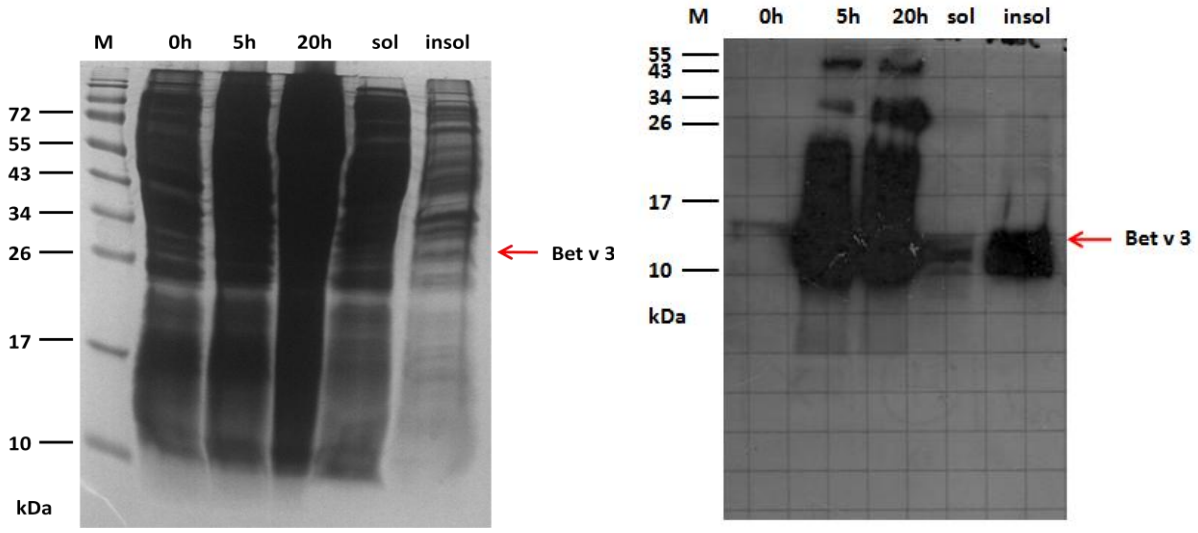


Figure 6: Left: Expression of Bet v 3 for 20h at 18°C. Fractions obtained before (0h) and after addition of IPTG (5h, 20h) were analysed by means of SDS PAGE (15%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 20h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3 with a molecular weight of 26 kDa

Right: Detection of HIS-tagged Bet v 3 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (5h, 20h). Sol, Insol, soluble and insoluble fractions obtained after 20h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 3 with a molecular weight of 26 kDa. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 20 seconds exposure to the fotofilm.

The testexpression of Bet v 3 at 18°C (Figure 6) also shows high amounts of Bet v 3 in the insoluble fraction and only low amounts in the soluble fraction.

Unfortunately only small amounts of Bet v 3 were expressed as soluble protein under all tested conditions. Therefore, Bet v 3 was purified from the insoluble fraction under denaturing conditions.

Testexpression of Bet v 4 at 37°C

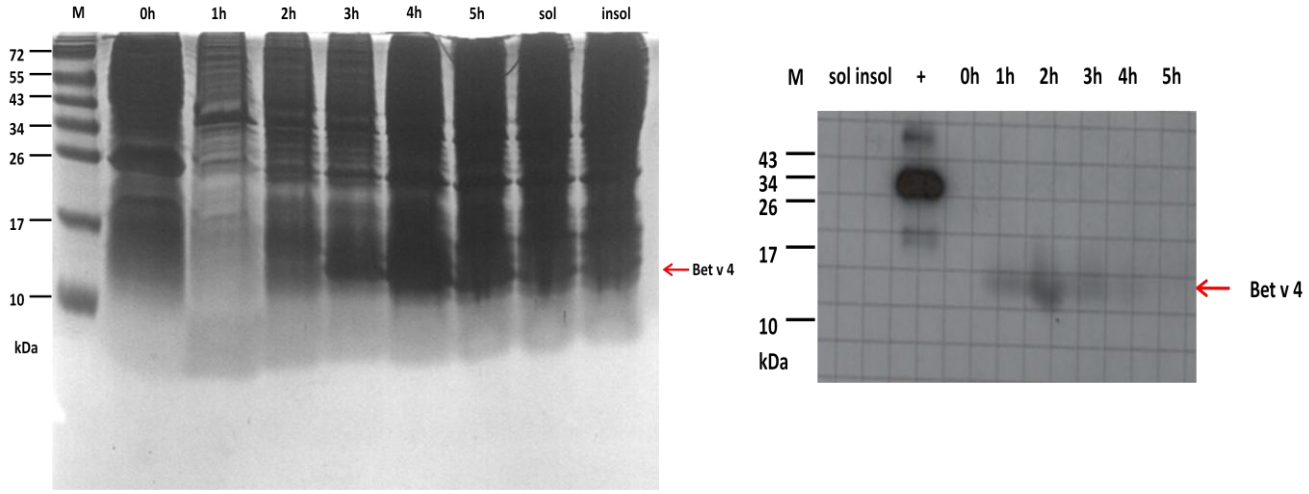


Figure 7: Left: Expression of Bet v 4 for 5h at 37°C. Fractions obtained before (0h) and after addition of IPTG (1h – 5h) were analysed by means of SDS PAGE (17%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 4 with a molecular weight of 12 kDa

Right: Detection of HIS-tagged Bet v 4 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (1h – 5h). Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 4 with a molecular weight of 12 kDa. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 20 seconds exposure to the fotofilm.

According to the immunoblot shown in Figure 7, the Bet v 4 protein (expected with a MW of 12 kDa) was found within the first 2 hours of IPTG-induced expression. The decreased concentration of Bet v 4 at the later timepoints could result from degradation of the protein. After 5h of induced expression and cell lysis, no his tagged protein was detected in the soluble and insoluble fraction.

To limit degradation, the duration of the next testexpression was reduced to 2 hours at 37°C.

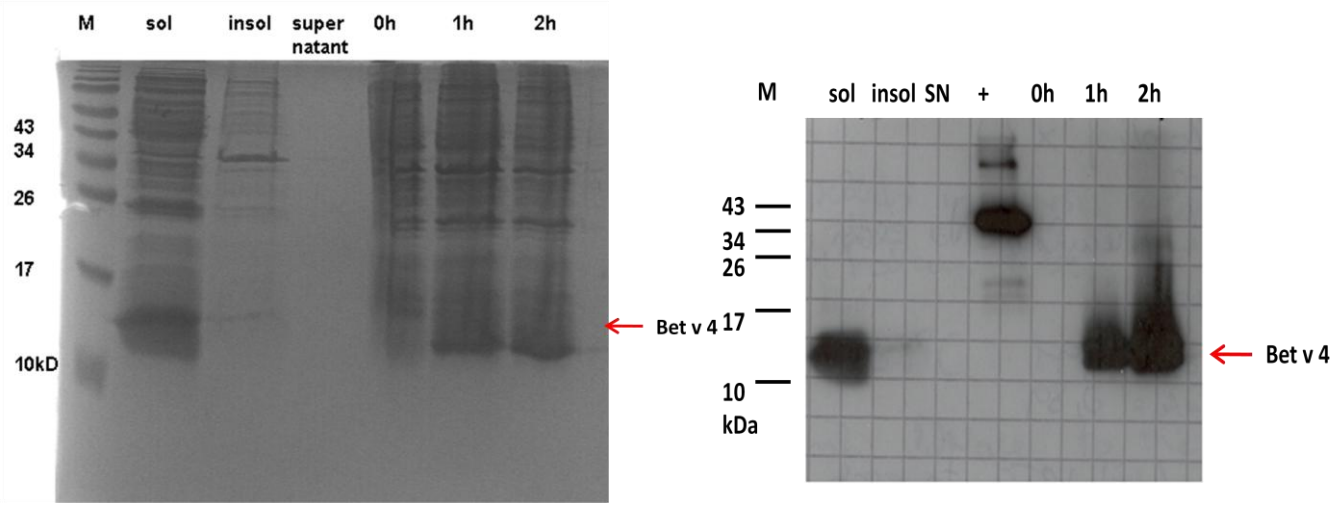


Figure 8: Left: Expression of Bet v 4 for 2h at 37°C. Fractions obtained before (0h) and after addition of IPTG (1h - 2h) were analysed by means of SDS PAGE (17%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 2h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 4 with a molecular weight of 12 kDa

Right: Detection of HIS-tagged Bet v 4 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (1h – 2h). Sol, Insol, soluble and insoluble fractions obtained after 2h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 4 with a molecular weight of 12 kDa. M, Fermentas PAGE Ruler™ protein ladder. SN, supernatant. A His-tagged protein was used as a positive control. 20 seconds exposure to the fotofilm.

After 2h expression at 37°C the majority of Bet v 4 was found in the soluble fraction (Figure 8), therefore the optimal conditions for the expression of Bet v 4 in *E.coli* were 2h at 37°C.

Testexpression of Bet v 6 at 37°C

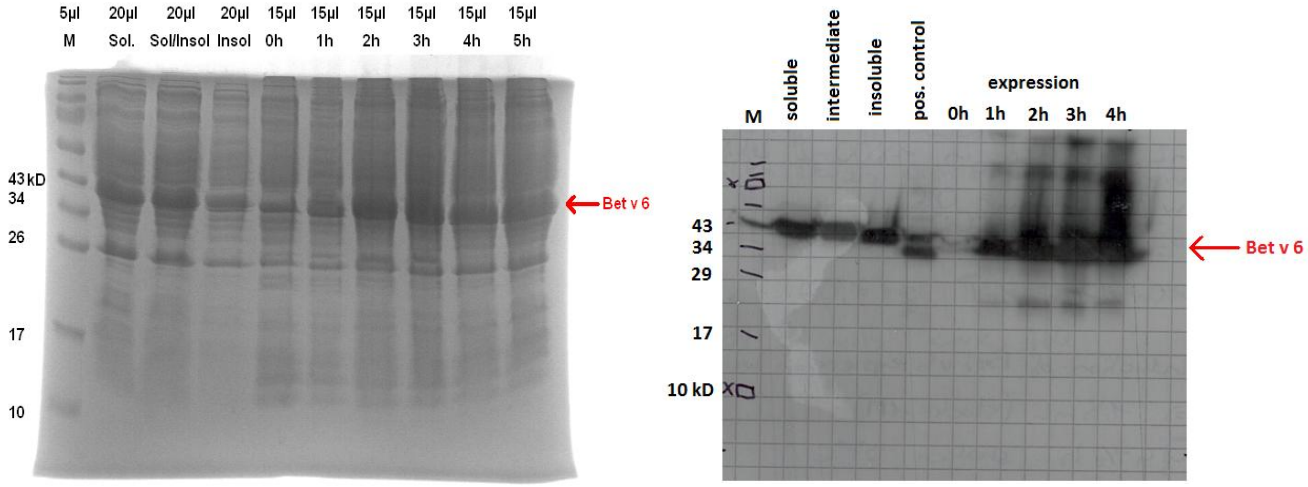


Figure 9: Left: Expression of Bet v 6 for 5h at 37°C. Fractions obtained before (0h) and after addition of IPTG (1h- 5h) were analysed by means of SDS PAGE (17%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 6 with a molecular weight of 36 kDa

Right: Detection of HIS-tagged Bet v 6 by means of Western blotting. Fractions obtained before (0h) and after addition of IPTG (1h - 4h). Sol, Insol, soluble and insoluble fractions obtained after 4h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 6 with a molecular weight of 36 kDa. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 20 seconds exposure to the fotofilm.

Bet v 6 (expected with a MW of 36 kDa) was found at all timepoints of IPTG induced expression (Figure 9). High amounts of Bet v 6 were found 2h after the addition of IPTG. After 5h of induced expression and cell lysis, the majority of the protein was found in the soluble fraction. Bet v 6 was also present in the urea-treated insoluble fraction.

The results of the western blot confirmed the data shown in Figure 9. Before the addition of IPTG no Bet v 6 was detected (0h). After induction, His- tagged Bet v 6 was expressed well at all time points (1h-4h). Highest amounts of Bet v 6 were found in the soluble fraction 4h after induction. Thus, the optimal time period for the expression of Bet v 6 in *E.coli* was 4h-5h at 37°C.

Testexpression of Bet v 7 at 37°C

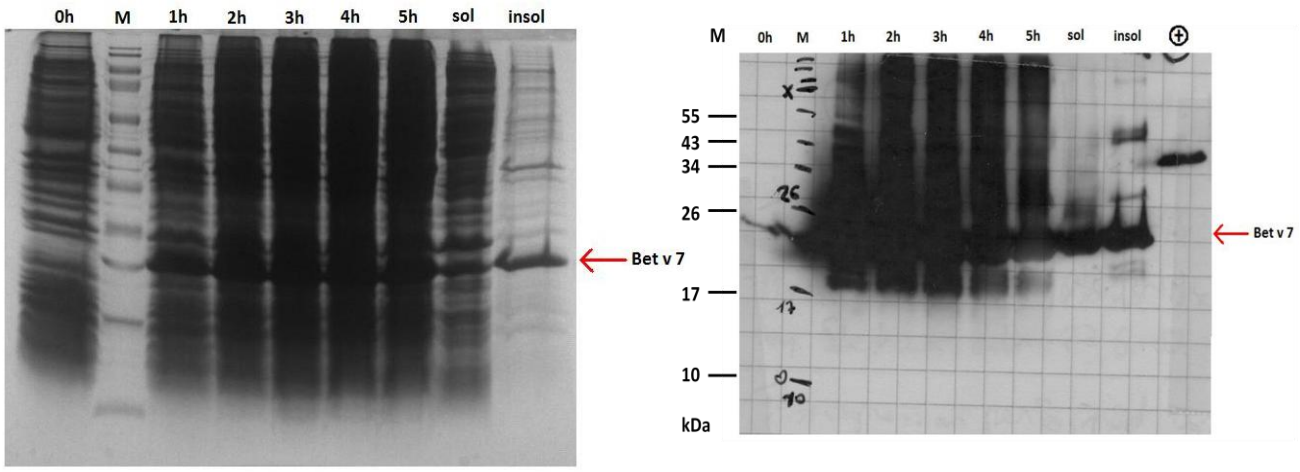


Figure 10: Left: Expression of Bet v 7 for 5h at 37°C. Fractions obtained before (0h) and after addition of IPTG (1h - 5h) were analysed by means of SDS PAGE (17%) and coomassie staining. M, Fermentas PAGE Ruler™ protein ladder. Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 7 with a molecular weight of 21 kDa

Right: Detection of HIS-tagged Bet v 7 by means of Western blot. Fractions obtained before (0h) and after addition of IPTG (1h – 5h). Sol, Insol, soluble and insoluble fractions obtained after 5h expression and cell lysis. Insoluble fraction after treatment with 5M Urea. The red arrow indicates Bet v 7 with a molecular weight of 21 kDa. M, Fermentas PAGE Ruler™ protein ladder. A His-tagged protein was used as a positive control. 30 seconds exposure to the fotofilm.

Bet v 7 (expected with a MW of 23 kDa) was found in high amounts at all timepoints of IPTG induced expression (Figure 10). After 5h of induced expression and cell lysis, the protein was found in the soluble and urea treated, insoluble fraction.

The results of the western blot confirmed the data shown in Figure 10. Before the addition of IPTG only low amounts of Bet v 7 were detected (0h). After induction, His- tagged Bet v 7 was expressed well at all timepoints (1h-5h). Sufficient amounts of Bet v 7 were found in the soluble fraction, therefore the optimal time period for the expression of Bet v 7 in *E.coli* was 4h-5h at 37°C

Large scale expression and affinity purification of recombinant birch pollen proteins

After determining the optimal expression conditions for each allergen, large scale protein expressions with 2 L *E.coli* BL21(DE3)pLysS cultures were performed. The cultured cells were broken up in lysis buffer and freeze-thaw cycles. Most proteins were purified from the soluble fraction, only Bet v 3 was purified from the insoluble fraction using 5M Urea.

All his-tagged proteins were separated from the lysate using His-Trap FF crude 1 mL columns. The column was equilibrated and loaded using the ÄKTA™ prime protein purification system. Bound proteins were eluted by an increasing imidazol gradient and fractions were collected. These were separated by SDS-PAGE and detected by coomassie staining.

<u>Purification of Bet v 3:</u>

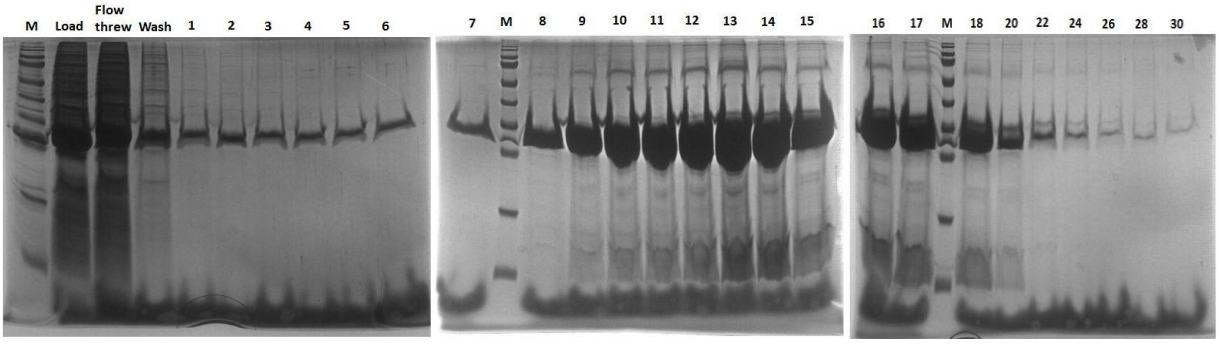


Figure 11: Eluted fractions (1-30) were separated by SDS- PAGE (15%) and stained with coomassie brilliant blue. Loaded protein sample (Load) flow through (Flow) and wash fractions were also analysed. M, Fermentas PAGE Ruler™ protein ladder.

HIS-tagged Bet v 3 with a molecular weight of 26 kDa was found in high concentrations in all eluted frations as depicted in Figure 11. The fractions 1-24 were pooled.

Purification of Bet v 4:

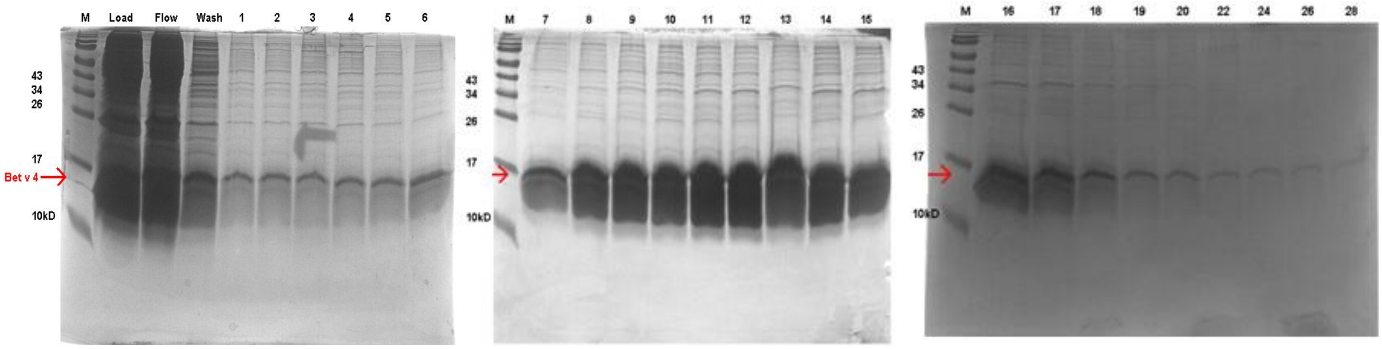


Figure 12: Eluted fractions (1-28) were separated by SDS- PAGE (17%) and stained with coomassie brilliant blue. Loaded protein sample (Load) flow through (Flow) and wash fractions were also analysed. M, Fermentas PAGE Ruler™ protein ladder.

His-tagged Bet v 4 (12 kDa) was detected in all eluted fractions (Figure 12). The fractions 7-20 contained high amounts of Bet v 4 and were pooled.

Purification of Bet v 6:

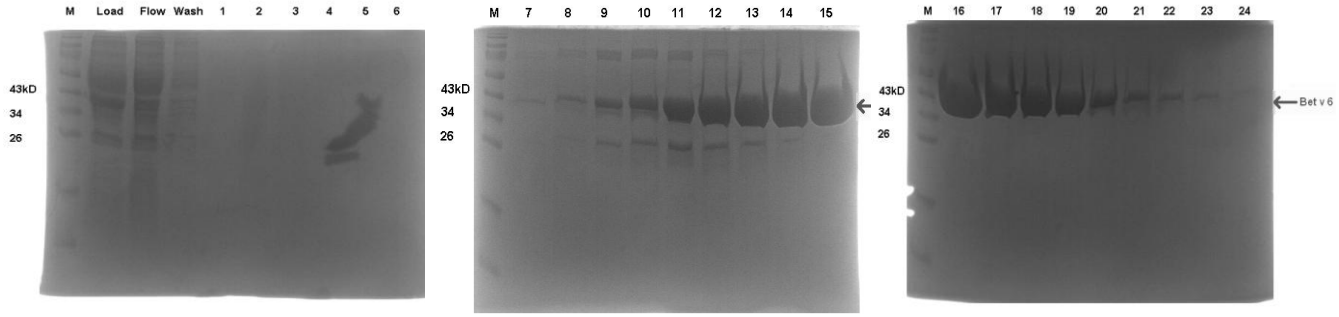


Figure 13: Eluted fractions (1-24) were separated by SDS- PAGE and stained with coomassie brilliant blue. Loaded protein sample (Load) flow through (Flow) and wash fractions were also analysed. M, Fermentas PAGE Ruler™ protein ladder.

Highest concentrations of His-tagged Bet v 6 (36 kDa) were found in the eluted fractions 11-20 shown in Figure 13. The fractions 15-24 contained pure Bet v 6 and were pooled.

Purification of Bet v 7:

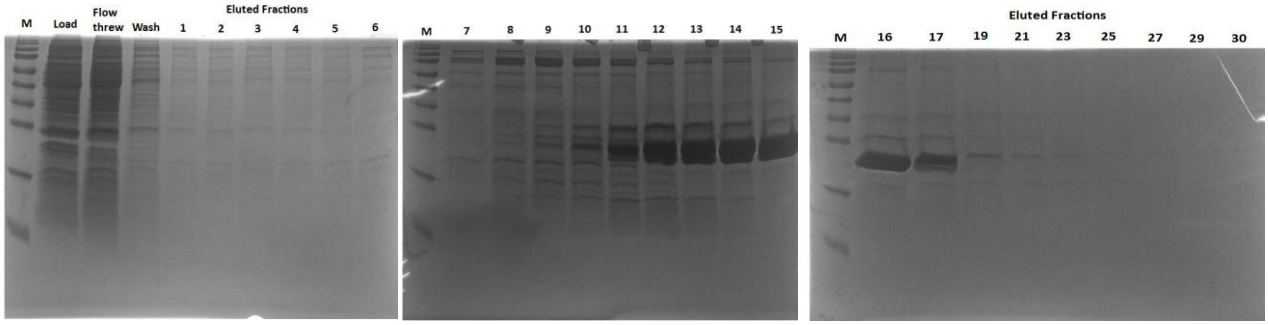


Figure 14: Eluted fractions (1-30) were separated by SDS- PAGE and stained with coomassie brilliant blue. Loaded protein sample (Load) flow through (Flow) and wash fractions were also analysed. M, Fermentas PAGE Ruler^{TM} protein ladder.

High concentrations of His-tagged Bet v 7 (21 kDa) were found in the fractions 11-17 and were pooled (Figure 14). These fractions contained Bet v 7 together with low amounts of unknown proteins with higher molecular weight.

The pooled fractions from all purified proteins were dialyzed against either PBS or Sodium phosphate buffer (100 mM pH=6), to remove toxic imidazol and to determine the optimal storage conditions that prevent protein precipitation. Samples were dialysed in semipermeable dialysis tubes with a cut off at 5-7kD.

For most proteins, dialysis against the Sodium phosphate buffer resulted in less protein precipitation and thus higher protein stability as summarized in Table 6.

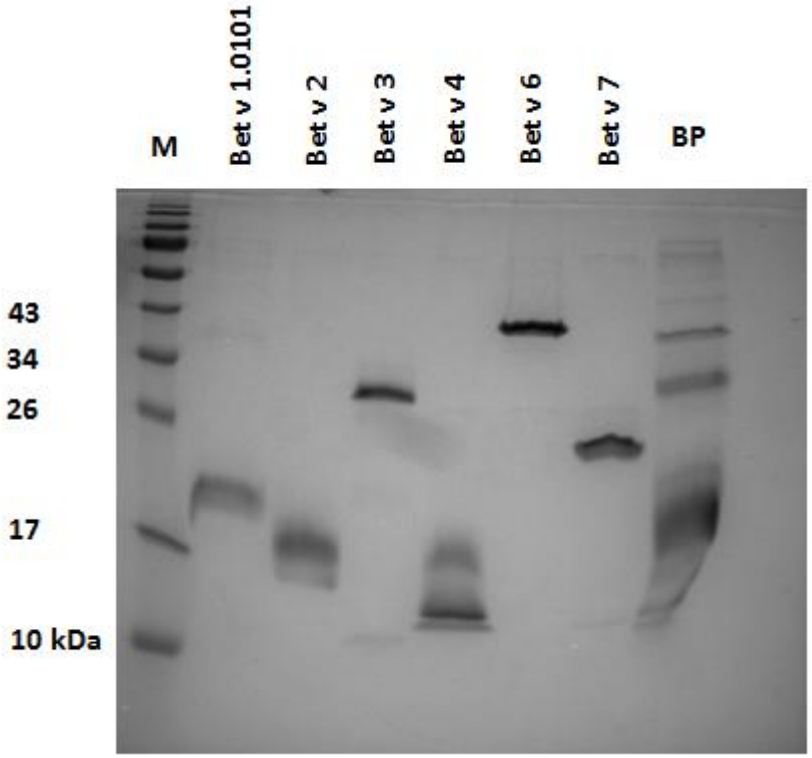


Figure 15: Purified recombinant birch pollen allergens separated by SDS-PAGE (15%) and coomassie stained. BP, birch pollen extract (50 µg), M, Fermentas PAGE Ruler™ protein ladder.

Figure 15 shows the purity of pooled protein fractions of all recombinant birch pollen allergens after dialysis on a coomassie stained SDS-PAGE gel. Lanes loaded with Bet v 3, Bet v 6 and Bet v 7 show one single protein band with the expected size of 26 kDa, 36 kDa and 21 kDa, respectively. The lane loaded with Bet v 4 showed the expected band at 12 kDa and a second band at approximately 20 kDa which is expected to be a dimer.

Endotoxin removal and determination of endotoxin levels of recombinant proteins

Several allergen batches were purified from 2 L cultures and dialyzed against different buffers as shown in Table 6. Endotoxins were removed from dialysed samples using EndoTrap® red 1 mL columns (Hyglos GmbH, Germany). Protein concentrations were determined using the Pierce Endogen BCA assay (Thermo Scientific Inc., Rockford, USA) and endotoxin levels were assessed by chromogenic LAL assays.

Allergen	Expression conditions	Form	Batch	Storage	Conc. [µg/mL]	Endotoxin lvl. [ng LPS/mg]
Bet v 3	4h 37°C	Insoluble	#1	PBS	900	16
Bet v 4	2h 37°C	Soluble	#1/10	PBS--> much preciptiation	1200	10
			#9/10	50mM NaP pH=6	1000	0,015
			#10/11	50mM NaP pH=6	960	13,6
Bet v 6	5h 37°C	Soluble	#12/09	PBS --> much prec.	670	1
			#10/10	100mM NaP ph=6	940	0,42
Bet v 7	3h 37°C	soluble	#1	PBS --> much prec.	290	2,2
			#2	250mM NaP pH=6	250	0,4
			#3	250mM NaP pH=6	550	0,45

Table 6: Summary of recombinant protein batches with expression time and temperature, fraction from which the protein was purified, dialysis buffer, protein concentration after purification and endotoxin level after LPS removal.

With the optimized expression protocols and optimal dialysis buffers it was possible to produce all minor birch pollen allergens in sufficient amounts as shown in Table 6. The endotoxin removal by the EndoTrap® red 1 mL columns effectively reduced LPS concentrations to satisfactory levels, in all proteins.

Humoral and cellular characterization of minor birch pollen allergens

IgE binding capacity of recombinant allergens

The IgE reactivity of the recombinant allergens was analyzed by means of ELISA. Allergens were coated on microtiter plates and incubated with sera from birch pollen-allergic patients. Sera from non-allergic donors and patients solely sensitized to Bet v 1 served as negative controls. Bound IgE Ab were detected using a murine anti-human IgE antibody.

All tested birch pollen-allergic patients displayed in Figure 16 and Figure 17 (P1, P2, P3, P6, P7, P8) showed IgE-reactivity to birch pollen extract (BP).

Recombinant Bet v 3 and Bet v 4 were clearly recognized by IgE antibodies from patient 2 (Figure 17). Patient 6 showed weak IgE binding to Bet v 6 and Patient 7 showed clear IgE binding to Bet v 6. Bet v 7 was recognized by IgE antibodies from patient 2 and patient 3. Non-allergic individuals and buffer controls were negative to BP and all tested allergens.

These results prove, that all recombinant allergens have maintained their IgE-binding capacity, indicating that these proteins have kept their natural conformation.

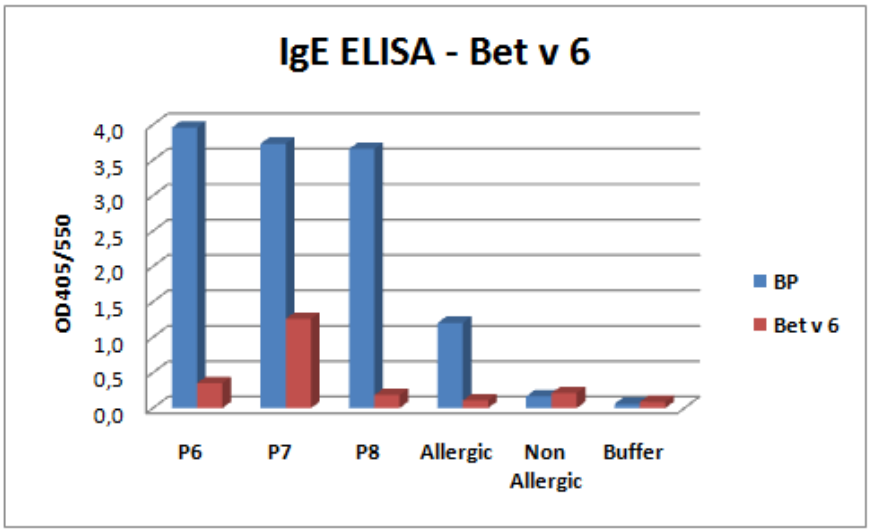


Figure 16: IgE reactivity to birch pollen extract (50 µl/mL) and Bet v 6 (5 µg/mL) of sera from three selected patients (P6-8), plasma from an allergic patient as positive control and plasma from a non allergic as negative control; OD= Optical density

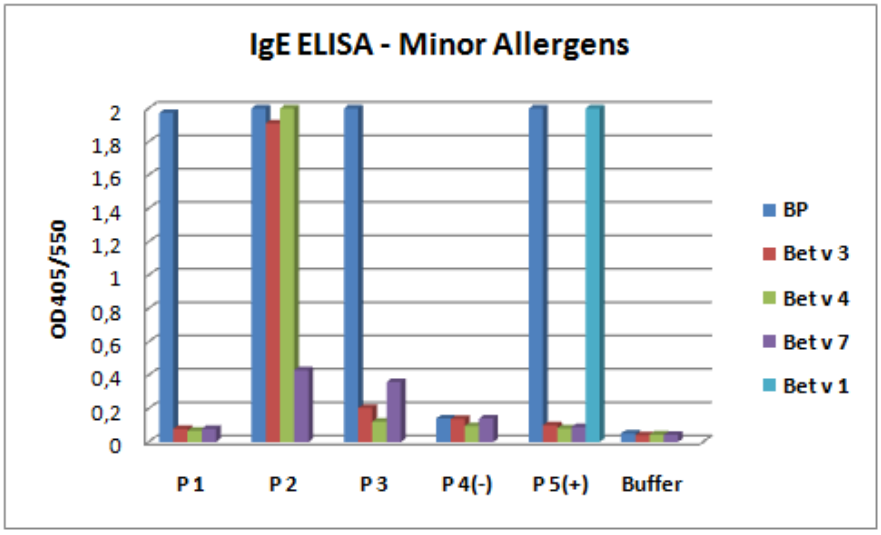


Figure 17: IgE reactivity to birch pollen extract (50 µl/mL), Bet v 3, Bet v 4, and Bet v 7 (5 µg/mL) of sera from three birch pollen allergic patients (P1-3), plasma from an allergic patient as positive control (P 5) and plasma from a non allergic as negative control (P 4); OD= Optical density

Proliferative responses of PBMC to recombinant birch pollen allergens

Primary responses were performed to test the activating capacity of the recombinant allergens on PBMCs. Allergens were incubated at different concentrations with PBMCs isolated from birch pollen-allergic donors. BP and IL-2 served as positive controls. Proliferation was measured by adding ^{3}H thymidine during the last 16 hours of culture.

Proliferation was determined after 6 days. Stimulation indices (SI) were calculated as the ratio between counts per minute (cpm) obtained in cultures stimulated with allergen and cpm from cultures incubated in medium alone. A SI greater than 2 was regarded as positive.

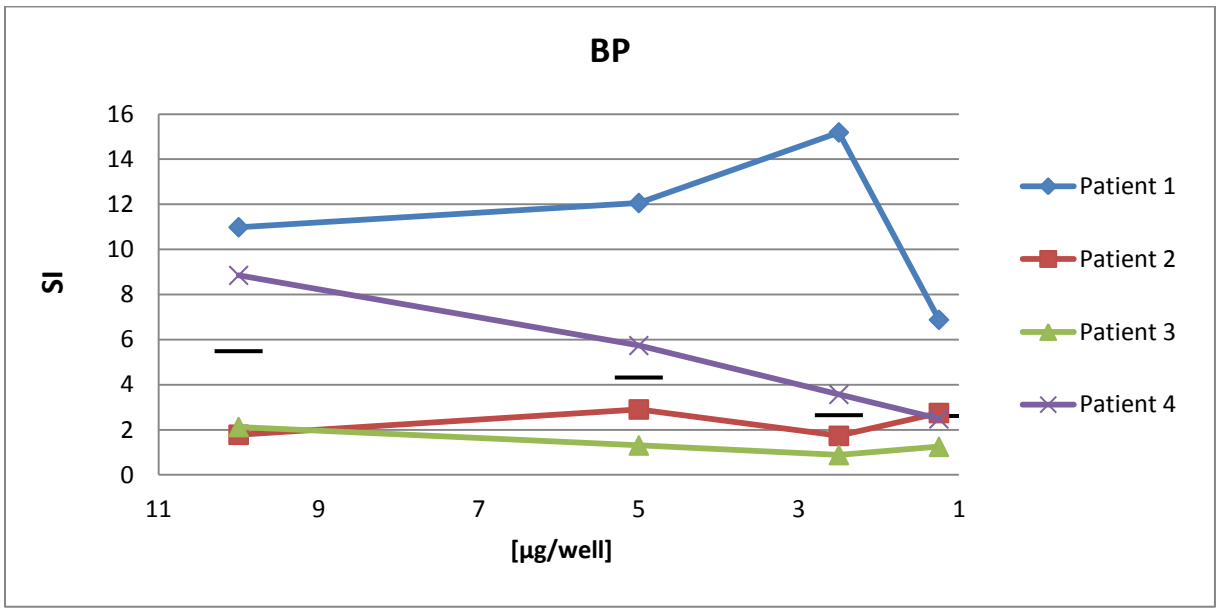


Figure 18: Allergen induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

BP extract was able to induce T-cell proilferation in all 4 patients (Figure 18). T-cells from two patients showed high responses with a SI > 5. Proliferative responses were dose dependend in all patients.

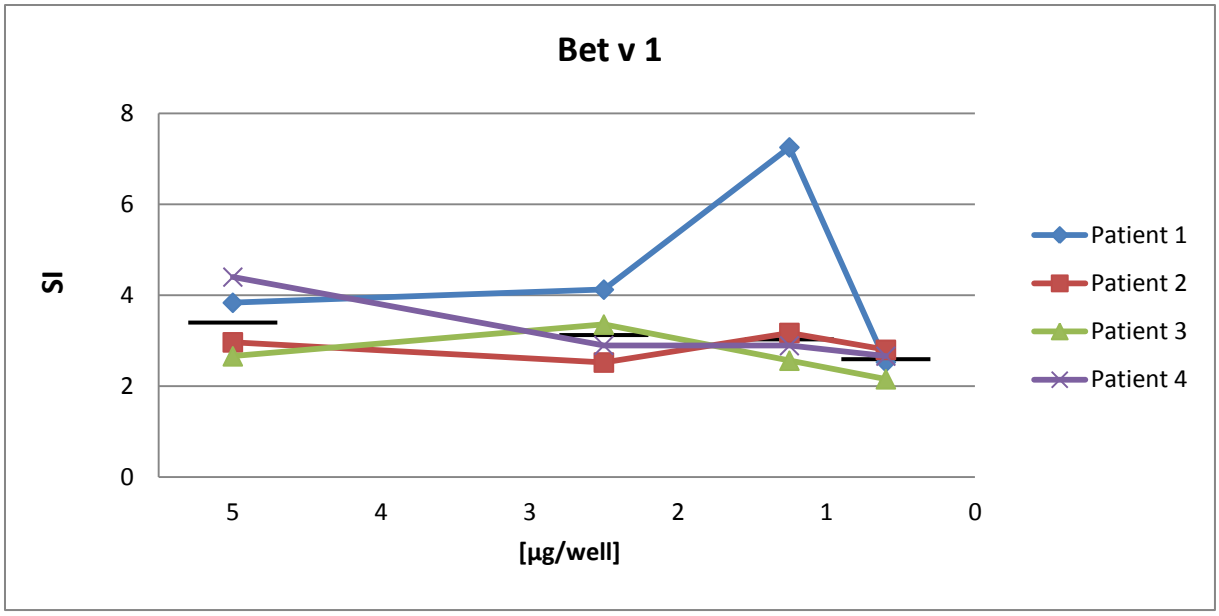


Figure 19: Bet v 1-induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

Incubation with the major Bet v 1 isoform rBet v 1.0101 induced T-cell proliferation in PBMCs from all patients under investigation (Figure 19). Overall, proliferative responses correlated with Bet v 1 concentration.

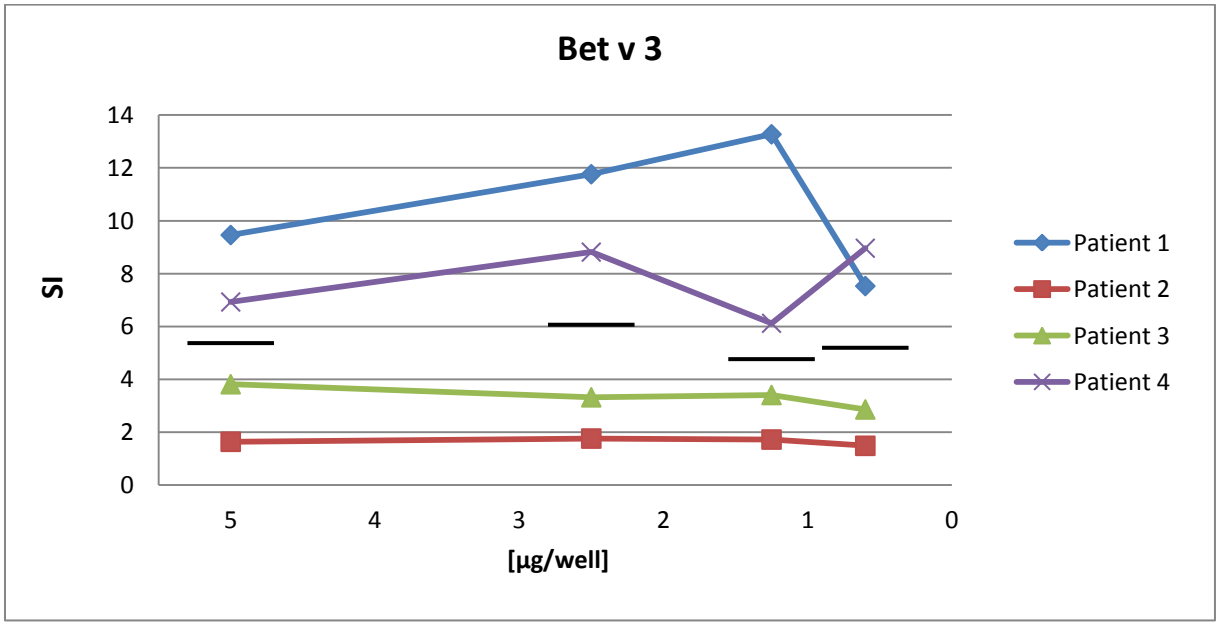


Figure 20: Bet v 3-induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

Recombinant Bet v 3 induced a detectable T-cell response in 3 of 4 patients as shown in Figure 20. T-cells from two patients reacted with a SI < 5 and one patient showed no T-cell reactivity to Bet v 3 (SI < 2). No clear dose-dependency was observed.

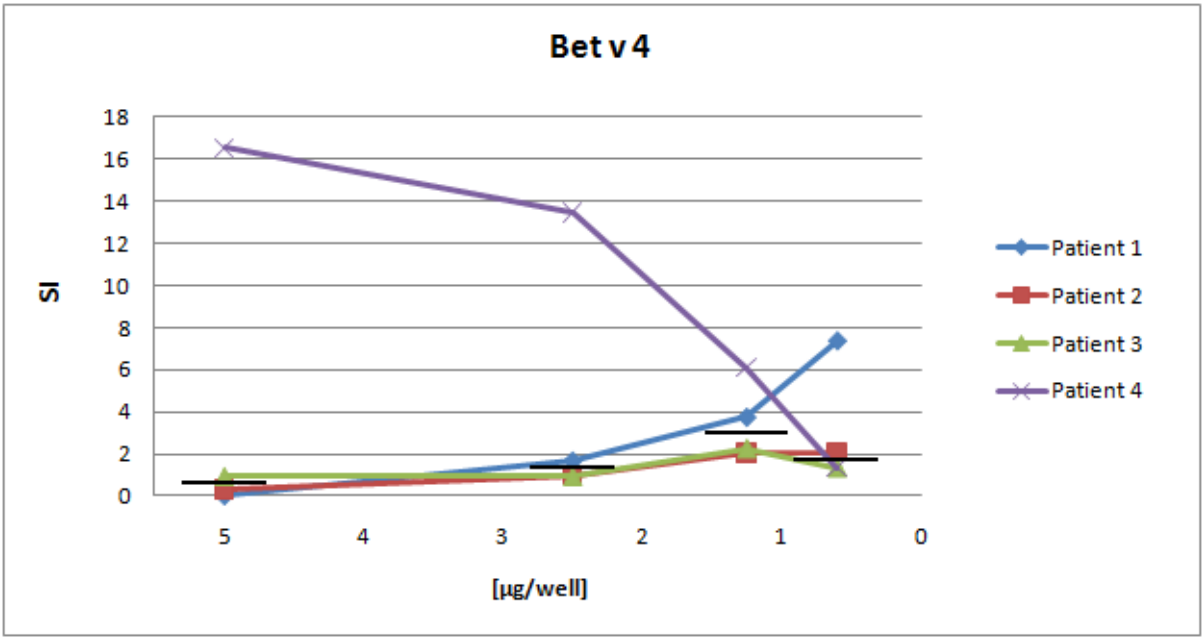


Figure 21: Bet v 4- induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

Incubation with recombinant Bet v 4 induced T-cell proliferation in all tested patients (Figure 21). Of note, 3 patients showed highest reactivity to low doses of Bet v 4 (1,25 µg/mL) whereas one patient responded to higher amounts (5 µg/mL), therefore no clear dose-dependency was observed.

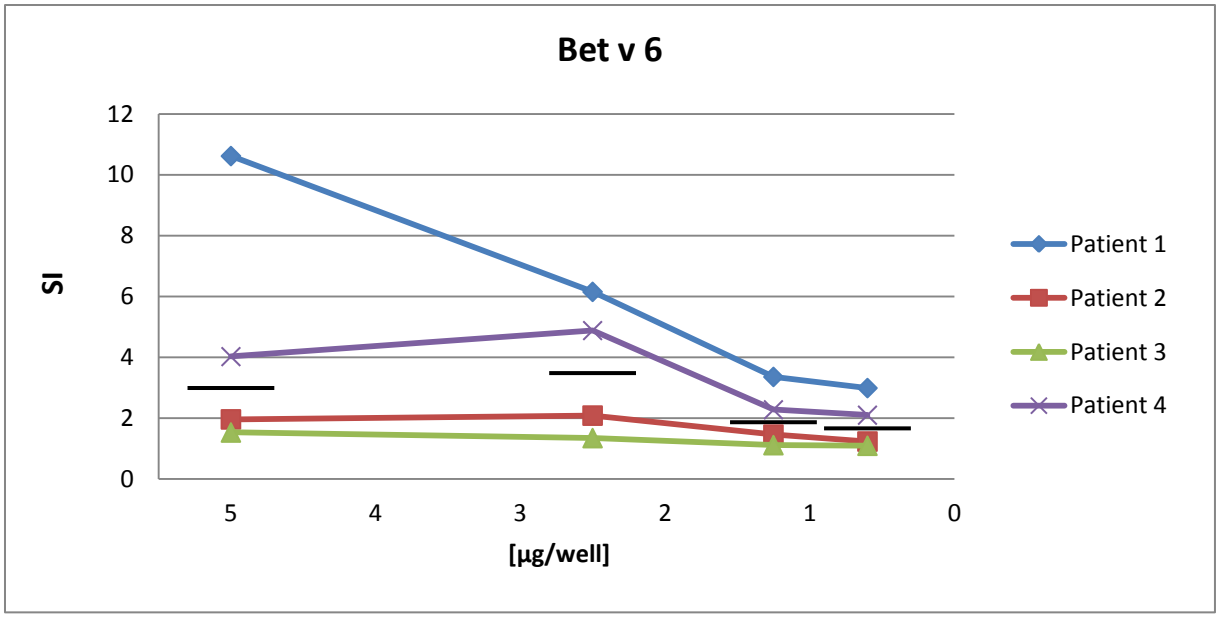


Figure 22: Bet v 6-induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

Recombinant Bet v 6 induced T-cell proliferation in 3 of 4 patients as shown in Figure 22. Two patients reacted to Bet v 6 with a SI > 4 , one patient reacted with a SI of 2 and one patient showed no reactivity to any concentration tested. The proliferative response correlated with the Bet v 6 concentration.

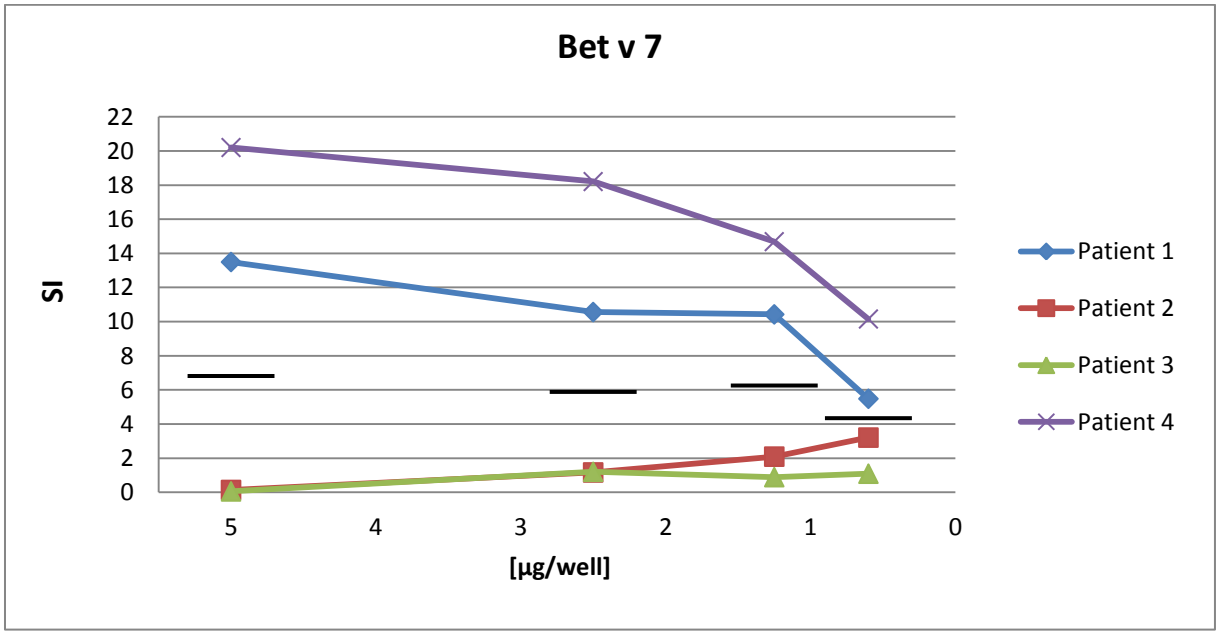


Figure 23: Bet v 7-induced T-cell proliferation. PBMCs isolated from 4 birch pollen-allergic donors were incubated for 7 days with titrated concentrations of recombinant allergens. Proliferation measured by ^{3}H thymidine incorporation. Results are mean values of triplicates; Black lines indicate the median; SI = stimulation index = stimulated/ unstimulated well;

Incubation with recombinant Bet v 7 induced remarkable T-cell proliferation in two patients (SI > 10) and one patient reacted with an SI > 2 (Figure 23). T-cell proliferation correlated with the concentration of Bet v 7 in the patients with strong responses. T-cells from the patient with a weak response, responded only to low Bet v 7 concentrations. One patient showed no T-cell reactivity to any concentration tested.

Together, PBMCs from 4 birch pollen-allergic patients were tested for proliferative T-cell responses to recombinant Bet v 1, Bet v 3, Bet v 4, Bet v 6, Bet v 7 and birch pollen extract (Figure 18-23). 50% of the individuals showed T-cell responses to all allergens. Birch pollen extract, Bet v 1 and Bet v 4 induced T-cell proliferation in all patients under investigation. Recombinant Bet v 3, Bet v 6 and Bet v 7 induced T-cell responses in 3 of 4 patients.

These results demonstrate the preserved T-cell activating capacity of the recombinant allergens and their non-toxicity to T-cells.

Isolation and characterization of birch pollen specific T-cell clones

Allergen induced proliferation of T-cell clones

In total, 25 birch pollen-reactive T-cell clones (TCC) were established from birch pollen specific T-cell lines and tested for their reactivity to the individual recombinant allergens. TCCs with a stimulation index (SI) > 5 were considered as specific. A summary is shown in Table 7.

13/25 TCCs (52%) reacted to recombinant Bet v 1.0101, the most abundant form of the major allergen and natural Bet v 1 (nBet v 1). Natural Bet v 1 is the allergen directly isolated from birch pollen containing all isoforms.

2/25 TCCs (8%) reacted with nBet v 1 but were Bet v 1.0101 negative.

One TCC (4%) was specific for the recombinant minor allergen Bet v 2.

9/25 TCCs (36%) reacted to BP extract but were negative for all tested recombinant allergens. One TCC also reacted to BP extract only and could not be tested with Bet v 3 and Bet v 7 because these allergens weren't available at that time.

TCC	BP	Bet v 1.0101	n Bet v 1	Bet v 2	Bet v 3	Bet v 4	Bet v 6	Bet v 7
Patient 1 #28	565*	680	823	1,7	n.t.	0,7	0,8	n.t.
Patient 1 #e	30	31	45	0,3	n.t.	0,4	0,4	n.t.
Patient 2 #10	22	29	12	0,3	n.t.	0,2	0,6	n.t.
Patient 2 #11	274	448	533	3,2	n.t.	1,3	0,5	n.t.
Patient 3 #105	16	2,5	3,4	20	n.t.	n.t.	n.t.	n.t.
Patient 3 #114	17	0,5	1,9	0,5	n.t.	0,2	0,7	n.t.
Patient 3 #221	91	1,1	75	0,9	n.t.	1	1	n.t.
Patient 3 #18	128	313	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 3 #145	80	196	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 3 #247	32	112	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 3 #352	8	15	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 3 #493	16	21	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 3 #134	34	0,1	1,1	1,1	0,7	0,7	0,7	0,7
Patient 3 #158	120	0,8	1,4	1	0,6	0,6	0,6	0,6
Patient 3 #160	240	2,1	0,9	0,8	1,8	1,8	1,8	1,8
Patient 3 #184	115	0,9	1,2	0,5	0,3	0,3	0,3	0,3
Patient 3 #250	129	0,9	8,4	0,7	1	1	1	1
Patient 3 #282	97	0,02	2,4	0,4	0,4	0,4	0,4	0,4
Patient 3 #294	132	0,9	0,7	0,3	0,3	0,3	0,3	0,3
Patient 3 #305	617	1,1	0,6	0,3	0,4	0,4	0,4	0,4
Patient 3 #320	176	1,1	1,1	0,7	0,6	0,6	0,6	0,6
Patient 3 #431	7	0,9	10	1	0,3	0,3	0,3	0,3
Patient 4 #46	23	44	113	0,9	n.t.	0,7	n.t.	n.t.
Patient 5 #26	230	5	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Patient 5 #44	146	2,1	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Table 7: Proliferative response of birch pollen specific TCCs to birch pollen allergens; nBet v 1, natural Bet v 1; n.t.= not tested; *SI are shown;

To characterize Bet v 1- specific TCCs further, 12 TCCs reactive to natural Bet v 1 were tested with several Bet v 1- isoforms available as recombinant proteins (Table 8). Moreover these TCCs were stimulated with a pannel of 50 synthetic, overlapping dodeca peptides representing the complete Bet v 1.0101 amino acid sequence (Table 8).

5/12 TCCs (42%) reacted to Bet v 1.0201
6/12 TCCs (50%) reacted to Bet v 1.1401
10/12 TCCs (83%) reacted to the Bet v 1 isoforms 1.0401, 1.0501, 1.0101 and 1.0601

The two clones specific for natural Bet v 1 only, didn't react to any tested isoform. All tested Bet v 1.0101 specific TCC were specific for one of the distinct T-cell activating regions in Bet v 1, described by Jahn-Schmid et al[43]. Figure 24 shows an alignment of the tested Bet v 1-isoforms and the mapped T-cell activating regions.

The TCC P4 #46 showed a significantly lower reaction to Bet v 1.1001 compared to the other tested isoforms. The alignment shows an exchange of leucine to methionine at the recognized epitope in this isoform.

The clones #18, #247 and #352 from patient 3 showed a comparable reaction to all isoforms and the alignment confirms the sequential identity of the recognized epitope.

The TCCs P3 #145, P3 #493, P1 #e and P1 #28 did not react to the isoforms Bet v 1.0201 and Bet v 1.1401. Here the alignment shows exchanges from valine to methionine, proline to alanine and isoleucine to leucine at the recognized epitopes in the non reactive isoforms (compared to Bet v 1.0101).

The cross reactivity of the TCCs with the Bet v 1-isoforms could be explained by their differing sequence homology at the recognized epitope (Figure 24). The data also indicates, that clone #10 from Patient 2 is polyclonal because he reacted to two distant Bet v 1 epitopes.

TCC	Bet v 1 isoforms							T-cell activating region
	1.0101	1.0201	1.0401	1.0501	1.1001	1.0601	1.1401	
Patient 1 #28	680	1,1	481	489	575	285	1,3	NYSVIEGGPIGDTLE
Patient 1 #e	82	1,8	377	279	439	156	1,6	NYSVIEGGPIGDTLE
Patient 2 #10	178	215	149	220	114	112	101	TSVIPAARLFKA + SILKISNKYHTK
Patient 2 #11	87	0,7	110	124	66	44	55	n.t.
Patient 3 # 221	1,1	0,4	1,3	0,3	0,6	0,2	0,6	n.t.
Patient 3 #18	42	111	106	51	117	26	75	APQAISSVENIEGNG
Patient 3 #145	330	0,6	547	441	649	200	0,3	FKYNYSVIEGGP
Patient 3 #247	77	117	206	199	222	42	162	APQAISSVENIEGNG
Patient 3 #352	42	143	157	93	163	27	55	APQAISSVENIEGNG
Patient 3 # 493	112	3,7	229	161	227	132	0,5	FKYNYSVIEGGP
Patient 3 #431	7	0,8	0,9	0,8	0,7	0,5	0,5	n.t.
Patient 4 # 46	38	79	85	68	6	35	64	TSVIPAARLFKA

Table 8: Epitope specificity and isoform reactivity of Bet v 1-specific TCCs; SI are shown

Figure 24: Alignment of the AA sequence of Bet v 1 isoforms. T-cell activating regions and important AA differences are highlighted.

Th subsets of allergen specific T-cell clones

Supernatants of 25 allergen-specific TCCs were assessed for the content of the signature cytokines IL-4 (T_H2) and IFN-γ (T_H1) upon specific stimulation (Table 9). The ratio of the determined T_H subsets are shown in Figure 25.

12/25 TCCs (48%) were specific for Bet v 1. From these 12 clones:

- 5/12 TCCs (42%) were T_H0 like cells.
- 1/12 TCCs (8%) belonged to the T_H1 subset.
- 6/12 TCCs (50%) belonged to the T_H2 subset.

2/25 TCCs (8%) responded to natural Bet v 1 but not to Bet v 1.0101. Both TCCs were T_H1 like cells.

10/25 TCCs (40%) were specific for BP extract only. From these 10 clones:

- 8/10 (80%) were T_H0 like cells.
- 2/10 (20%) belonged to the T_H1 subset.
- None of the TCCs only specific for BP extract were T_H2 like cells.

One TCC reacted to Bet v 2 and belonged to the T_H1 subset.

These results, illustrated in Figure 25, show that a majority of the Bet v 1.0101 reactive clones are T_H2 like cells. In contrast, for TCCs non reactive to Bet v 1.0101 the dominant subset were T_H0 like cells and none of these clones belonged to the T_H2 subset.

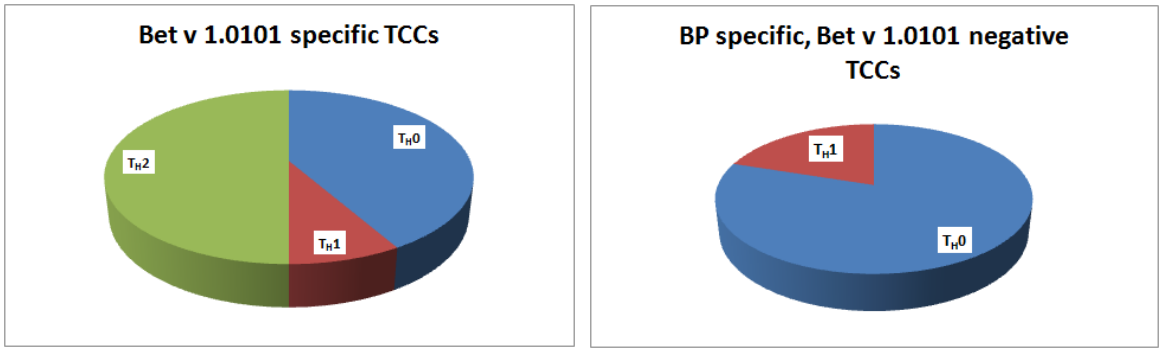


Figure 25: T_H subset ratio in Bet v 1.0101 positive and negative TCCs. Subsets determined by key cytokines in the supernatant. T_H2= IL-4 : IFNγ >5, T_H1= IL-4 : IFNγ <0,2, T_H0= IL-4 : IFNγ 0.2 to 5

TCC	IL-4 [pg/mL]	IFNg [pg/mL]	Th subset *	Specificity
Patient 1 #28	247	30	Th2	Betv 1.0101
Patient 1 #e	102	12	Th2	Betv 1.0101
Patient 2 #10	12	0	Th0	Betv 1.0101
Patient 2 #11	153	0	Th2	Betv 1.0101
Patient 3 # 105	0	384	Th1	Betv 2
Patient 3 # 114	0,79	1740	Th1	BP
Patient 3 # 221	0	384	Th1	nBetv 1
Patient 3 #18	1	18	Th 0	Betv 1.0101
Patient 3 #145	229	136	Th 0	Betv 1.0101
Patient 3 #247	1	9	Th 0	Betv 1.0101
Patient 3 #352	0	63	Th 1	Betv 1.0101
Patient 3 # 493	234	367	Th 0	Betv 1.0101
Patient 3 #134	5	5	Th 0	BP
Patient 3 #158	1	11	Th 0	BP
Patient 3 #160	3	11	Th 0	BP
Patient 3 #184	2	9	Th 0	BP
Patient 3 #250	2	7	Th 0	BP
Patient 3 #282	4	14	Th 0	BP
Patient 3 #294	4	3	Th 0	BP
Patient 3 #305	282	9682	Th 1	BP
Patient 3 #320	1	8	Th 0	BP
Patient 3 #431	2	150	Th 1	nBetv 1
Patient 4 # 46	30.29	0	Th2	Betv 1.0101
Patient 5 #26	62	0	Th2	Betv 1.0101
Patient 5 #44	235	2	Th2	Betv 1.0101

Table 9: Cytokine patterns of allergen- stimulated TCCs; *Determination of the T_H subset: T_H2= IL-4 : IFNγ >5, T_H1= IL-4 : IFNγ <0,2, T_H0= IL-4 : IFNγ 0.2 to 5

Surface marker expression of allergen specific T-cell clones

The following surface markers were analysed on 19 TCCs by flow cytometry:

- CD3, CD4 and to select the T_H cell population
- TCR α/β to determine the T-cell receptor form
- CLA (cutaneous lymphocyte associated antigen) to stain for skin homing T-cells
- CD161 and CCR6 to stain for memory T-cells

In addition, TCCs were stained for the expression of surface markers suggested to be specific for T_H1 and T_H2 cells:

- T-cell immunoglobin domain, mucin domain 3 (TIM-3)[64] and CCR5[65] for T_H1 cells
- Chemoattractant receptor of T_H2 cells (CrTh2)[66] and CCR3[65] for T_H2 cells

Figure 26 shows a representative example of one TCC analyzed by flow cytometry. This clone was defined as $CD4^+$ $TCR\alpha/\beta^+$ CLA^- $CCR6^-$ $CD161^-$ $CrTh2^+$ $CCR3^-$ $TIM3^-$ $CCR5^-$.
The results were then compared to the cytokine profile in the collected supernatants.

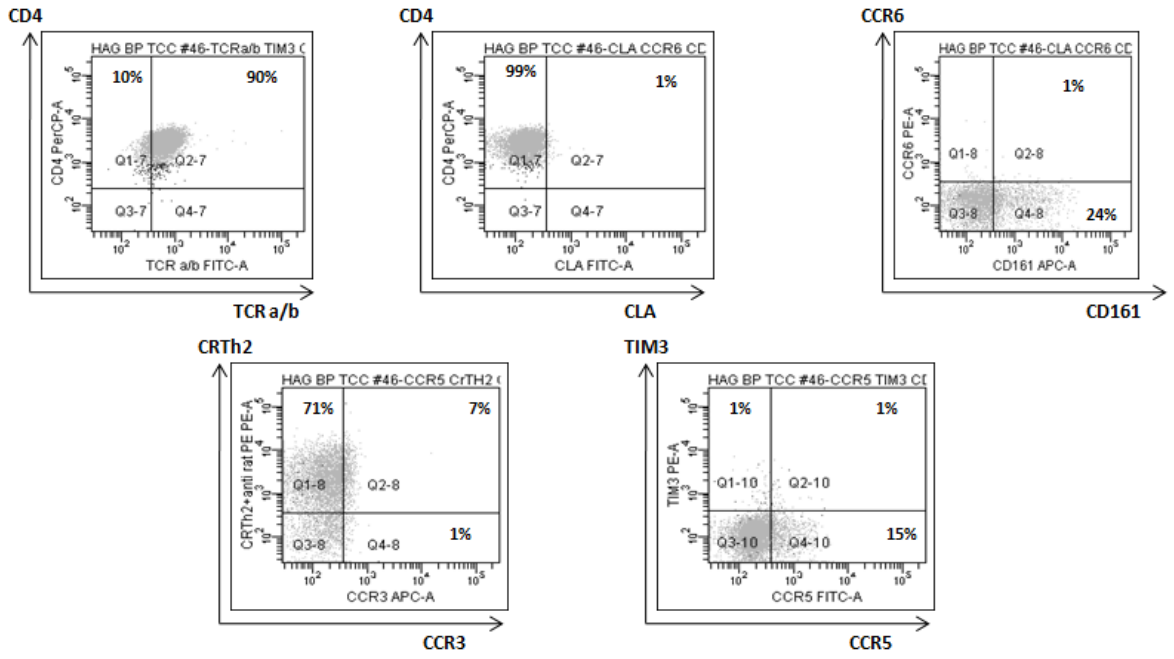


Figure 26: Surface marker expression of allergen-specific TCCs. The expression of CD4, TCR α/β, CLA, CCR6, CD161, CCR3, CrTh2, TIM3 and CCR5 was analyzed by flow cytometry.

19 TCCs were characterized by flow cytometry as shown in Table 10. As expected, all TCCs expressed the surface markers CD3, CD4 as well as TCR α/β (not shown) and none of the tested TCC expressed CLA (not shown).

10 TCCs were of the T_H0 subtype according to the cytokine data. From these 10 clones:

2/9 (22%) TCCs expressed CCR6 (1 TCC was not tested)
9/9 (100%) TCCs expressed CD161 (1 TCC was not tested)
2/9 (22%) TCCs expressed CCR6 and CD161

7/10 (70%) TCCs expressed CRTH2
1/10 (10%) TCCs expressed CCR3
1/10 (10%) TCCs expressed CRTH2 and CCR3

2/10 (20%) TCCs expressed TIM3
10/10 (100%) TCCs expressed CCR5
2/10 (20%) TCCs expressed TIM3 and CCR5

4 TCCs were of the T_H1 subtype according to the cytokine data. From these 4 clones:

3/4 (75%) TCCs expressed CCR6
4/4 (100%) TCCs expressed CD161
3/4 (75%) TCCs expressed CCR6 and CD161

2/4 (50%) TCCs expressed CRTH2
1/4 (25%) TCCs expressed CCR3
1/4 (25%) TCCs expressed CRTH2 and CCR3

0/2 (0%) TCCs expressed TIM3 (2 TCCs were not tested)
2/2 (100%) TCCs expressed CCR5
0/2 (0%) TCCs expressed TIM3 and CCR5

5 TCCs were of the T_H2 subtype according to the cytokine data. From these 5 clones:

0/5 (0%) TCCs expressed CCR6
5/5 (100%) TCCs expressed CD161
0/0 (0%) TCCs expressed CCR6 and CD161

4/5 (50%) TCCs expressed CRTH2
0/5 (25%) TCCs expressed CCR3
0/5 (25%) TCCs expressed CRTH2 and CCR3

1/2 (0%) TCCs expressed TIM3 (3 TCCs were not tested)
2/2 (100%) TCCs expressed CCR5 (3 TCCs were not tested)
1/2 (50%) TCCs expressed TIM3 and CCR5

However, the expression of surface markers did not correlate with the production of the signature cytokines IL-4 (T_H2) and IFNγ (T_H1) .

In 9/19 (47%) cases the results from the T_H1 and T_H2 markers were inconclusive.

In 5/10 (50%) conclusive cases, the results from the surface markers didn't correspond to the cytokine patterns detected in the supernatants.

For example, CrTh2 was expressed in high levels (>50%) by 4 TCCs. One of them produced low amounts of both signature cytokines and was therefore defined as T_H0. One produced more IFNγ than IL-4 and was considered a T_H1 like clone. One TCC produced no IFNγ and low amounts of IL-4 and the last one produced significant amounts of IL-4. Both were defined as T_H2 like clones.

TCC	Memory T-cells		Th2 marker		Th1 marker		Specificity	IL-4 [pg/mL]	IFNγ [pg/mL]	Th subset (cytokines)
	CCR6	CD161	CRTH2	CCR3	TIM3	CCR5				
Patient 1 #28	6	12	23	n.t.	n.t.	n.t.	Betv 1.0101	247	30	Th2
Patient 1 #e	4	30	3	n.t.	10	51	Betv 1.0101	102	12	Th2
Patient 2 #10	1	83	79	n.t.	n.t.	n.t.	Betv 1.0101	12	0	Th2
Patient 2 #11	6	14	27	n.t.	n.t.	n.t.	Betv 1.0101	153	0	Th2
Patient 3 # 114	21	81	2	n.t.	n.t.	n.t.	BP	0,79	1740	Th1
Patient 3 # 221	32	60	8	n.t.	n.t.	n.t.	n Bet v 1	0	384	Th1
Patient 3 #18	n.t.	n.t.	2	5	1	82	Betv 1.0101	1	18	Th 0
Patient 3 #145	23	19	37	2	24	94	Betv 1.0101	229	136	Th 0
Patient 3 #247	1	12	15	1	2	61	Betv 1.0101	1	9	Th 0
Patient 3 #134	1	75	24	8	1	60	BP	5	5	Th 0
Patient 3 #158	1	65	39	1	1	80	BP	1	11	Th 0
Patient 3 #160	3	76	15	1	1	36	BP	3	11	Th 0
Patient 3 #250	2	23	39	1	1	54	BP	2	7	Th 0
Patient 3 #282	1	67	5	0	2	42	BP	4	14	Th 0
Patient 3 #294	17	93	57	16	14	57	BP	4	3	Th 0
Patient 3 #305	5	79	20	1	2	95	BP	282	9682	Th 1
Patient 3 #320	9	82	5	1	0	52	BP	1	8	Th 0
Patient 3 #431	86	87	87	10	6	98	n Bet v 1	2	150	Th 1
Patient 4 # 46	1	25	78	8	2	15	Betv 1.0101	30.29	<0	Th2

Table 10: Surface marker expression of allergen specific TCCs. Numbers indicate the percentage of positive cells; n.t.= not tested

TCR family typing

The family type of the T-cell receptor α and β chain was determined from 13 TCCs to confirm the monoclonality of the T-cell clones. Figure 27 shows one representative example of a TCR family typing.

As seen in Table 11, 8/13 (62%) TCCs were monoclonal according to the TCR family typing. In 5/13 (38%) of the characterized T-cell clones, the ß chains consisted of two or more families which indicated that the cultured cells originated from at least two cells.

The most frequent β-chain families were:

β2 (4/13 TCCs = 31%)
β14 (3/13 TCCs = 23%)
β7 (2/13 TCCs = 15%)

The most frequent α-chain families were:

α18 and α4 (4/13 TCCs = 31%)
α2 (3/13 TCCs = 23%)
α1, α8 and α20 (2/13 TCCs =15%)

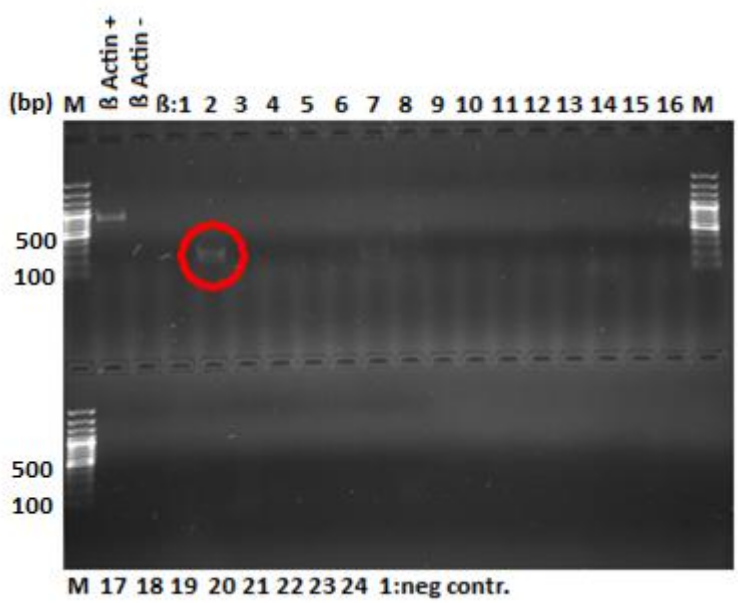


Figure 27: TCR family typing ß-chain of TCC P4 #46.M, 100bp marker; ßactin with (+) and without (-) template as control; Each lane represents one ß-chain family. Positive for ß-chain family 2.

TCC	ß chain	α chain	Result
Patient 1 #28	2,14		polyclonal
Patient 1 #e	2,14	1	polyclonal
Patient 2 #10	1, 14	2,17,18	polyclonal
Patient 3 #18	6	1,8,18,20	monoclonal
Patient 3 #158	3, 20	4	polyclonal
Patient 3 #160	17	4	monoclonal
Patient 3 #184	7	14	monoclonal
Patient 3 #247	5.2	15,20	monoclonal
Patient 3 #282	9	4,18	monoclonal
Patient 3 #305	7	2,8	monoclonal
Patient 3 #320	13	4	monoclonal
Patient 3 #431	2, 15	2,3,18	polyclonal
Patient 4 #46	2		monoclonal

Table 11: T-cell receptor family typing of allergen-specific TCCs. Isolated and transcribed cDNA from T-cell clones was used as a template for the PCR. TCC; TCCs expressing more than one β-chain families were considered as polyclonal

Discussion

The usage of allergen extracts and purified natural allergens for diagnosis and therapy of type I allergy, bears several problems. Exact standardization, batch to batch variablilty and sensitization of the patient with other allergens are some of them.[6] Therefore, the production and characterization of recombinant allergens plays an important role in the field of allergology.

Previous studies have identified Bet v 3, Bet v 4, Bet v 6 and Bet v 7 as minor birch pollen allergens through their ability to bind IgE antibodies from less than 50% of allergic patients. The Ca-binding allergens Bet v 3 and Bet v 4 were discovered by Seiberler S. et al and Twardosz et al. respectively, by IgE immunoscreening of a birch pollen cDNA expression library.[51,67] The influence of Ca^{2+} on IgE epitopes and their cross reactivity to other proteins were tested by immunoblot and immunoblot inhibition analyses.[57,68-70] Bet v 6 was characterized by Karamloo et al. Cross-reactivity was asseseed by immunoblot inhibition analyses and allergenicity was tested using immunoblot and basophil histamine release experiments.[58,59] The immunological cross reactivity to Bet v 7 was demonstrated in immunoblot and ELISA inhibition experiments by Cadot et al.[61,63]

The present study focused on expression and purification of the recombinant minor allergens Bet v 3, Bet v 4, Bet v 6 and Bet v 7 and their humoral and cellular characterization.

Expression of eukaryotic genes in prokaryotes often leads to the formation of so called inclusion bodies. These protein aggregates can form due to differences in the procaryotic microenvironment, the absence of folding mechanism or overexpression. The isolation of these protein aggregates requires harsh denaturation steps which may result in the loss of the native structure of the protein. The loss of correct protein folding is often accompanied by a loss of IgE binding and therefore often has a direct impact on the allergenic properties[71]. Therefore, the major goal was to express the minor allergens as soluble recombinant proteins.

This aim was successfully achieved for Bet v 4, Bet v 6 and Bet v 7 (Figure 7-10). Although Bet v 3 was expressed under several different conditions (Figure 4-6) only small amounts were obtained as soluble protein. Therefore, Bet v 3 was purified from the insoluble fraction under denaturing conditions.

All His-tagged recombinant allergens were purified by Ni- affinity chromatography (Figure 11-15) and rebuffered by dialysis to avoid interference of imidazol with downstream applications. Dialysis against sodium phosphate buffer showed the least protein precipitation and thus higher protein stability for most proteins (Table 6).

To eliminate LPS-related effects in cell culture experiments, LPS concentrations were successfully reduced to negligible levels (Table 6).

Allergen recognition by IgE antibodies from allergic patients is an indicator for allergenicity and correct protein folding. Therefore, IgE binding to all minor allergens was assessed by ELISA experiments with sera from birch pollen allergic patients (Figure 16, 17). Only sera from patients with high CAP classes were used which makes statements on the prevalence of the allergens impossible. However, all recombinant proteins were recognized by IgE antibodies, indicating the correct folding of the allergens. These results were particularly important to prove the remaining IgE binding capacity of the Bet v 3 protein after the denaturation steps included in the isolation.

The minor allergens Bet v 3-7 have mostly been characterized at the molecular level in previous studies[51-61,63]. In this study we additionally wanted to test their T-cell activating capacity. For this purpose, we employed PBMC and allergen-specific TCCs expanded from peripheral blood of birch pollen allergic patients.

Studying the proliferative responses of PBMCs to recombinant allergens confirms their T-cell activating capacity and ensures that they are not toxic for T-cells. All recombinant minor allergens were able to induce proliferation in PBMCs from birch pollen allergic patients.

It is still not known, why an overwhelming majority of BP allergic patients are sensitized to Bet v 1[32]. All allergens showed comparable PBMC responses when used in high concentrations (ranging from 25 µg/mL to 3,1 µg/mL). A major difference between major and minor allergens ist their abundance in birch pollen. Bet v 1 accounts for 10% of the total protein content in *B. pendula* pollen as shown in Figure 1[31]. The high quantity of Bet v 1 inhaled each spring might increase the chances of T- and B- cell responses. Additionally the low abundance of minor allergens in birch pollen could be the cause of the low sensitization rate in allergic patients.

In experiments with PBMCs or T-cell lines, antigen specific proliferation often originates from a small number of specific T-cells. T-cell cloning is an important system to characterize these cells that are potential targets for therapeutic interventions in allergic diseases. It allows the analysis of the cytokine response and the expression of surface markers of one TCC specific for one epitope. On the downside, large numbers of TCC must be generated to draw proper conclusions.

We isolated BP-specific TCCs from allergic patients using BP extract. The majority (52%) were specific for Bet v 1. All tested TCC specific for the main isoform Bet v 1.0101, reacted to one of the distinct T-cell-activating regions in Bet v 1, described by Jahn-Schmid et al.[43]. Their cross reactivity with several Bet v 1-isoforms could be explained by their identical sequence of the recognized T-cell epitope (Figure 24).

None of the TCCs reacted with the allergens Bet v 3-7 and only one TCC was specifc for Bet v 2. The concentration of the minor allergens in birch pollen and the sensitization rate of allergic patients is very low compared to Bet v 1, therefore these results were not surprising. Still 36% of all TCCs were not reactive to any of the tested allergens but responded to BP extract. These clones might be specific for a yet unknown protein in birch pollen.

The ratio of T_H1 and T_H2 cells and their produced cytokines play a major role in allergic diseases. In order to classify TCCs into T_H1/T_H2 cells, their cytokine patterns in response to specific stimulation were measured and the T_H subset was determined accordingly. The majority (58%) of Bet v 1 specific TCCs were T_H2–like cells. They expressed high levels of IL-4 and negligible levels of IFNγ. No Bet v 1-specific TCC belonged to the T_H1 subset and the remaining 42% were T_H0 like cells. In contrast, Bet v 1-negative, birch pollen-reactive TCCs belonged mostly to the T_H0 subset (80%), synthesizing comparable levels of IL-4 and IFNγ. The remaining 20% were T_H1 like cells. According to the present data it might be possible that the specificity of T-cells to the major allergen Bet v 1 correlates with an increased differentiation of T-cells to the T_H2 subset.

Allergen-specific TCCs are very suitable to characterize the expression of homing markers and surface markers defining their subset. All clones expressed the coreceptor $CD4^+$ as well as CD3 and TCRα/β defining them as T-helper cells with a α/β TCR. None of the clones expressed the skin homing marker CLA which was expected, as none of the patients showed skin manifestations origining from an allergic disease.

We also tried to determine the phenotype of TCCs by specific markers for T_H-subsets. The surface markers TIM-3 and CCR5 are preferentially expressed by TH1 as cited by Sabatos et al. and Bonecci et al. respectively[64,65]. CrTH2 and CCR3 were used to determine Th2 cells by Cosimi et al. and Bonecci et al. respectively[65,66]. Unfortunately the results were often inconclusive and contradictive to the cytokine levels measured in the supernatant. Therefore, we decided to rely solely on the cytokine-levels for the determination of the T_H subtype.

After the limiting dilution steps during T-cell cloning, it is possible to have different cells in the same well or clones of the same T-cell in different wells. To adress this problem, hypervariable regions of the TCR α and β chain were mapped by a PCR-based method to fingerprint each TCC (Table 11). This was especially important for the discrimination of clones that could not be distinguished by their cytokine levels or allergen specificity.

In summary, I was able to produce endotoxin-free batches (>5 mg) of recombinant Bet v 3, Bet v 4, Bet v 6 and Bet v 7 with confirmed IgE reactivity. For the first time this study demonstrates the T-cell stimulating capacity of these minor allergens. Now we are equipped with a pannel of characterized minor allergens which will help to examine the differences between Bet v 1 and minor allergens. Furthermore it is now possible to determine their prevalence of recognition in a large population of allergic patients. Although no TCC specific

for the characterized allergens was isolated in this study, the stored TCC are valuable in the search for new proteins with immunological value in birch pollen.

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Appendix

Zusammenfassung

Allergische Erkrankungen haben in urbanen Regionen epidemische Ausmaße angenommen und beeinträchtigen die Lebensqualität der betroffenen Personen. Pollen von windbestäubenden Pflanzen wie der Europäischen Weiss-Birke (*Betula verrucosa*) gehören in Mittel- und Nordeuropa zu den potentesten Allergenquellen. Birkenpollen enthält das gut charakterisierte Hauptallergen Bet v 1 sowie mehrere Nebenallergene, die von weniger als 50% der Birkenpollen-Allergiker erkannt werden.

Diagnose und Therapie von Typ I Allergien hängen von der Qualität der verwendeten Allergenextrakte ab. Dabei können Defizite wie Chargenvariabilität oder unterschiedliche Konzentrationen verschiedener Komponenten auftreten. Diese können durch die Verwendung rekombinanter Allergene überwunden werden.

Die wichtigsten Ziele dieser Arbeit waren i) die Expression, Aufreinigung und Charakterisierung der Birkenpollen-Nebenallergene Bet v 3, Bet v 4, Bet v 6, Bet v 7 und ii) die Isolierung und Charakterisierung von T-Zell-Klonen (TCC) spezifisch für Birkenpollen Proteine.

Alle Nebenallergene wurden in *E.coli* mit einem His-Tag exprimiert und mittels Ni-Affinitätschromatographie aufgereinigt. Der LPS-Gehalt wurde auf vernachlässigbare Mengen reduziert. Die korrekte Faltung und Allergenizität wurde durch IgE ELISA-Experimente mit Seren von Birkenpollen-Allergikern getestet. Die Fähigkeit Birkenpollen-spezifische T-Zellen zu aktivieren wurde durch Proliferationsassays mit mononukleären Zellen des peripheren Blutes (PBMC), allergen-spezifischen T-Zell-Linien (TCL) und T-Zell-Klonen von Birkenpollen-Allergikern ermittelt. Alle rekombinanten Allergene konnten IgE-Antikörper binden und Proliferation in PBMC induzieren.

Allergen-spezifische TCC wurden aus Birkenpollen-spezifischen TCL isoliert und anhand von Proliferationsassays charakterisiert. Marker für T-Zellen und spezifische T-Zell Subtypen wurden mittels Durchflusszytometrie analysiert. Zusätzlich wurden Zytokinkonzentrationen in Überständen von aktivierten Allergen-spezifischen TCC durch Bead-Arrays bestimmt. Unsere Ergebnisse zeigen, dass die Mehrzahl der mit Birkenpollenextrakt expandierten TCC und TCL spezifisch für Bet v 1 waren und diese mehrheitlich zum T_H2 Subtyp gehörten. TCC die nicht mit Bet v 1 reagierten gehörten großteils zum T_H0 Subtyp. Ein TCC war spezifisch für das Nebenallergen Bet v 2. Kein Klon reagierte mit den rekombinanten Allergenen Bet v 3-7.

Im Rahmen dieser Diplomarbeit wurden endotoxinfreie Chargen von Bet v 3, Bet v 4, Bet v 6 und Bet v 7 hergestellt und deren IgE-Reaktivität bestätigt. Zum ersten Mal konnte die T-Zell-aktivierende Fähigkeit dieser Nebenallergene gezeigt werden.

In Zukunft könnten die produzierten und charakterisierten Allergene helfen, die immonologischen Unterschiede zwischen Bet v 1 und den identifizierten Nebenallergenen im Birkenpollen aufzuklären. Darüber hinaus könnten die eingefrorenen TCC hilfreich bei der Suche nach neuen Proteinen von immunologischen Wert im Birkenpollen sein.

Abstract

Allergic diseases have reached epidemic dimensions in urban areas and reduce the patients´ quality of life. Pollen from wind pollinated plants is among the most potent allergen sources. Pollen from the European white birch *Betula verrucosa* contain the well characterized major allergen Bet v 1 and several minor allergens which are recognized by less than 50% of birch pollen-allergic patients.

Diagnosis and therapy of Type I allergy depends on the quality of the employed allergen extracts. Disadvantages such as batch to batch variability or standardization of the concentration of different components in allergen extracts may be overcome by the use of recombinant allergens.

The major aims of this thesis were i) the expression, purification and characterization of the minor birch pollen allergens Bet v 3, Bet v 4, Bet v 6, Bet v 7 and ii) the isolation and characterization of T-cell clones (TCC) specific for proteins in birch pollen.

All minor allergens were expressed in *E.coli* with a His-tag and purified by Ni- affinity chromatography. The LPS content was reduced to negligible levels. Correct folding and allergenicity of the allergens was tested by IgE ELISA experiments conducted with sera from birch pollen-allergic patients. The ability of the allergens to activate birch pollen-specific T-cells was assessed in proliferation assays using peripheral blood mononuclear cells (PBMC), allergen-specific T-cell lines (TCL) and TCC derived from birch pollen-allergic patients. All recombinant minor allergens were able to bind IgE antibodies and induced proliferation in PBMC from birch pollen-allergic patients.

Allergen specific TCC were expanded from birch pollen-specific TCL and characterized in proliferation assays. Various subset-specific markers as well as molecules up regulated by specific activation were analyzed by flow cytometry. Cytokine levels in the supernatants of allergen-activated TCC were determined by cytokine bead arrays. Our results demonstrate that the majority of the TCC isolated from TCL expanded with birch pollen extract were specific for Bet v 1 and belonged to the T_H2-like subset. TCC non-reactive with Bet v 1 belonged to the T_H0-subset. None of the clones reacted with the recombinant minor allergens Bet v 3-7. One TCC was found to be Bet v 2-specific.

In summary, endotoxin free batches of recombinant Bet v 3, Bet v 4, Bet v 6 and Bet v 7 were produced and their IgE reactivity was confirmed. For the first time, the T-cell activating capacity of these minor allergens was shown.

In the future, the produced and characterized allergens may help to examine the differences between Bet v 1 and minor allergens. Furthermore, the stored TCC are valuable in the search for new proteins with immunological value in birch pollen.

Abbreviations

Amp	ampicillin
APCs	antigen presenting cells
BCA	bicinconinic acid
cDNA	complementary DNA
CIAP	Calf Intestine Alkaline Phosphatase
kDa	kilo Dalton
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetraacetate
ELISA	enzyme-linked immunosorbent assay
EtBr	Ethidium Bromide
HIS	histidine
HRP	horseradish peroxidase
IFN γ	Interferon γ
Ig	Immunoglobulin
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IS	Immune system
LPS	lipopolysaccharide
M	molar
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MW	molecular weight
Ni	Nickel
NK-cells	natural killer cells
PAGE	polyacrylamide gelelectrophoresis
PAMPS	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
SI	stimulation index
TCC	T-cell clones
TCL	T-cell lines
TCR	T-cell receptor
TLRs	Toll like receptors
TNFα	Tumor necrosis factor α
U	Units

Restriction sites used in pHis parallel 2 vector:

4851 AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC CGCAAGGAAT
4901 GGTGCATGCA AGGAGATGGC GCCCAACAGT CCCCCGGCCA CGGGGCCTGC
4951 CACCATACCC ACGCCGAAAC AAGCGCTCAT GAGCCCGAAG TGGCGAGCCC
5001 GATCTTCCCC ATCGGTGATG TCGGCGATAT AGGCGCCAGC AACCGCACCT
5051 GTGGCGCCGG TGATGCCGGC CACGATGCGT CCGGCGTAGA GGATCGAGAT
5101 CTCGATCCCG CGAAATTAAT ACGACTCACT ATAGGGGAAT TGTGAGCGGA
5151 TAACAATTCC CCTCTAGAAA TAATTTTGTT TAACTTTAAG AAGGAGATAT
5201 ACATATGTCG TACTACCATC ACCATCACCA TCACGATTAC GATATCCCAA
5251 CGACCGAAAA CCTGTATTTT CAGGGCGCCA TGGGATCCGG AATTCAAAGG
5301 CCTACGTCGA CGAGCTCAAC TAGTGCGGCC GCTTTCGAAT CTAGAGCCTG
5351 CAGTCTCGAG CACCACCACC ACCACCACTG AGATCCGGCT GCTAACAAAG
5401 CCCGAAAGGA AGCTGAGTTG GCTGCTGCCA CCGCTGAGCA ATAACTAGCA
5451 TAACCCCTTG GGGCCTCTAA ACGGGTCTTG AGGGGTTTTT TGCTGAAAGG
5501 AGGAACTATA TCCGGAT

Restriction sites for BamHI and Not I in red

Media and buffer

10x TBE solution:
54 g Tris base
27.5 g Boric acid
20 mL 0.5 M EDTA solution / or: 4.65 g (Na_4EDTA)
Fill up to 500 mL with ddH_2O.

LB Amp medium:
5 g NaCl
10 g peptone
5 g yeast extract
Fill up to 1000 mL with ddH_2O
Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 mg/mL) under laminar flow/sterile conditions and keep in fridge.

LB medium:
5 g NaCL
10 g peptone
5 g yeast extract
Fill up to 1000 mL with ddH_2O
Autoclave and store at RT.

LB Amp plates:
5 g NaCL
10 g peptone
5 g yeast extract
15 g Agar
Fill up to 100 mL with ddH_2O
Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 mg/mL) and pour plates under laminar flow/sterile conditions.

LB plates:
5 g NaCL
10 g peptone
5 g yeast extract
15 g Agar
Fill up to 100 mL with ddH_2O
Autoclave and pour plates under laminar flow/sterile conditions.

10x PBS:
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4
pH = 7.4 (HCl/NaOH)
Fill up to 1000 mL with ddH_2O.

Coomassie Brilliant Blue:
1 g Coomassie brilliant blue G-250
500 mL methyl alcohol
100 mL acetic acid
400 mL dd H_2O
Dissolve Brilliant Blue in methyl alcohol first O/N
and then add the other substances because otherwise
the Brilliant Blue won't dissolve properly.

Destaining solution:
100 mL methyl alcohol
100 mL acetic acid
800 mL ddH_2O

Lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole):
6.057 g Tris
29.22 g NaCl
2.04 g imidazole
Fill up to 1000 mL with ddH_2O pH = 7.5 with HCl

Elution buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 500 mM imidazole):
6.057 g Tris
29.22 g NaCl
34.04 g imidazole
Fill up to 1000 mL with ddH_2O pH = 7.5 with HCl
1 mg/mL DNase stock solution:

Buffer: 10 mM Tris/Hcl pH = 7.5, 150 mM NaCl, 1 mM MgCl
For 25 mL of buffer:
0.03 g Tris
0.219 g NaCl
0.00508 g MgCl
Dissolve 2 mg of DNase in 1 mL of buffer and when DNase has dissolved completely add 1 mL of glycerol.

Carbonate buffer (pH = 9.6):
1.965 g Na_2CO_3
2.645 g $NaHCO_3$
Ad 500mL of ddH_2O

Tissue culture media:
UCØ medium:
500 mL Ultra Culture medium (Lonza Group Ltd., Basel, Switzerland)
5 mL Glutamin (2.937 g/100 mL)
2.5 mL β-mercaptoethanol (35 µL/50 mL)
1 mL Gentamycin (10 g/118 mL)

N_2 medium
500 mL RPMI 1640 buffered with 25mM Hepes (Gibco®,Invitrogen GmbH)
20% FCS (PAA Laboratories GmbH)
10% DMSO
1 mL Gentamycin (10 g/118 mL)
(Note: Add FCS to RPMI1640 medium and then add DMSO dropwise whilst gently shaking!)

Curriculum vitae

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C. Walterskirchen, S. Mutschlechner, S. Deifl, B. Nagl, S. Scheurer, G. Zlabinger, B. Bohle
Identification and characterization of non-allergenic proteins in birch pollen
Poster presentation