universität wien

MASTERARBEIT

Titel der Masterarbeit

„Purification of seed storage proteins from tree nuts and peanut and their range of cross-reactivity – implication for the nut allergic patient“

Verfasserin

Christiane Agreiter, BSc

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 066 830
Studienrichtung lt. Studienblatt:	Masterstudium Molekulare Mikrobiologie und Immunbiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Manuela Baccarini

Contents

DANKSAGUNG

Der experimentelle Teil dieser Arbeit wurde am Institut für Pathophysiologie und Allergieforschung, des Zentrums für Pathophysiologie, Infektiologie und Immunologie, der Medizinischen Universität Wien durchgeführt.

Assoc. Prof. Univ. Doz. Dr. Karin Hoffmann-Sommergruber danke ich für die Ermöglichung meiner Arbeit in der Arbeitsgruppe für Molekulare Allergologie sowie für die wissenschaftliche Betreuung und Begutachtung meiner Masterarbeit.

Ich danke auch Priv.-Doz. Dr. Merima Bublin für die Zeit und Energie, die sie in die Betreuung meiner Arbeit gesteckt hat.

Ich danke außerdem Univ. Prof. Dr. Manuela Baccarini für die Begutachtung und Beurteilung dieser Arbeit.

Weiters möchte ich mich bei meinen LaborkollegInnen für die vielen Hilfestellungen und auch für die netten Kaffeepausen bedanken! Ein besonderer Dank geht an Nina, die mir immer wieder bei meinen kleineren und größeren Problemen mit der Chromatographie beistand.

Ein ganz besonderer Dank geht an meine Freunde die immer für mich da sind und an mich glauben.

Der größte Dank gebührt meinen Eltern die meine Studienzeit finanziert haben und mir auch in schwierigen Zeiten den Mut und die Kraft gegeben haben weiter zu machen.

LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
CD	Circular dichroism
CD	Cluster of differentiation
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DBPCFC	Double blind placebo controlled food challenge
DMF	Dimethylformamid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FPLC	Fast protein liquid chromatography
FT-IR	Fourier transform infrared
GM-CSF	Granulocyte macrophage colony stimulating factor
HIC	Hydrophobic interaction chromatography
HPLC	High pressure liquid chromatography
IEF	Isoelectric focusing
IEX	Ion exchange chromatography
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
mM	Milli molar
NBT	Nitroblue tetrazolium
NHS	Normal human serum
nsLTP	Non-specific Lipid Transfer Protein

OD	Optical density
pI	Isoelectric point
RAST	Radioallergosorbent test
SDS – PAGE	Sodiumdodecylsulfate-polyacrylamide gel-electrophoresis
TEMED	N, N, N‘, N‘-tetramethylethylenediamine
T_H	T helper cell
TNF-α	Tumor necrosis factor-α

1 Introduction

1.1 Hypersensitivity reactions

In 1906 the Austrian scientist and paediatrician Clemens von Pirquet coined the term „allergy“ for the first time [1]. Treating his patients he observed that some of them developed symptoms like skin rash or lymph node swelling when they were given an antiserum. He termed this reaction „serum sickness“.
Von Pirquet proposed the term “allergy“ from the Greek words *allos* (other) and *ergon* (work) because he recognized that serum sickness is caused by an external agent. For this external agent he proposed the name “allergen“ [2].

In 1963 Gell and Coombs established a classification scheme for hypersensitivity reactions. They proposed four classes, Type I to Type IV [3]. This classification is shortly summarized in Table 1.

Type of hypersensitivity	**Pathologic immune mechanisms**	**Mechanisms of tissue injury and disease**
Immediate hypersensitivity: Type I	IgE antibody	Mast cells and their mediators (vasoactive amines, lipid mediators, cytokines)
Antibody mediated hypersensitivity: Type II	IgM, IgG antibodies against cell surface or extracellular matrix antigens	Opsonization and phagocytosis of cells Complement- and Fc-receptor-mediated recruitment and activation of leukocytes Abnormalities in cellular functions
Immune complex mediated hypersensitivity: Type III	Immune complexes of circulating antigens and IgM or IgG antibodies	Complement- and Fc-receptor-mediated recruitment and activation of leukocytes
T cell mediated hypersensititvity: Type IV	1. $CD4^+$ T cells (delayed-type hypersensitivity) 2. $CD8^+$ CTLs (T cell-mediated cytolysis)	1. Macrophage activation, cytokine-mediated inflammation 2. Direct target cell killing, cytokine-mediated inflammation

Table 1: Classification of hypersensitivity reactions according to Gell and Coombs.
(according to: Abbas, A.K., Lichtman, A.H. Cellular and Molecular Immunology. 6th edition, 2007) [4].

Type I hypersensitivity reaction is also called immediate hypersensitivity because the reaction occurs within minutes. An antigen is recognized by IgE which is bound to FcεRI on mast cells. This leads to degranulation of mast cells and to symptoms like angiocœdema, urticaria, hay fever or allergic asthma.
In the healthy body the reason for this reaction is prevention of parasitic affection by nematodes [5-7].

Type II hypersensitivity reaction is also known as an antibody-mediated cytotoxic hypersensitivity. It is caused by IgM and IgG antibodies [4].
These antibodies bind to host cells and form complexes which activate the complement system. The complement protein C5 is synthesized. Moreover, C5 serves as a chemoattractant for polymorphonuclear leukocytes (PMNs). PMNs release hydrolytic neutrophil enzymes which lead to tissue injury. Type II hypersensitivity reactions occur within hours up to 24 hours. The evolutionary meaning of this reaction is to react on small extracellular pathogens which can be killed by PMNs [6, 7].

Type III hypersensitivity reaction is also known as an immune-complex mediated reaction. It is due to IgM and IgG antibodies. This type of hypersensitivity is a response to soluble antigens. They are bound to antibodies in tissues. An increasing number of these antigen-antibody-complexes leads to an inflammatory response and consequently to tissue injury [4] .
This reaction develops within hours or weeks. A typical example for type III hypersensitivity is the Arthus reaction. It was described in 1903 by Maurice Arthus [8]. He injected horse serum intradermally into a rabbit. The rabbit showed symptoms like œdema and hemorrhage and neutrophil infiltration of the skin. In healthy individuals this reaction developed to deal with circulating viral particles as viral particles in the blood are bound by antibodies and therefore cannot enter and damage a cell [6, 7].

Type IV hypersensitivity reaction is also called delayed-type hypersensitivity. It takes about 24 to 48 hours to develop [4]. Naive T-cells develop to memory T

cells after antigen exposure. In further encounters memory T cells differentiate to effector T cells very quickly and host cells are killed by apoptosis and cytotoxicity. Typical symptoms are contact dermatitis or local inflammation [9]. The evolutionary meaning of this reaction is response to pathogens by cytotoxic T-cells [6, 7].

1.1.1 Type I hypersensitivity

Type I hypersensitivity reactions are mostly linked with atopy. Atopy is the ability to produce specific IgE against an allergen. The word originates from the Greek *atopos* meaning “out of place“ and was introduced in 1922 by Coca and Cooke [10].

The mechanism of an allergic reaction can be divided into three phases: sensitization, immediate reaction and late phase reaction [4]

During sensitization the allergen is taken up by a dendritic cell. The dendritic cell migrates into the lymphoid tissue where the peptide/MHCII-complex (peptide/major histocompatibility complex II) is presented to naïve $CD4^+$ cells. This leads to differentiation of the T cell to a T_H2 cell (T helper 2 cell).

Three signals are necessary for interaction of APC (antigen presenting cell) and T-cell: an antigen specific signal (binding of MHCII/peptide and T-cell receptor), costimulation (mainly B7-1 and B7-2) and cytokines which determine the differentiation of T cells. The cytokines IL-4 (interleukin-4) and IL-33 lead the differentiation into the direction of T_H2 cells [11-13]. T_H2 cells interact with B cells. IL-4 leads to class switch reaction from IgG to IgE. IgE antibodies bind to FcεRI (fragment crystallisable epsilon receptor I) receptors on mast cells.

When the allergen enters the body it binds to allergen specific IgE, which is bound to FcεRI on the mast cells and cross-links them [14]. This leads to a process called “degranulation” which is the release of mediators like histamine, lipid mediators like prostaglandins and leukotrienes, proteases like tryptase and chymase, cytokines like TNF-α (tumor necrosis factor-α), GM-CSF (granulocyte macrophage-colony stimulating factor), macrophage inflammatory protein-1α, IL-3, IL-4, IL-5, IL-6 and IL-10 for example. This gives rise to symptoms like vasodilation, bronchoconstriction or inflammation [11, 15, 16]. Two to four hours

after allergen contact, during the late phase reaction, these mediators act on eosinophils, T_H2 cells and basophils. This leads to contraction of smooth muscle cells, asthma, œdema and chronic inflammation [16].

1.2 Food allergy

In general, adverse reactions to food are grouped into “non-toxic reactions” and “toxic reactions”. “Toxic reactions” occur in all individuals in the same way whereas “non-toxic reactions” only occur in susceptible individuals. In the group “non-toxic reactions” “food allergies” and “food intolerances” are found. In contrast to “food allergies” “food intolerances” are not immune-mediated. This classification scheme is summarized in Figure 1 [17].

Food allergy is an adverse immunological response to food proteins which is mediated by IgE leading to symptoms like urticaria, angiocœdema, rhinitis, asthma, emesis, diarrhoea or anaphylactic shock [4].

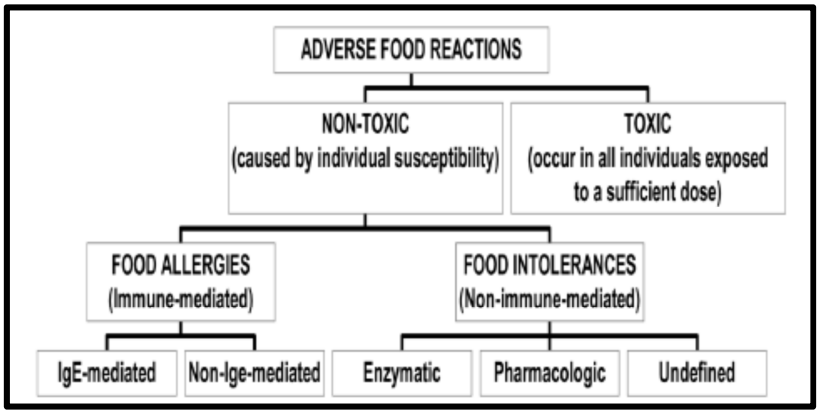

Figure 1: Classification of adverse food reactions. (taken from: Asero, R. et al. Mol Nutr Food Res, 2007: 51 (1), 135-47) [17]

About 2.3% of children and about 0.4 to 1.4% of adults are affected by tree nut or peanut allergy [18]. Hypersensitivity reactions to nuts can be life threatening since it can induce an anaphylactic shock [19].

1.3 Plant food allergens

Plant food allergens are classified into protein superfamilies and protein families. Most of the allergens belong to a few families and superfamilies like cupin superfamily or prolamin superfamily [20].

1.3.1 Prolamin superfamily

Proteins belonging to the prolamin superfamily have eight conserved cysteine residues. According to that they have four α-helices stabilized by disulfide bonds resulting in a conserved 3 dimensional structure. The overall sequence similarity between different members of the prolamin superfamily is rather low [21].

This superfamily consists of the families of non-specific lipid transfer proteins (nsLTPs), 2S albumins and cereal α-amylase and protease inhibitors [20].

1.3.1.1 Non-specific Lipid Transfer Proteins

Non-specific Lipid Transfer Proteins are basic proteins with an isoelectric point of about 9 [22]. Their molecular mass is 9-10 kDa and their primary structure consists of 91 to 95 amino acids [22].

Moreover, nsLTPs possess four α-helices which are kept together by four disulfide bridges built by eight conserved cysteine residues. Accordingly, the four α-helices are linked by short flexible loops. This forms a large internal hydrophobic cavity which is able to bind lipids [22, 23] .

According to the eight conserved cysteines there are two consensus sequences: T/SXXDR/K (residues 43-47) and PYXIS (residues 81-85) [23].

Non-specific lipid transfer proteins are synthesized with a signal peptide of 21-27 amino acids on the N-terminus. This signal peptide is responsible for arranging the co-translational insertion of the peptide into the lumen of the endoplasmic reticulum. Because of the missing endoplasmic reticulum retention signal (KDEL) at the C-terminus the nsLTPs enter the secretory pathway [22].

The name of the nsLTPs refers to their ability to transfer a broad range of lipids between membranes *in vitro* which was shown first by Kader et al. [22] in 1996.

Nowadays it is thought that nsLTPs play a role in plant defence against bacteria and fungi as it was demonstrated by Wang et al.[24] for nsLTP from mung bean seeds.

Non-specific lipid transfer proteins were first described in 1975 by Kader [25]. The first allergenic nsLTPs were described in *Rosaceae* fruits by Lleonart et al. [26] in 1992. Moreover, it was recognized that the reason for this type of allergy was different than cross-reaction to pollen and food allergens and the symptoms reported by nsLTP allergic patients were severe. In contrast, patients suffering from birch pollinosis, who react to birch pollen allergen homologues, show milder symptoms [27].

Asero et al. [28-30] showed that cross-reaction between taxonomically unrelated plant foods can be observed due to sensitization by nsLTPs. Consequently they deduced that nsLTPs could be important panallergens.

According to their resistance to proteolytic digestion, nsLTPs keep their allergenicity even in the gastrointestinal tract. After 2 hours treatment with pepsin at acidic pH they are still unaffected and after 24 hours treatment still 40% of the protein is stable [31].

Gaier et al. [32] showed that the nsLTP form peach (Pru p 3) gets denaturated when it is heated up to 95°C at a pH of 7.5. The denatured Pru p 3 was unable to refold when the temperature was cooled down to 25°C. At a pH of 3 Pru p 3 was not completely denatured when it was heaten up to 95°C. These data indicate that Pru p 3 is more stable under acidic conditions than at neutral pH. Heat treatment at acidic pH mimics conditions at food processing and shows that Pru p 3 is correctely folded in processed peach products.

Non-specific lipid transfer proteins also show a high resistance to heat treatment up to 100°C [33-35]. Therefore, they keep their allergenic potential even in plant products which are heated during procession.

1.3.1.2 2S albumins

2S albumins are seed storage proteins belonging to the prolamin superfamily. Their name arises from their sedimentation coefficient [36]. Moreover, 2S

albumins serve the plant as a source of nutrients which are needed for germination and seedling growth [37].

2S albumins are encoded by a multigene family and therefore contain many isoforms. Typically, 2S albumins are synthesized as a precursor polypeptide with a molecular mass of 18-21 kDa. This peptide is transported into the lumen of the endoplasmic reticulum where it folds. Further, the protein is transported into the vacuole where it is processed to a polypeptide of 12-14 kDa. In most cases this polypeptide is processed to a large (8-10 kDa) and a small (3-4 kDa) subunit which are held together by two intermolecular disulfide bonds. These disulfide bonds are formed between the cysteine-residues 1-5 and 2-3. In the large subunit two intra-molecular disulfide bonds are formed by the cysteine-residues 4-7 and 6-8.

The three dimensional structure of 2S albumins shows a scaffold built by five α-helices and a C-terminal right-handed superhelix. In addition the scaffold is stabilized by the four disulfide bonds [38].
A distinct region which can be seen when the α-helices III and IV are connected is called „hypervariable region“. Here preferred IgE-recognition takes place and therefore it is very important in relation to allergenicity [39].

An allergic reaction to 2S albumins can lead to severe symptoms like angiocœdema and anaphylactic shock [40]. 2S albumins are very stable to proteolytic attack and heat treatment and therefore they keep their allergenicity in the gut and they are resistant in food processing [41, 42].
2S albumins have high structure similarity but even though cross-reaction is rare because the variability is mainly in the IgE-binding site (hypervariable region) [43-45].

1.3.2 Nuts as allergen sources

1.3.2.1 Hazelnut (*Corylus avellana*)

In Europe allergy to hazelnut is quite frequent [46]. Hazelnut allergy often develops in patients suffering from birch pollinosis due to cross-reactivity between the major birch pollen allergen Bet v 1 and the hazelnut homologue Cor a 1. This occurs frequently in Central and North Europe were birch trees are abundant [47]. Cor a 2, the profilin from hazelnut, is homologous to birch pollen profilin Bet v 2. Cor a 2, as other profilins, is not resistant to heat treatment and protease digestion. In central Europe it is a minor allergen [48-51]. Schocker et al. [52] described a patient with hazelnut allergy without cross-reacting IgE antibodies to birch pollen allergens. In this case the sensitizing agent is the hazelnut nsLTP, Cor a 8, which was described by Pastorello et al. [53] in 2002. Allergy to Cor a 8 can also develop due to cross-reaction with other nsLTPs, especially those from fruits from the *Rosaceae* family (peach, cherry, apricot). This cross-reaction mainly happens in the Mediterranean Area where those fruits are consumed frequently and birch trees are not endemic [52]. Cor a 9 is a 11S globulin found to be a hazelnut allergen. It consists of a basic and an acidic subunit linked by disulfide bonds [54]. Cor a 11 is a vicilin of hazelnut which was found to be a minor allergen by Lauer et al [55]. It has two potential N-glycosylation sites (Asn38 and Asn254) of which only one is glycosylated in the mature protein. Cor a 12 and Cor a 13 both are oleosins with a hydrophilic N-terminal amino acid stretch, a highly conserved hydrophobic central domain and a C-terminal flanking sequence consisting of an amphipathic alpha helix [56]. Cor a 14, the 2S albumin of hazelnut was described by Garino et al. [57] in 2010. In Table 2 there is a summary of all known hazelnut allergens and their properties.

Allergen name	Species	Biochemical name	Sequence accession number	Molecular mass [kDa]	Isoelectric point
Cor a 1	*Corylus avellana*	Pathogenesis-related protein, PR-10	Q08407	17	5.43
Cor a 2	*Corylus avellana*	Profilin	Q9AXH5	14	4.90
Cor a 8	*Corylus avellana*	nsLTP	Q9ATH2	9	9.30
Cor a 9	*Corylus avellana*	11S globulin (legumin)	Q8W1C2	40	6.46
Cor a 10	*Corylus avellana*	Luminal binding protein	Q9FSY7	70	4.97
Cor a 11	*Corylus avellana*	7S globulin (vicillin)	Q8S4P9	48	6.05
Cor a 12	*Corylus avellana*	17 kDa oleosin	Q84T21	17	10.54
Cor a 13	*Corylus avellana*	14-16 kDa oleosin	Q84T91	14-16	9.98
Cor a 14	*Corylus avellana*	2S albumin	D0PWG2	15-16	6.59

Table 2: Overview of the properties of hazelnut allergens according to the International Union of Immunological Societies (IUIS, www.allergen.org). The allergen used in this study is marked grey.

1.3.2.2 Walnut (*Juglans Regia*)

The first described walnut allergen was the precursor of the 2S albumin Jug r 1 [58]. Jug r 1 has a molecular mass of about 12 kDa without signal peptide and an isoelectric point of 5,75. It consists of a small and a large subunit [59]. In Figure 2 the structure of Jug r 1 can be seen.

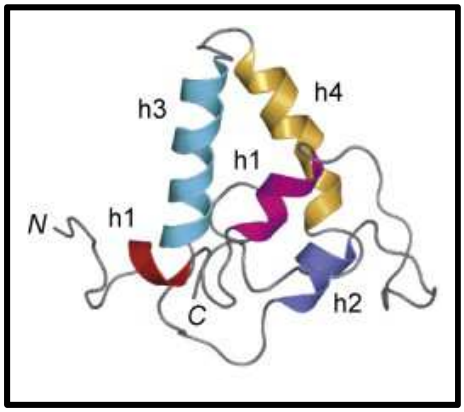

Figure 2: The structure of Jug r 1. The α-helices 1-4 are coloured in red, violet, blue and yellow. Loops and coiled regions are grey. This structure of natural Jug r 1 is represented as a single polypeptide chain. (taken from: Sordet, C. et al. Peptides, 2009. 30 (7), 1213-21) [59]

A linear IgE-reactive epitope of Jug r 1 was described by Robotham et al. [60] in 2002. It consists of 12 amino acids at the position 33 to 44: QGL**RGEE**ME**E**MV. They showed that the amino acids RGEE at the positions 36 to 39 are the core amino acids for IgE-binding. In addition the glutamic acid residue at position 42 is necessary for maximum IgE-binding.

Until today three more allergens have been described in walnut, the 7S vicilin-like globulin Jug r 2 [61], the nsLTP Jug r 3 [62] and Jug r 4 which is an 11S legumin-like globulin [63, 64]. A summary of these allergens and their properties is listed in Table 3.

Jug r 2 is the 7S globulin (vicilin) of English walnut with a molecular mass of 69.9 kDa and a pI of 6.19. As a seed storage protein it plays an important role as nitrogen reserve but it is also involved in plant defense mechanisms [61].

The nsLTP of walnut, Jug r 3, was described in 2004 by Pastorello et al. [62]. Like nsLTPs in other plants it has a molecular mass of about 9 kDa. Its isoelectric point is 9.45. A periodic acid-Schiff stain showed that there is no glycosylation. In the study of Pastorello et al. only patients without birch pollinosis reacted to Jug r 3. Patients reacting to Jug r 3 often show severe symptoms. Moreover, Jug r 3 is a true food allergen as it can be the sensitizing agent [62].

Jug r 4, the 11S globulin of walnut, is encoded by a multigene family. After translation the peptide is proteolytically processed into two subunits, the acidic and the basic subunit whereas the acidic subunit has a molecular mass of about 35-40 kDa and the molecular mass of the basic subunit is 20-30 kDa [65, 66].

Allergen name	**Species**	**Biochemical name**	**Sequence accession number**	**Molecular mass [kDa]**	**Isoelectric point**
Jug r 1	*Juglans regia*	2S albumin	P93198	15.5	5.75
Jug r 2	*Juglans regia*	7S globulin, vicillin	Q9SEW4	44	6.19
Jug r 3	*Juglans regia*	nsLTP	C5H617	9	9.20
Jug r 4	*Juglans regia*	11S globulin, legumin	Q2TPW5	58.1	6.80

Table 3: Overview of the properties of walnut allergens according to the IUIS (www.allergen.org). The allergens used in this study are marked grey.

1.3.2.3 Brazil Nut (*Bertholletia excelsa*)

The Brazil nut belongs to the family of *Lecythidaceae* and to the order of *Ericales* [67]. Its major allergen is the 2S albumin Ber e 1 with a molecular mass of 12 kDa and an isoelectric point of 6.4 [68]. Ber e 1 is rich in sulfur containing amino acids as it contains about 30% methionine and cysteine residues in its amino acid sequence [69]. In Table 4 the two known allergens from Brazil nut and their properties are summarized.

Ber e 1 is synthesized through an 18 kDa precursor which is post-translationally processed to a 15 kDa intermediary form. This is further processed to the 12 kDa protein. The 12 kDa peptide is later processed to the large (9 kDa) and the small (3 kDa) subunit (see Figure 3).

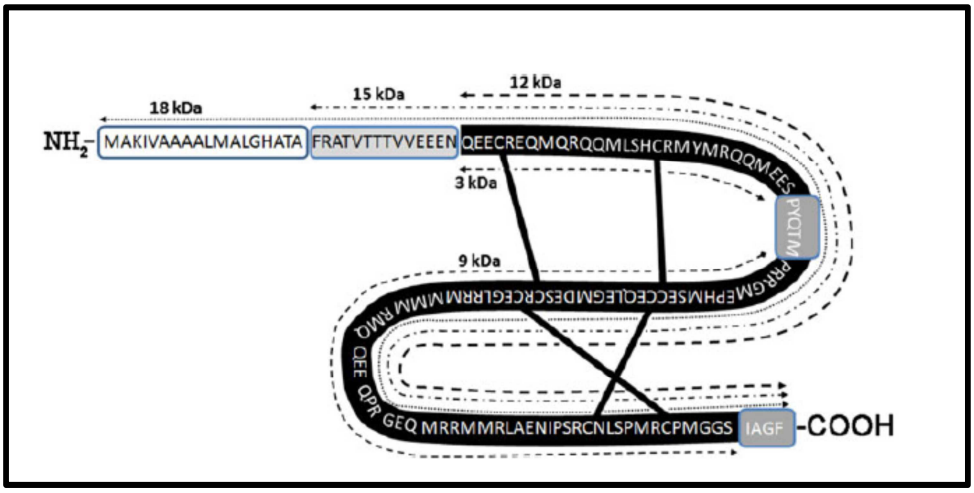

Figure 3: Post-translational processing of Ber e 1 precursor. The mature short and large subunits are shown in black. In the white and grey boxes at the N-terminus the aminoterminal signal sequence and the pre-prosignal sequence are shown. The linker (grey box between small and large subunit) and the C-terminal sequence (grey box) are removed during processing. (taken from: Alcocer, M. et al. Biotechnol Lett, 2012, 34(4), 597-610) [67]

The protein is post-translationally modified and stored in protein bodies which are in the hypocotyls of the embryo [68]. This accumulation of Ber e 1 is linked with the accumulation of the corresponding mRNA [70]. In addition, there are three DNA box sequences which are important for the regulation of this accumulation [71].

Ber e 1 is resistant to heat treatment up to 65°C at a pH of 2.2. This also shows a high tolerance of acidic environment. At 95°C Ber e 1 is significantly unfolded but if it is cooled again it renatures. In addition Ber e 1 is very resistant to chemical-induced unfolding as it stays stable during a treatment with 4.5 M guanidinium chloride [72, 73].

In a simulated gastric fluid model Ber e 1 stays intact for 30 minutes. Other proteins from Brazil nut were completely digested by pepsin after a few seconds [72]. If the two subunits of Ber e 1 are reduced and alkylated they are digested within 30 seconds [74]. This experiment shows how important the disulfide bridges are for the stability of the protein.

A molecular model for the structure of Ber e 1 has been established using the structure of napin 2S albumin as a template [75].

Three isoforms of Ber e 1 have been characterized using CD (circular dichroism) and FT-IR spectroscopy (Fourier transform infrared spectroscopy) [76]. In 1983 Hide [77] described four cases of Brazil nut allergy. In 1991 the

same group together with Arshad et al. [78] reported twelve cases of allergy to Brazil nut in an atopic population from the Isle of Wight (UK).
It was shown that patients with a positive skin prick test to Brazil nut reacted strongly with Ber e 1 [72]. This supports former investigations showing that Ber e 1 is the major allergen of Brazil nut [78-80].
As Ber e 1 is stable in acidic environment and due to heat treatment it is able to reach the gastrointestinal tract as an intact protein with its allergenic potential [39].

Bartolome et al. [81] identified Ber e 2, a 11S globulin seed storage protein in Brazil nut. It is also known as excelsin and was the second protein in history which was crystallized [82]. Ber e 2 was found to have a molecular mass of 29 kDa and an isoelectric point of 6.14.

Allergen name	**Species**	**Biochemical name**	**Sequence accession number**	**Molecular mass [kDa]**	**Isoelectric point**
Ber e 1	*Bertholletia excelsa*	2S albumin	P04403	9	5.96
Ber e 2	*Bertholletia excelsa*	11S globulin	Q84ND2	29	6.14

Table 4: Overview of the properties of Brazil nut allergens according to the IUIS (www.allergen.org). The allergen used in this study is marked grey.

1.3.2.4 Peanut (*Arachis hypogaea*)

In peanut the 2S albumins Ara h 2, Ara h 6 and Ara h 7 are important allergens as allergic reaction to them often leads to anaphylaxis [83]. Ara h 2 has a molecular mass of 17.9 kDa and an isoelectric point of 5.51 whereas the molecular mass of Ara h 6 is 14.8 kDa and the isoelectric point is 5.49 (see Table 5).
There is a high degree of homology between Ara h 2 and Ara h 6 [84, 85]. It was shown by cross-inhibition experiments that Ara h 2 and Ara h 6 are partially identical and significant cross-reaction between these two allergens was demonstrated [42].

Ara h 2 is characterized by two additional loops at the N- and C-terminus, compared to other 2S albumins (see Figure 4). Moreover, an internal loop contains a tandem repeat of a proline rich hexapeptide DPYSPS. In addition it shows a putative N-glycosylation site Asn88-Gln-Ser which however does not seem to be glycosylated [86].

A further difference between Ara h 2 and other 2S albumins is that Ara h 2 is not processed after translation and therefore consists of one subunit instead of a small and a large subunit.

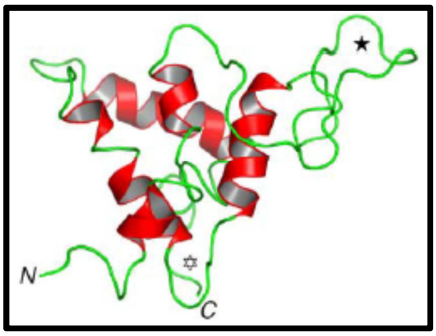

Figure 4: The structure of Ara h 2. The α-helices are coloured in red, the loops and coiled regions are shown in green. The additional loops at the N- and C-terminus are marked with stars. (taken from: Barre, A. et al. Immunol Lett, 2005. 100(2), 153-58) [86]

Ara h 6 belongs to 2S albumins of peanut. A purification protocol and characterization of Ara h 6 was published by Suhr et al. [87] in 2004. Purified Ara h 6 is resistant to pepsin digestion and heat treatment.

Ara h 1, the peanut vicilin, is a major peanut allergen. The homotrimeric protein belongs to the cupin superfamily and is recognized by more than 90% of individuals sensitized to peanuts [20, 88, 89]. Ara h 3 is a legumin from peanut with a molecular mass of 60 kDa. It consists of a basic and an acidic subunit [90]. Ara h 4 was also described as a legumin with about 60 kDa [91]. Sequence analyses showed a high sequence similarity between these two 11S globulins and nowadays Ara h 3 and Ara h 4 are believed to be isoforms [92]. Ara h 5 is the peanut profilin. It is cross-reactive with the profilins of cherry, pear and celery. [93]. Ara h 8 belongs to the PR-10 family and is a clinically relevant allergen as it is cross-reactive with Bet v 1 [94]. Ara h 9 is the nsLTP from peanut which existence was first published by Asero et al. [29] in 2002. Later it was defined as a minor allergen leading to severe symptoms in allergic individuals [95]. Ara h 10 and Ara h 11 are oleosins which are involved in lipid

storage. They were described by Pons [96] in 2005. Ara h 12 and Ara h 13 are both defensins with a molecular mass of about 5 kDa.

Peanut was thought to be a rich source of protein and its production is cheap [97]. Yet, peanut allergy is increasing, especially in western countries and an allergic reaction to peanut can occur by ingesting only a small amount of peanut proteins [98, 99].
Roasted peanuts are more allergenic than boiled ones. The reason therefore is the Maillard reaction in which the amino groups are glycosylated and proteins are cross-linked and thus keep their intact structure in the digestive tract [100].
In Table 5 all known peanut allergens and their properties are listed.

Allergen name	Species	Biochemical name	Sequence accession number	Molecular mass [kDa]	Isoelectric point
Ara h 1	*Arachis hypogaea*	Vicilin-type 7S globulin	P43238	64	6.62
Ara h 2	*Arachis hypogaea*	2S albumin	Q6PSU2	17.3	5.51
Ara h 3	*Arachis hypogaea*	Legumin-type 11S globulin	O82580	60.37	5.68
Ara h 4	*Arachis hypogaea*	Legumin-type 11S globulin	Q9SQH7	61.01	5.48
Ara h 5	*Arachis hypogaea*	Profilin	Q9SQI9	15	4.58
Ara h 6	*Arachis hypogaea*	2S albumin	Q647G9	15.8	5.49
Ara h 7	*Arachis hypogaea*	2S albumin	Q9SQH1	15	5.95
Ara h 8	*Arachis hypogaea*	Pathogenesis-related protein, PR-10	Q6VT83	17	5.03
Ara h 9	*Arachis hypogaea*	nsLTP	B6CEX8	9.1	9.45
Ara h 10	*Arachis hypogaea*	16 kDa oleosin	Q647G5	16	9.61
Ara h 11	*Arachis hypogaea*	14 kDa oleosin	Q45W87	14	10.08
Ara h 12	*Arachis hypogaea*	Defensin	-	5.1	-
Ara h 13	*Arachis hypogaea*	Defensin	-	5.5	-

Table 5: Overview of the properties of peanut allergens according to the IUIS (www.allergen.org). The allergens used in this study are marked grey.

1.4 Cross-reactivity in tree nut and peanut allergens

Cross-reactivity occurs when epitopes from different proteins have a certain structural similarity. An antibody to one epitope then can also bind to the similar epitope from the other protein [101].

In IgE-binding epitopes two different forms can be distinguished. First there are linear epitopes which consist of amino acids which are neighbours in the primary structure. The second ones are conformational epitopes which are formed by amino acids which are further apart in the amino acid sequence but are in close proximity in the folded protein [102].

To identify linear epitopes the techniques used are quite straightforward whereas identifying conformational epitopes is more difficult. Consequently, more linear epitopes are identified than conformational ones [103]. Yet, IgE-antibodies bind rather to conformational epitopes on the protein [104]. The recognition of conformational epitopes by IgE-antibodies was shown in studies by Niemi [105] and Padavattan [106].

The structural homology is often more important in determining cross-reactivity than the botanical relationship. In peanut and tree nuts 2S albumins, nsLTPs, vicilins, legumins and profilins are found. These proteins can be responsible for a possible cross-reactivity. But even high similarity does not necessarily mean that allergens are cross-reactive [107, 108].

Cross-reaction has to be distinguished from co-sensitization. Co-sensitization is when a patient is sensitized to different allergens by independent sensitization events [109].

1.5 The aim of the study

The aim of this master thesis was to establish purification protocols for 2S albumins from tree nuts and peanut. The purification protocol already found in the literature was adapted and modified. Moreover, the IgE-binding properties of the purified allergens were investigated. Finally, the cross-reactivity between the 2S albumins from different nuts were studied as this has a clinical importance for nut allergic patients.

2 Material and Methods

2.1 Chemicals

Material	Supplier
PlusOne DryStrip cover fluid SP Sepharose™ Fast Flow	Amersham Biosciences, Uppsala, Sweden
AKP Mouse Anti-Human IgE	BD Biosciences, Franklin Lakes, NJ USA
BCIP CHAPS DTT Glycine NBT TRIS ultra pure	Biomol GmbH, Hamburg, Germany
Acetonitrile Coomassie Brilliant Blue Sodium acetate SDS ultra pure TEMED Urea	Carl Roth GmbH and CO.KG, Karlsruhe, Germany
Phenyl Sepharose™ Fast Flow Q Sepharose™ Fast Flow IPG-Buffer 3-10 IPG-Buffer 4-7	GE Healthcare, Uppsala, Sweden
APS Glycerol	Life Technologies, Inc., Gaithersburg, MD, USA
Acetone Acrylamide Ammonium acetate Ammonium sulfate Bis-acrylamide Bromophenolblue DMF	Merck, KGaA, Darmstadt, Germany

HCl, 37% Isopropanol Iodoacetamide Methanol n-Hexane NaCl $NaHCO_3$ NaOH MES Sodium acide Thiourea	
BSA	PAA, Pasching, Austria
Complete EDTA-free Protease Inhibitor Cocktail Tablets	Roche Diagnostics GmbH, Mannheim, Germany
Ponceau S PVPP SIGMAFAST™ p-Nitrophenyl phosphate Tablets Tween 20	Sigma-Aldrich Co., St. Louis, MO, USA
Agarose	StarLab GmbH, Ahrensburg, Germany
Page Ruler Prestained Protein Ladder	Thermo Scientific
Acetic Acid Ethanol absolute	VWR, Fontenay-sous-Bois, France

2.2 Allergens

In addition to the purified allergens the following allergens were used in this study: the 2S albumin of hazelnut, Cor a 14, provided by Maria Kühebacher, the 2S albumins Ara h 2/6 from peanut provided by Priv.-Doz. Dr. Merima Bublin, the Brazil nut 2S albumin Ber e 1 provided by Johanna Altmann, Jug r 1/3 and a batch of Jug r 1/2 from walnut were contributed by Tamara Stary. Jug r 1/2 and Jug r 1/3 means that there is a Jug r 1 enriched batch containing impurities of Jug r 2 and Jug r 3, respectively.

2.3 Nuts

The nuts used in this study were bought at a market in Vienna (Naschmarkt).

2.4 Patients‘ sera

Forty-four sera from nut allergic patients with a convincing case history and positive IgE values for tree nuts were used for IgE-ELISA and Immunoblot. Twenty-one out of the 44 patients showed IgE-binding to walnut, 9 to hazelnut and 14 to both, walnut and hazelnut allergens.

2.5 Establishment of purification protocols for 2S albumins and non-specific lipid transfer proteins (nsLTPs) from tree nuts and peanut

2.5.1 Extraction of proteins from walnut

Fifty g of nuts were cut and homogenized with a knife, a mixer and a mortar. N-hexane was used to defat the nuts in a relation of 1:5 (w/v). Therefore, 250 ml of n-hexane was added to the flour and they were stirred for one hour. Afterwards the mixture was filtered and then the rest of n-hexane was removed through centrifugation for 10 min at 10°C and 1,000 g. This procedure was repeated three times.

The defatted nuts were dried over night at room temperature. Next, 300 ml of extraction buffer was added to 20 g of defatted, dried nuts. Subsequently, the suspension was stirred for one hour at room temperature and for 15 hours at 4°C. Afterwards the suspension was filtered. The filtrate was centrifuged for 50 min at 4°C and 18,000 rpm. The supernatant was filtered again.

Extraction buffer

20 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 5.6
0.4 g/l natrium azide
3% (w/v) PVPP (polyvinylpyrrolidone)
1 Complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Mannheim, Germany) per 50 ml

2.5.2 Extraction of proteins from peanut

Peeled peanuts were frozen at -80°C for four hours. Afterwards they were ground using a mixer and defatted using n-hexane. Subsequently, they were stirred in n-hexane for one hour and the mixture was filtered and centrifuged for 10 min at 10°C at 1,000 g to remove n-hexane. This procedure was repeated three times. The defatted nuts were dried at room temperature over night. On the next day the nuts were stirred in extraction buffer for one hour at room temperature and for 15 hours at 4°C.

Before the extract was filtered, the mixture was centrifuged for 20 min at 4°C with 18,000 rpm to get out the whole extract.

Extraction buffer

100 mM ammoniumacetate
pH 5.0
dissolved in MilliQ

2.5.3 Chromatography

2.5.3.1 Ion Exchange Chromatography (IEX)

Using IEX proteins can be separated according to their net surface charge. The principle is based on a charged immobile phase and charged sample molecules. The proteins bind to the column due to ionic interactions.
Proteins below their pI (isoelectrical point) are positively charged and bind to the negatively charged column material of a cation exchanger.
In anion exchange chromatography the column material is positively charged. The proteins above their pI are negatively charged and consequently can bind to the immobilized phase of an anion exchanger.
The net charge of a protein depends on the pH of its environment. Therefore, to separate the proteins a pH gradient can be used. The shift in pH changes the charge of the protein and it can be eluted.
First the column is washed with buffer A, the loading buffer, before the sample is loaded. Next the unbound molecules are washed away using buffer A. Then elution can start by establishing a gradient between buffer A and buffer B. The eluate is collected in fractions for further analysis [110].

The protein extract with a pH of 5.6 in case of walnut and a pH of 5.0 in case of peanut was loaded onto a cation exchange column (dimension: 7 ml). SP-sepharose with a binding capacity of 120 mg BSA/ml column material was used. The proteins of walnut were eluted with an NaCl gradient (buffer A: 20

mM MES, pH 5.6, buffer B: 20 mM MES, pH 5.6 + 1 M NaCl). For the gradient 0-100% of buffer B was used in 84 min with a flow rate of 1 ml/min.
The proteins of peanut were also eluted with a different buffer system (buffer A: 50 mM NaAc/Ac, pH 5.0, buffer B: 50 mM NaAc, pH 5.0 + 1 M NaCl). For the gradient 0-100% of buffer B was used in 84 min with a flow rate of 1 ml/min. The fraction size was 4 ml and the absorbance was recorded by 280 nm.

For anion exchange chromatography the prepacked column HiTrap^{TM}Capto^{TM}Q (GE Healthcare, Uppsala, Sweden) was used (dimension: 1 ml, binding capacity: 100 mg BSA/ml column material). As buffer A 20 mM Tris/HCl, pH 7.5 was used and as buffer B 20 mM Tris/HCl, pH 7.5 with 1 M NaCl was used. The volume of the column was 1 ml. First the column was equilibrated with 5 ml of buffer A, buffer B and Buffer A again, respectively. Subsequently, the sample was loaded. For elution a gradient of up to 50% buffer B in a total volume of 20 ml with a flow rate of 1 ml/min was used. As a final elution step 10 ml of 100% buffer B was used. The column was washed with 10 ml of buffer A, 5 ml of MilliQ. The absorbance was recorded by 280 nm.

2.5.3.2 Hydrophobic Interaction Chromatography (HIC)

HIC separates the proteins according to their surface hydrophobicity. Hydrophobic groups like phenyl-, octyl- or butyl- are attached to the chromatography matrix.
Hydrophobic side chains of amino acids can interact with the hydrophobic groups in the column if the sample is in a buffer with high salt concentration because salts can increase the hydrophobic interactions. Consequently HIC is ideal after ammonium sulfate precipitation. For elution the salt concentration decreases and therefore the hydrophobic interactions are reduced. Accordingly the proteins can no longer bind to the column and are eluted [111].

As column material for hydrophobic interaction chromatography phenyl-sepharose was used (dimension: 10 ml, binding capacity: 14 mg BSA/ml column material) Buffer A was 15 mM MES, pH 5.6, with 3 M ammonium sulfate and buffer B was 15 mM MES, pH 5.6. For elution a gradient of up to 100%

buffer B in a total volume of 100 ml with a flow rate of 1 ml/min was established. The fraction size was 3.5 ml and the absorbance was recorded by 280 nm.

2.5.3.3 Gel filtration

Using gel filtration molecules can be separated according to their size. Smaller molecules can diffuse into the porous matrix and are therefore retained. Larger molecules do not enter the pores of the matrix and pass by. Therefore, large molecules are eluted first, followed by smaller molecules. The molecules are eluated isocratically. This means that the solvent system does not need to change composition during elution [112].

For gel filtration the column HiPrep 16/60 Sephacryl S-300 High Resolution with a dimension of 120 ml was used.
The pumps and the fractionator were washed with MilliQ. Then the column was washed with half a column volume MilliQ. Flow rate was kept at 0.5 ml/min maximum in order not to exceed the pressure limit in the system.
Subsequently, the column was equilibrated with three column volumes of the buffer.
In case of peanut 20 mM NaAc/Ac, pH 4.5 was used.
Then the sample was loaded and we used one column volume of buffer for elution. Fractions of 2 ml were collected for further analysis.
Afterwards, the pumps and the fractionator were washed with MilliQ and the column was washed with half a column volume of MilliQ. Then pumps and fractionator were washed with 20% ethanol. The column was washed with 3 column volumes of 20% ethanol over night. It is important to fill the reservoir with 20% ethanol to prevent drying of the column and to keep the pressure at the correct level. The absorbance was recorded by 280 nm.

2.5.4 Dialysis

Dialysis is used for desalting samples or for changing the buffer. The sample is put into a dialysis tube with pores of a distinct size. The pores should be that

small that the target molecules cannot pass them but large enough that salts and non-target proteins can go through.
The buffer in which the dialysis tube with the sample is put should be a hundred times more than the volume of the sample.
In our case dialysis was performed at 4°C for 15 hours under constant stirring.

The supernatant after ammonium sulfate precipitation was taken and put into a dialysis tube (Spectra/Por molecularporous membrane, Spectrum, Rancho Dominguez, CA; MWCO 1,000). The dialysis tube was closed with clips and put into a beaker with 15 mM MES buffer, pH 5.6 and stirred for 15 hours at 4°C.

Then the sample was concentrated using Millipore Centrifugal Filter Units.

2.6 Physicochemical characterization of 2S albumins and nsLTPs from tree nuts and peanut

2.6.1 SDS-PAGE and Coomassie stain

SDS-PAGE is used to separate proteins according to their molecular mass.
SDS is a detergent which is responsible for proteins to unfold and according to that it masks the charge of proteins. Therefore, the proteins get negatively charged in proportion to their molecular mass.
The gel for SDS-PAGE consists of a stacking gel and a separation gel. These two gels differ in their pH and their pore size. The size of the sample protein can be determined using a reference marker with known molecular masses [113].

Coomassie Brilliant Blue R-250 is a dye which binds to aromatic amino acids. If the environment is acidic the dye binds to proteins through ionic bindings between its sulfonic groups and the protein's amino groups. According to that also Van der Waals forces are responsible for the interaction. The bands which are visualized are the stabilized anionic form of Coomassie Brilliant Blue R-250. The dye which has not bound to protein is removed using a destainer solution [114, 115].

30% Reagent C	**Lower Buffer**	**Upper Buffer**
29.2% Acrylamid	1.5 M Tris/HCl, pH 8.8	0.5 M Tris/HCl, pH 6.8
0.8% Bis-Acrylamid	0.4% SDS	0.4% SDS
dissolved in MilliQ	dissolved in MilliQ	dissolved in MilliQ

15% Separation Gel	**16% Separation Gel**	**4,5% Stacking Gel**
2.5 ml Reagent C	2.7 ml Reagent C	300 µl Reagent C
1.25 ml Lower Buffer	1.25 ml Lower Buffer	500 µl Upper Buffer
1.25 ml MilliQ	1 ml MilliQ	1.2 ml MilliQ
2.5 µl TEMED	2.5 µl TEMED	1 µl TEMED
25 µl 10% APS	25 µl 10% APS	20 µl 10% APS

4x Sample Buffer

200 mM Tris/HCL, pH 6,8
300 mM DTT
4% SDS
40% Glycine
Bromophenolblue
dissolved in MilliQ

Electrophoresis Buffer

250 mM Tris pure
192 mM Glycine
1% SDS
dissolved in MilliQ

Coomassie Brilliant Blue Stain

0.125% CBB R-250
50% Methanol
10% Acetic Acid
dissolved in MilliQ

Coomassie Destainer

20% Methanol
15% Acetic Acid
dissolved in MilliQ

For SDS-PAGE a 15% separation gel and a 4.5% stacking gel were used.
First the glass plates were put in the casting frame. For analytical gels the space between the two plates is 0.75 mm. For preparative gels and gels for 2D electrophoresis this space is 1.5 mm.
The components of the separation gel were put together and poured between the two glass plates. Then the unpolymerized gel was overlaid with isopropanol to get a plain surface of the separation gel.
When the separation gel was polymerized the isopropanol was poured off and the slot was washed with MilliQ to be sure that no isopropanol is left.
Subsequently, the mix of the components of the stacking gel was poured between the glass plates. Quickly the comb was inserted.
The gels for 2D electrophoresis only consist of the separation gel.
Before starting the gel electrophoresis the glass plates with the gels were put into the electrophoresis chamber and the comb was removed. Moreover the slots were washed with buffer to remove small residues of the gel in the slots.

The samples were prepared by adding 4x sample buffer and heating up to 95°C for 5 minutes. The DTT in the sample buffer reduces the proteins and the heat denatures them.
The samples were applied slowly to the gel and the upper and lower tank was filled up with electrophoresis buffer. The gel coaster was connected with the electrical current and the gel was run at 160 V until the blue front line has reached the bottom of the gel.

The gel was put into a plastic dish and Coomassie Brilliant Blue staining solution was added. The gel was stained for one hour with shaking. After the staining solution was removed a destaining solution was put into the plastic dish. The destainer was changed 3 times. When the background was clear and the bands were visible the gel was taken out of the destainer and scanned.

2.6.2 Determination of protein concentration

To determine the concentration of proteins we used bicinchoninic acid (BCA) assay. This is a method based on Biuret reaction (reduction of Cu^{++} to Cu^{+} by protein in alkaline environment containing sodium potassium tartrate). First a chelate of copper and protein is built. This can be seen as a light blue colour. Next BCA reacts with the Cu^{+} which was formed in the previous step. A chelate of two molecules of BCA and one cuprous ion is built. This reaction can be seen by purple colour. The complex of BCA and Cu^{+} exhibits absorbance at 562 nm. The absorbance increases with increasing protein concentration. As a reference protein bovine serum albumin (BSA) was used [116, 117].

Reagent A

Sodium Carbonate
Sodium Bicarbonate
Bicinchoninic Acid
Sodium Tartrate in 0.1 M Sodium hydroxide

Reagent B

4% Cupric Sulfate

BSA (Bovine Serum Albumin, 2 mg/ml in 0.9% saline and 0.05% sodium azide) was used as standards in the following concentrations: 0, 50, 100, 150, 200 and 250 µg/ml.

The Working reagent consisting of 50 parts of Reagent A and 1 part of Reagent B was prepared.

Subsequently, 25 µl of each sample was put into a polystyrene microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) as duplicates in different dilutions.

Afterwards 200 µl of Working reagent was added to each sample and the plate was incubated for 30 min at 37°C. The OD was measured at 562 nm using SpectraMaxPlus (Molecular Devices, Sunnyvale, CA). To analyse the data the software SoftMaxPro 4.8 (Molecular Devices, Sunnyvale, CA) was used.

2.6.3 2D-Electrophoresis

2D-Electrophoresis is a combination of isoelectric focusing (IEF) and SDS-PAGE. The proteins are separated in two subsequent steps.

The first dimension is IEF. Here the proteins are separated according to their pI. If the pH corresponds to the pI of the protein the positive and negative charges of the molecule outweigh each other. Therefore, in IEF the protein migrates to that point in the gel where the pH equals the pI of the protein.

CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in IEF Sample Buffer is a zwitterionic detergent which is important for solubilisation of proteins. Urea is used as denaturating agent. Thiourea improves the solubilisation of the proteins.

The next step is equilibration of the gel from IEF. DTT in the equilibration solution I is a reducinig agent which breaks up the disulfide bridges in the proteins. To prevent a re-oxidation the iodoacetamide in the equilibration solution II is used to alkylate the SH-groups.

Subsequently, the strip from IEF is put between the glass plates on the upper edge of the gel and a layer of agarose sealing solution is added. SDS is used for unfolding the proteins and in addition it masks the charge of the proteins.

The second dimension is SDS-PAGE (see 2.6.1.). Here the proteins are separated according to their molecular mass [118, 119].

1% Bromophenolblue

50 mM Tris
1% bromophenolblue
dissolved in MilliQ

IEF Sample Buffer

7 M Urea
2 M Thiourea
2% CHAPS
0.5% IPG Buffer 3-10
0.002% bromophenolblue
dissolved in MilliQ
65 mM DTT added directly before use

SDS-PAGE Equilibration Stock Solution I

50 mM Tris/HCl, pH 8.8
6 M Urea
2% SDS
30% Glycerol (98%)
0.002% Bromophenolblue
65 mM DTT added directly before use

SDS-PAGE Equilibration Stock Solution II

50 mM Tris/HCl, pH 8.8
6 M Urea
2% SDS
30% Glycerol (98%)
0.002% Bromophenolblue
25 mg/ml iodoacetamide

Agarose Sealing Solution

0.5% Agarose
0.002% Bromophenolblue
dissolved in SDS Electrophoresis Buffer

15% Separation Gel

6.75 ml Reagent C
3.38 ml Lower Buffer
2.25 ml H_2O
6.75 µl TEMED
67.5 µl 10% APS

Ten µg per sample were used. First the proteins had to be precipitated. Therefore, 3 volumes of 100% acetone were added to the protein solution and the mixture was incubated at -20°C for 30 minutes. Moreover the samples were centrifuged for 30 minutes at 4°C and 14,000 rpm. Subsequently, the supernatant was removed and 50 µl of 90% acetone were added to the pellet. Centrifugation for 10 minutes at 4°C and 14,000 rpm was done before the supernatant was removed and the pellet was dried.
For solubilisation of the samples 125 µl of IEF-SB (pH range 3-10) with 1 M DTT (65 µl 1 M DTT to 1 ml of IEF-SB) were added to each sample and the pellet was resuspended. After the samples were shaken at 300 rpm at 22°C for 30 min they were centrifuged for 10 min at room temperature at full speed.

Then separation by the first dimension was performed. Therefore, the sample was put in the middle of a stripholder and the strip was put onto the sample. Moreover the strip was covered with 400 µl of mineral oil (Plus One, Dry Strip Cover Fluid). Subsequently, the stripholder with the strip was put into the machine (Ettan^{TM}IPGphor^{TM} , Amersham Biosciences, Uppsala, Sweden) and the isoelectric focusing was started.
Before starting the second dimension of separation the strip was put into Equilibration Solution I for 15 min and was rotated on a Rock'n'Roller. Then this step was repeated using Equilibration Solution II.
The marker (PageRuler Prestained Protein Ladder Part No. 26619, Thermo Scientific) was put on a 3x3 mm square of Whatman Paper and put at the side where the plus pole is found on the strip. Finally an agarose layer sealed the upper front of the gel.

The first minutes the gel was run at 16 mA until a blue front was visible. Then it was run at 40 mA.
To interpret the results the gel was stained using Coomassie Brilliant Blue.

2.6.4 Mass spectrometry

Mass spectrometry is a method to determine the molecular masses of proteins. First the sample must be ionized. The ions are separated according to the ratio of molecular mass to their charge in an electromagnetic field. The ions are deflected and hit the analyser. Then the mass spectrogram is recorded. The distribution of the measured masses gives conclusion about the molecular mass of the sample and the relative concentration of the analytes [120].

The mass spectrometry was performed at the CD-lab by Dr. Gabriele Gadermaier (Department of Molecular Biology, University of Salzburg).
For sequence analysis 5 µg of the sample were digested using Proteoextract Trypsin Digestion Kit (Calbiochem, San Diego, USA). The peptides were separated by capillary RP-HPLC (Waters, Milford, USA; precolumn Waters Nanoease Symmetry300 trap column, separating column Waters Nanoease Atlantis dC18, connected via a 10 port stream select valve). The flow rate was 300 nl/min. The peptides were eluted with an ACN gradient (solvent A: 0.1% v/v formic acid, 5% v/v ACN; solvent B: 0.1% v/v formic acid, 95% v/v ACN). For the gradient 5-95% of solvent B was used in 90 min.
The RP-HPLC was directly coupled with the mass spectrometer (Global Ultima Q-Tof, Waters, Manchester, UK).
For ionisation electrospray ionisation (ESI; Waters nanoflow spray head) was performed. The infusion rate was 1 µl/min. Nitrogen was used as desolvation gas and a capillary voltage of 3.4 kV was used.
The instrument was calibrated with the fragment ions of [Glu]-fibrinopeptide B (Sigma).
For data acquirement Data Directed Analysis (DDA) was used. Survey and fragment spectra were analysed using the software PLGS version 2.2.5 (Waters). Sequences were identified using a mini database comprising the

trypsin and sample sequences and a combined SwissProt/Trembl database were used.

2.7 Immunological Characterization of 2S albumins and nsLTPs from tree nuts and peanut

2.7.1 Immunoblot

Immunoblot is a method where separated proteins adsorbed on a nitrocellulose membrane are recognized by a primary antibody. Detection is usually performed by a conjugated secondary antibody.

Proteins from a polyacrylamide gel after SDS-PAGE are blotted on a membrane using an electrical field. The proteins are in this case negatively charged and therefore migrate to the anode.

Before the primary antibody or a patient's serum is added unspecific binding sites are blocked to avoid non-specific binding.

The secondary antibody is linked to an enzyme like alkaline phosphatase (AP). AP reacts with added NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and makes protein bands visible on the membrane [121].

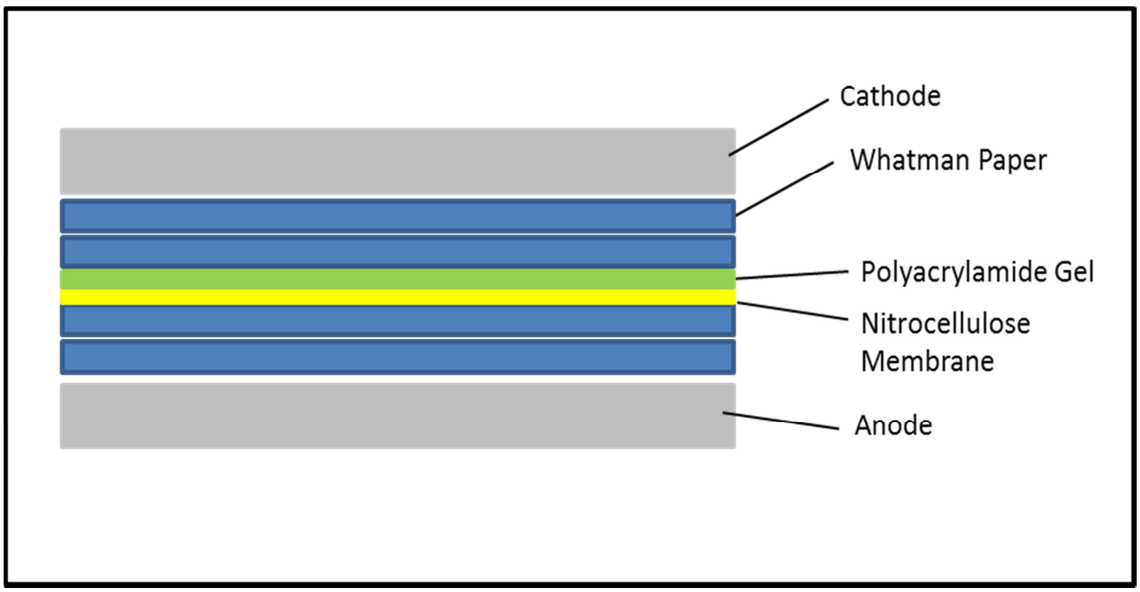

Figure 5: Composition of the blotting sandwich for Immunoblot.

Transfer Buffer	**AP-Buffer**
250 mM Tris pure	100 mM Tris pure
1.92 M Glycine	pH 9.5
20% Methanol	100 mM NaCl
	5 mM $MgCl_2$ x $6H_2O$

For immunoblot the proteins were separated by SDS-PAGE (see 2.4.1). Then the gel, the membrane and the Whatman paper were put into 1x transferbuffer. Subsequently, the blotting-sandwich was put together. At the bottom next to the anode, two Whatman paper were put. Then the membrane, followed by the gel was placed onto the Whatman paper. At the top next to the cathode again two Whatman paper were put.

Then blotting was started with 10 V for 30 min.

Afterwards the membrane was taken out from the blotting-sandwich and air dried.

The bands of the marker were marked with a pen to make sure to keep the reference sizes even though the bands of the marker fade away.

Next step was to saturate the membrane two times for 15 min each. Therefore TBST containing 3% BSA was used. Then the sera-dilution with the anti-human IgE antibody was put onto the membrane and it was incubated at 4°C over night.

On the following day the membrane was washed for one hour with TBST. The TBST should be changed every 20 min. After washing the membrane for a short time with MilliQ and AP-Buffer, 10 ml of AP-Buffer with 60 µl NBT and 60 µl BCIP was put on the membrane. Then the membrane was put into the dark until bands could be seen. Then the reaction was stopped with water. As negative control sera of non-allergic subjects (NHS) were used.

2.7.2 IgE-ELISA

IgE-ELISA is a method for allergen detection by using IgE antibodies. A plate is coated with an antigen which is immobilized by binding to the solid phase. Incubation with patients' sera follows. The allergen captures specific IgE antibodies in the sera. For detection a secondary antibody which binds to human IgE is used. The secondary antibody is linked to an enzyme, in our case it is alkaline phosphatase. When substrate is added the enzyme converts it. This can be measured as change of OD (405 nm) (optical density at 405 nm) [122].

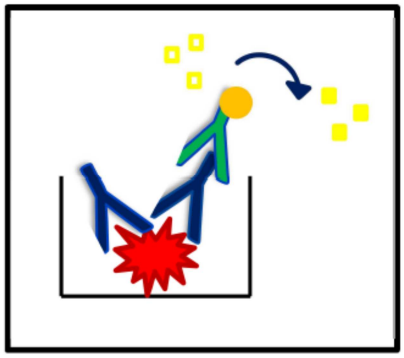

Figure 6: The principle of an IgE-ELISA. The plate is coated with an allergen (red) which is recognized by specific antibodies (blue) from patients' sera. A mouse anti-human-IgE-antibody (green) binds to the primary antibody. This antibody is linked to AP (alkaline phosphatase, orange). If a substrate (yellow) is added AP converts it and this can be detected in a change of the OD at 405 nm.

Coating Buffer	**Washing Buffer**	**Blocking Buffer**
25 mM $NaHCO_3$	TBS	TBST
pH 9.5	0.5% Tween-20	3% BSA

Dilution Buffer

TBST

0.5% BSA

The 96 well plate (MaxiSorp Immunoplate, Nunc, Roskilde, Denmark) was coated with 100 µl of allergen dilution with a concentration of 1 µg/ml. We used duplicates to later calculate the mean. Then the plate was incubated for one hour at 37°C. After washing three times with washing buffer 200 µl blocking buffer were put into each well. The plate was incubated for two hours at room temperature.

In the meantime the sera were diluted 1:5 to 1:10, depending on the sera. Alkaline phosphatase conjugated mouse anti-human-IgE antibody (BD Biosciences, Franklin Lake, NJ USA) was put into the serum-dilution. The anti-human-IgE antibody should be diluted 1:1,000.

After blocking the plate was washed three times with washing buffer. Then 100 µl of the serum-antibody-dilution was put into each well. The plate was incubated over night at 4°C.

On the following day the serum-antibody-dilutions were recovered and the plate was washed 7 times with washing buffer. Subsequently, 100 µl of the substrate (nitrophenyl phosphate tablets, Sigma-Aldrich) were put into each well. The OD was measured at 405 nm at different time points whereas the value was

considered as positive when $Mean_{Serum} > Mean_{NHS} + 3*Standard\ Deviation_{NHS}$. NHS (normal human serum) is serum from nonallergic individual.

2.7.3 IgE-Inhibition ELISA

First the 96 well plate (MaxiSorp Immunoplate, Nunc, Rosklide, Denmark) was coated with allergen diluted in coating buffer. Therefore the plate was incubated at 37°C for one hour. Then it was washed for three times with washing buffer. The blocking buffer was added and the plate was incubated at room temperature for two hours.

The sera from non-allergic individuals (NHS) and not inhibited serum were diluted 1:10 and the allergens were added in different concentrations to inhibit the sera.

The incubation of sera with allergens were put on a Rock'n'Roller for two hours. Afterwards they were put on the plate and the plate was incubated over night at 4°C.

Next day the plate was washed three times with washing buffer and one hour incubation with the alkaline phosphatase-linked mouse anti human IgE-antibody followed. Then substrate was added and the OD at 405 nm was measured at different time points.

3 Results

3.1 Establishment of purification protocols for 2S albumins

3.1.1 Purification of 2S albumin from peanut

For extraction of peanut 2S albumin Ara h 6 from protein extract cation exchange chromatography was performed. The pI of the protein is 6.13 and the buffer for chromatography had a pH of 5.6. Therefore, the protein is existent as cation which could bind to the negatively charged column material of a cation exchanger.

Fractions obtained by chromatography were analysed by SDS-PAGE. Fractions F8-F11, F15 and F16 were pooled (see Figure 7) because on Coomassie stained SDS-PAGE these fractions displayed protein bands at 15 kDa. The fractions F8-F11 were eluted with 38.6%-51.5% of buffer B while the fractions F15-F16 were eluted with 60%-60.7% of buffer B. In Figure 7 the blue line shows the absorption, the green line the gradient and on the x-axis the fraction numbers and fraction size are marked.

Subsequently, hydrophobic interaction chromatography (phenyl-sepharose) was performed and fractions were analysed by SDS-PAGE. Fractions with enriched proteins at approximately 15 kDa (F19-F26) were pooled. They were eluted with 42.5%-60% buffer B, were pooled (see Figure 8).

Next gel filtration (see Figure 9) was done to separate the proteins according to their size. The fractions were analysed by Coomassie stained SDS-PAGE and pools were built. Pool A contained fractions F11-F15, for Pool B fractions F16-F19 were pooled.

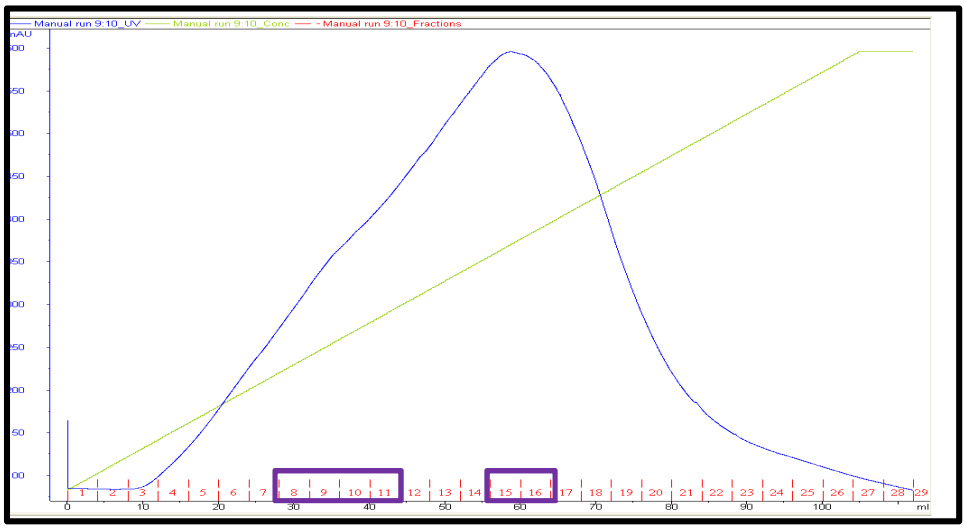

Figure 7: Chromatogram of peanut protein extract separation by a cation-exchange column (SP-Sepharose). The marked fractions were pooled. Blue line incicates the absorption, green line the gradient. On the x-axis fraction numbers and fraction size can be seen.

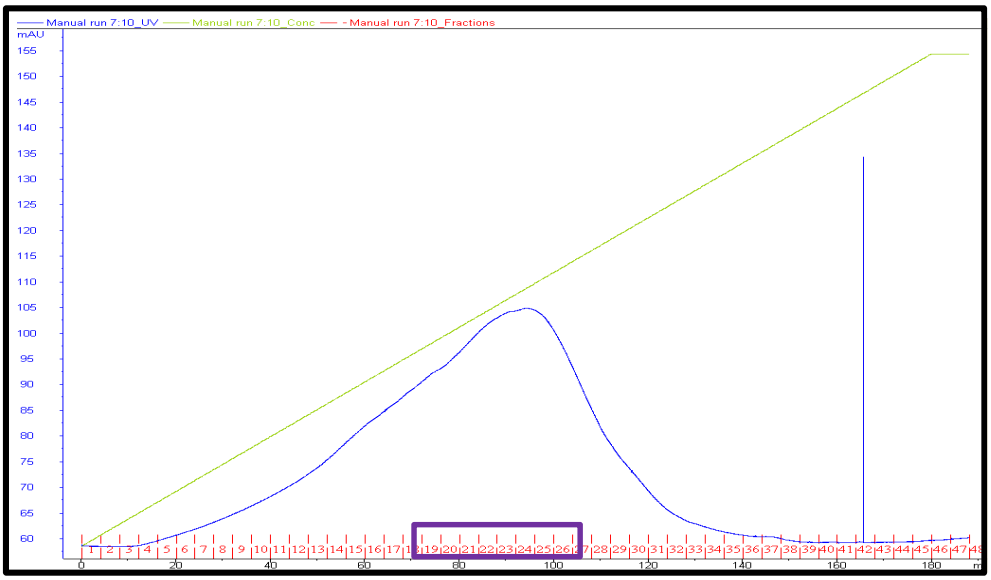

Figure 8: Chromatogram of peanut pool after cation-exchange chromatograpyh loaded onto a hydrophobic interaction column. The marked fractions were pooled. For more information see Figure 7.

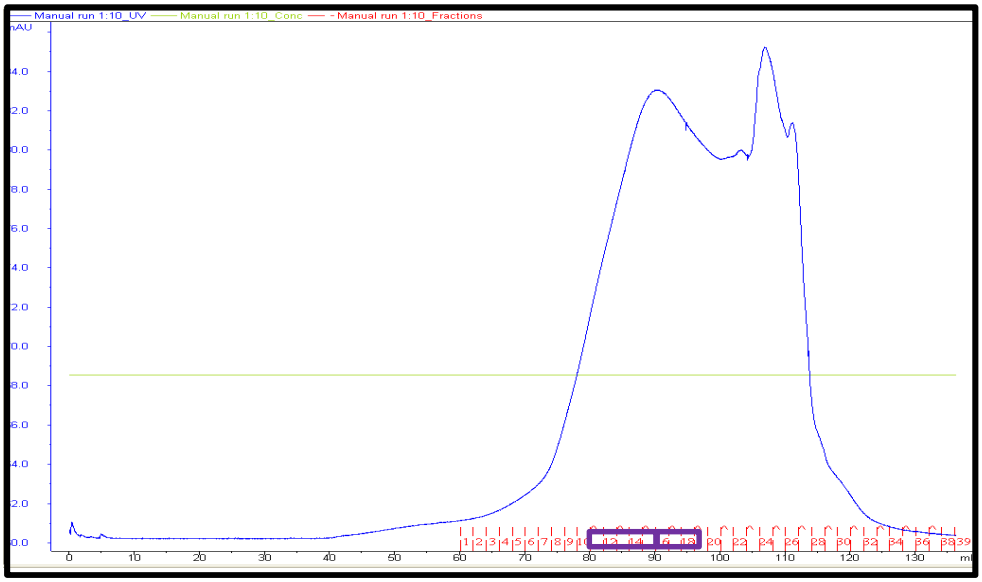

Figure 9: Chromatogram of peanut pool after hydrophobic interaction chromatography loaded onto gel filtration column. The marked fractions were pooled. For more information see Figure 7.

3.1.2 Purification of 2S albumin from walnut

Walnut protein extract was loaded on a cation exchange column (SP-sepharose) because the 2S albumin has a pI of 5.75 and the buffer used had a pH of 5.6 and therefore the protein existed as a cation (see Figure 10).

The fractions were analysed by Coomassie stained SDS-PAGE and fractions F6-F11, eluted with 32.4%-64.9% buffer B, were pooled because on Coomassie stained SDS-PAGE these fractions contained proteins migrating at approximately 15 kDa. This pool was used for hydrophobic interaction chromatography (phenyl-sepharose, see Figure 11). Here again we analysed the fractions using Coomassie stained SDS-PAGE and the proteins from F15-F20 appeared at about 15 kDa, were pooled. The fractions F15-F20 were eluted using 32%-45.6% of buffer B. The Coomassie stained SDS-PAGE of this final pool can be seen in Figure 13 at approximately 13 kDa.

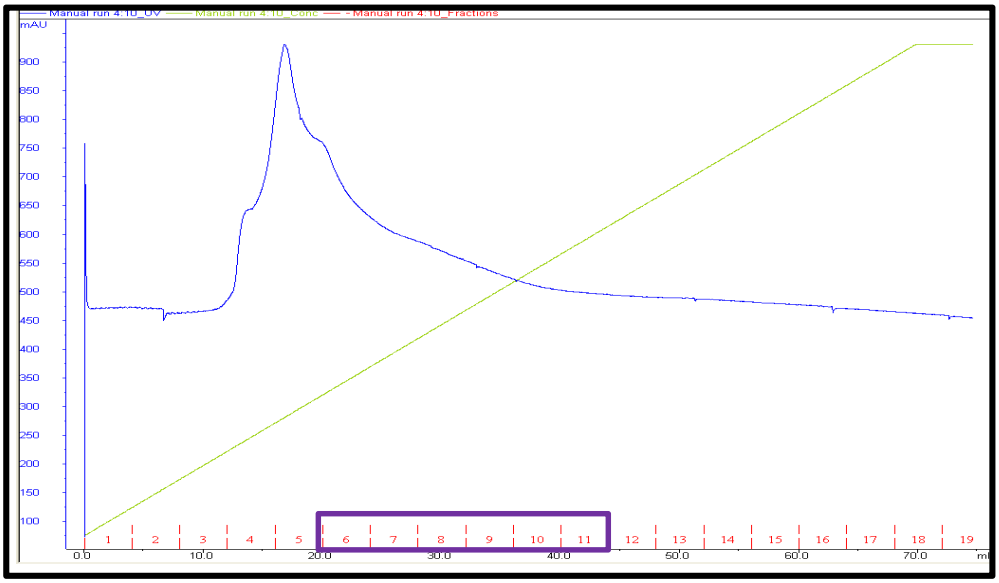

Figure 10: Chromatogram of walnut protein extract loaded onto a cation-exchange column (SP-sepharose). The marked fractions were pooled. For more information see Figure 7.

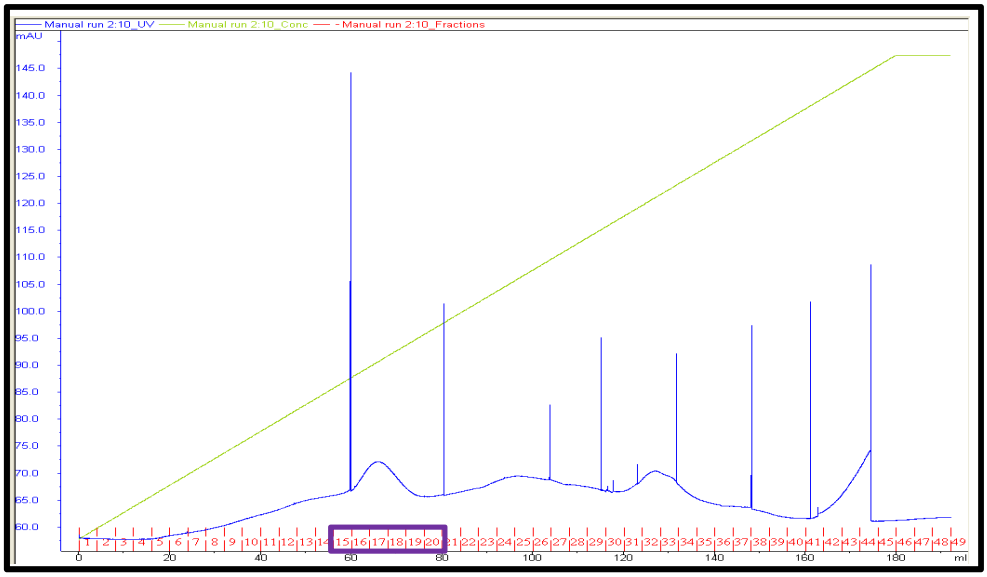

Figure 11: Chromatogram of walnut pool after cation-exchange chromatography loaded onto a hydrophobic interaction column (phenylsepharose). The marked fractions were pooled. For more information see Figure 7.

3.1.3 Purification of Jug r 4, 11S globulin from walnut

A walnut extract prepared by Gerlinde Hofstetter, BSc (Department of Pathophysiology and Allergy Research, Medical University of Vienna) was used for 20%, 70% and 90% ammonium sulfate precipitation.

The fraction obtained by 90% ammonium sulfate precipitation was applied on the anion exchange column CaptoQ (see Figure 12). The pI of Jug r 4 is 6.8 and the pH of the used buffer was 7.5. Therefore, the target protein existed as anion and could bind to the positively charged column material of the anion exchanger.

Two peaks could be seen. F4 was chosen for further experiments because on Coomassie stained SDS-PAGE it showed up between 10 kDa and 15 kDa. This might be one subunit of Jug r 4.

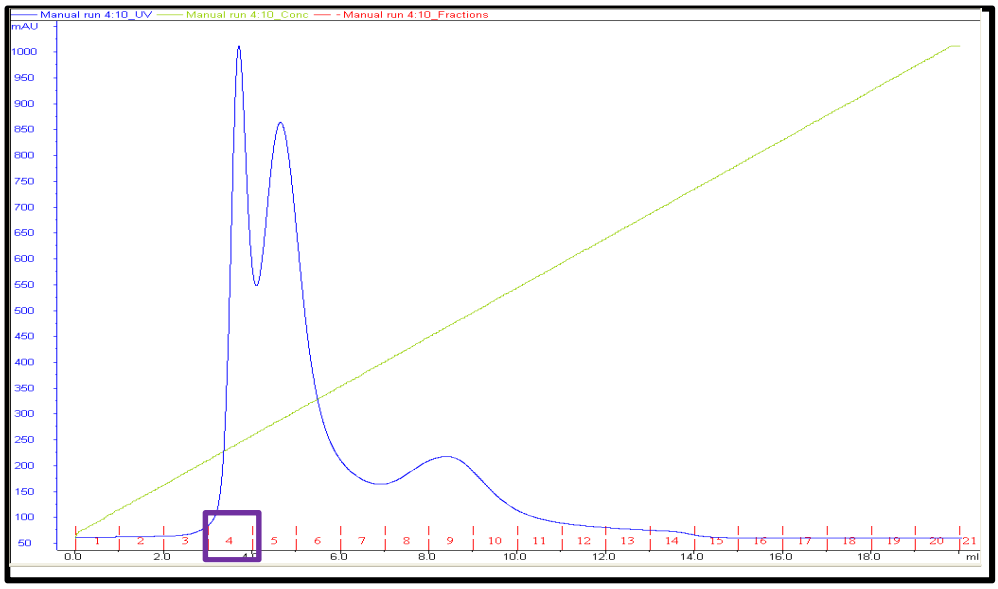

Figure 12: Chromatogram of the fraction of 90% ammoniumsulfate precipitation loaded on a anion-exchange column (CaptoQ). Fraction 4 was taken for further experiments. For more information see Figure 7.

3.2 Physicochemical characterization of purified 2S albumins from tree nuts and peanut

3.2.1 SDS-PAGE of protein extracts and purified allergens

In Figure 13 Coomassie stained SDS-PAGE analysis of protein extracts from walnut and peanut can be seen (lane 1 and lane 4, respectively). In addition also the 2S albumins of walnut and peanut (Jug r 1 and Ara h 6) were run on the gel (lane 2 and lane 6 respectively) . The allergens Jug r 1/2, Ara h 2/6 and Ber e 1 can also be seen (lane 3, lane 5 and lane 7, respectively).

Unexpectedly, the two Jug r 1 enriched batches do not appear at the same molecular mass (see lane 2 and lane 3). Ara h 2/6 splits up in some bands between about 12 and 17 kDa (see lane 5).

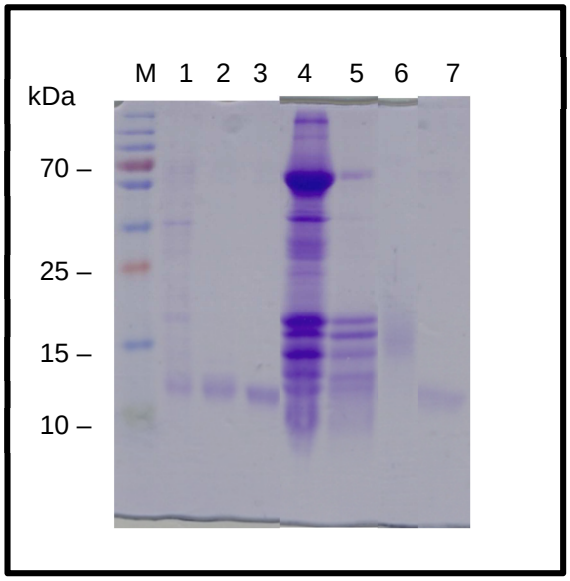

Figure 13: Coomassie stained SDS-PAGE of protein extracts and purified allergens. The 15% gel was run under reducing conditions. M = Marker, Lane 1 = walnut protein extract, Lane 2 = 2S albumin from walnut, Lane 3 = Jug r 1/2, Lane 4 = peanut protein extract, Lane 5 = Ara h 2/6, Lane 6 = 2S albumin from peanut, Lane 7 = Ber e 1.

3.2.2 Protein concentrations, total amounts and yield of nut protein extracts and purified allergens

The protein concentration of all extracts and purified allergens was determined using BCA assay and the total amount was calculated. This is summarized in Table 6.

Protein	Concentration [µg/ml]	Total [mg]
Peanut protein extract	2 300	115
Walnut protein extract	1 690	84.5
Cor a 14	315	0.3
Ara h 6	480	0.7
Ara h 2/6	541	0.3
Jug r 1/2	130	2.9
Jug r 1/2	130	1.3
Jug r 4	59	0.1
Ber e 1	319	0.5

Table 6: Protein concentration and total amounts of protein extracts and purified allergens.

Using the purification protocols, 0.7 mg Ara h 6 was obtained form peanut protein extract which corresponds to 0.6% of total peanut protein extract. From walnut, 1.3 mg of Jug r 1/2 was obtained which is 1.5% of total walnut protein extract.

3.2.3 2S Albumin from peanut

The final Pool B (Ara h 6) of peanut was characterized by mass spectrometry (see Figure 14). A total mass of 16.908 kDa was identified while in the literature a value of 15.8 kDa is found. The sequence of Ara h 6 is shown in Figure 15. Peptides identified by mass spectrometry reach from amino acid residue 41 to residue 54 (RVNLKPCEQHIMQR), from residue 79 to residue 91 (CCDELNEMENTQR) and from residue 112 to residue 135 (QMVQQFKRELMNLPQQCNFRAPQR). The identified peptides are marked in Figure 14 by coloured lines and in Figure 15 by coloured letters. The coverage of the peptide sequence is 59.16% of the entire sequence.

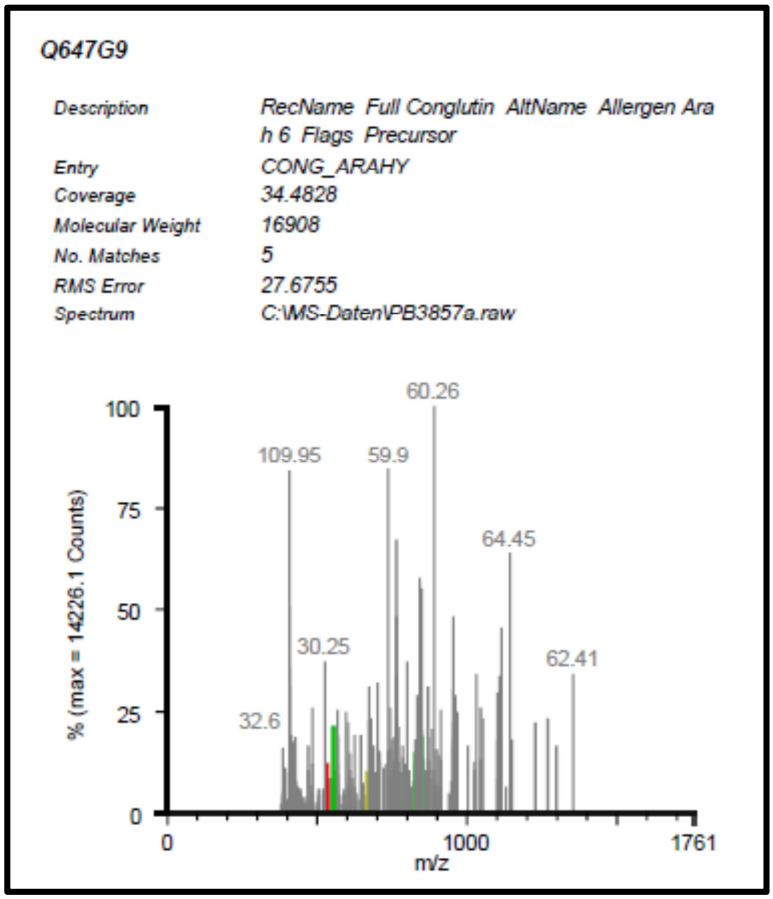

Figure 14: Analysis of 2S albumin of peanut enriched batch by mass spectrometry. The mass spectrogramm shows the percentage of counts and the ratio of the mass to the charge of the peptide fragments identified by mass spectrometry.

```
        10         20         30         40         50         60
MAKSTILVAL LALVLVAHAS AMRRERGRQG DSSSCERQVD RVNLKPCEQH IMQRIMGEQE
        70         80         90        100        110        120
QYDSYDIRST RSSDQQQRCC DELNEMENTQ RCMCEALQQI MENQCDRLQD RQMVQQFKRE
       130        140
LMNLPQQCNF RAPQRCDLDV SGGRC
```

Figure 15: Sequence of Ara h 6. Peptides identified by mass spectrometry are marked in different colours.

3.2.4 2S albumin form walnut

The final batch was characterized by 2D-electrophoresis (see Figure 16). Six spots of about 14 kDa and a pI of about 5 to 8.5 can be seen on the 2D gel. In the literature the molecular mass of Jug r 1 is indicated with 15.5 kDa and the pI with 5.75, therefore, 2 spots seem to correspond to Jug r 1. If the other spots

are isoforms of Jug r 1 or other proteins remains to be determined by additional analyses.

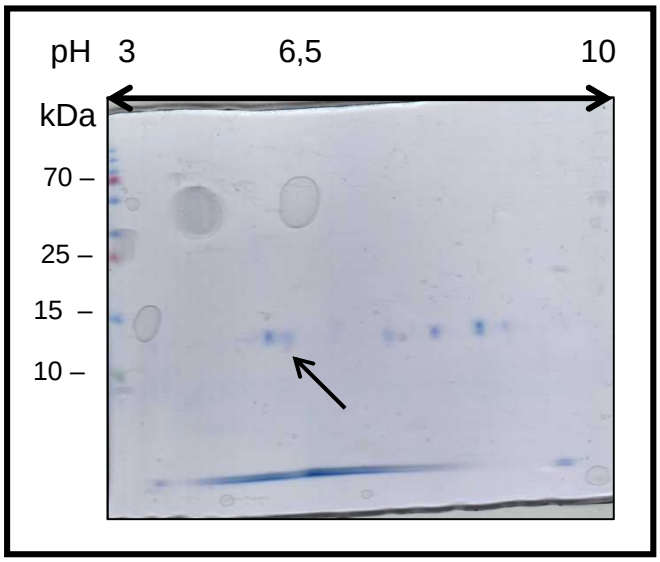

Figure 16: 2D Electrophoresis of final batch of walnut 2S albumin purification. The 15% gel was run under reducing conditions. Spots of about 14 kDa and a pI of about 5 to 6 are marked by the arrow.

The final Jug r 1 enriched pool was characterized by mass spectrometry (see Figure 17). The peptides identified in the sequence of Jug r 1 corresponds to amino acid residue 46 to residue 57 (QQNLNHCQYYLR), to residue 99 to residue 119 (RQQQQQGLRGEEMEEMVQSAR) and to residue 120 to residue 131 (DLPNECGISSQR). The identified peptides are marked by cloured lines in Figure 17 and by coloured letters in Figure 18. The coverage of the total sequence is 81.8%.

As the Jug r 1 enriched pool is contaiminated with Jug r 2, also peptides in the sequence of Jug r 2 were identified by mass spectrometry. These peptides reach from amino acid residue 69 to residue 85 (PRDPEQRYEQCQQQCER) and from residue 130 to residue 139 (CQIQEQSPER). The identified peptides are marked by coloured letters in Figure 20.

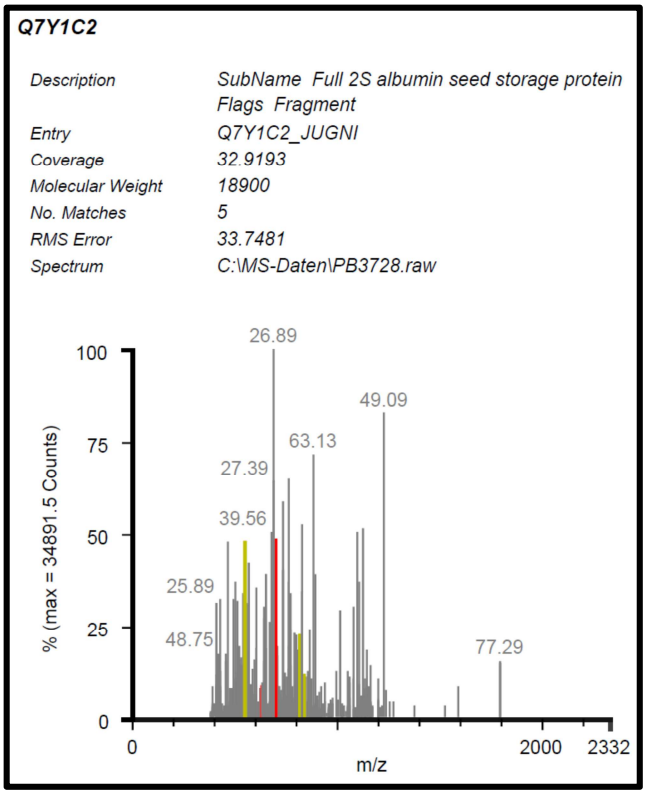

Figure 17: Analysis of Jug r 1 enriched batch Jug r 1/2 Batch 1 by mass spectrometry. The mass spectrogramm of Jug r 1 shows the percentage of counts and the ratio of the mass to the charge of the peptide fragments used.

```
        10         20         30         40         50         60
AALLVALLFV ANAAAFRTTI TTMEIDEDID NPRRRGEGCR EQIQRQQNLN HCQYYLRQQS

        70         80         90        100        110        120
RSGGYDEDNQ RQHFRQCCQQ LSQMDEQCQC EGLRQVVRRQ QQQQGLRGEE MEEMVQSARD

       130
LPNECGISSQ RCEIRRSWF
```

Figure 18: Sequence of Jug r 1. Peptides identified by mass spectrometry are marked in different colours.

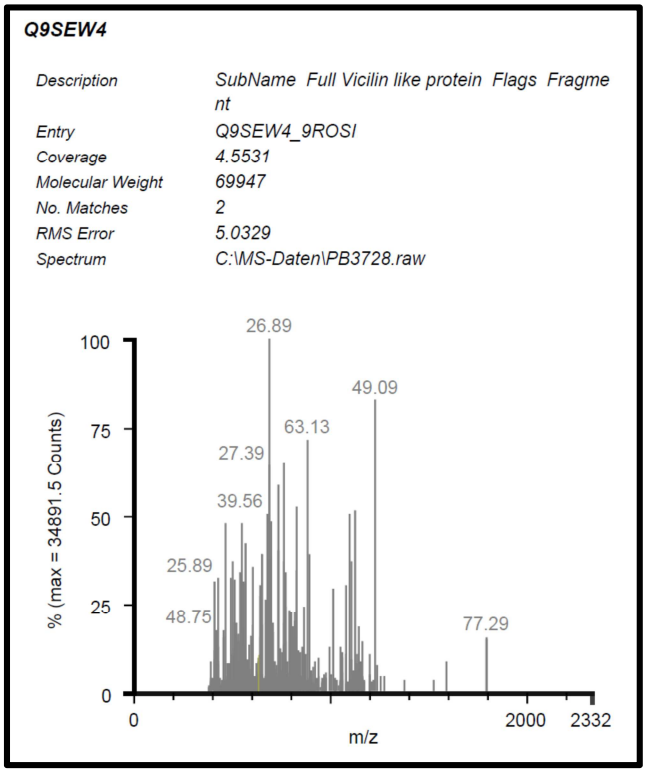

Figure 19: Analysis of Jug r 1 enriched batch Jug r 1/2 Batch 1 by mass spectrometry. The mass spectrogramm shows the percentage of counts and the ratio of the mass to the charge of identified peptides from walnut vicilin, Jug r 2, found in Jug r 1 enriched batch.

```
     10         20         30         40         50         60
RGRDDDDEEN PRDPREQYRQ CQEYCRRQGQ GQRQQQQCQI RCEERLEEDQ RSQEERERRR

         70         80         90        100        110        120
GRDVDDQNPR DPEQRYEQCQ QQCERQRRGQ EQTLCRRRCE QRRQQEERER QRGRDRQDPQ

        130        140        150        160        170        180
QQYHRCQRRC QIQEQSPERQ RQCQQRCERQ YKEQQGRERG PEASPRRESR GREEEQQRHN

        190        200        210        220        230        240
PYYFHSQSIR SRHESEEGEV KYLERFTERT ELLRGIENYR VVILDANPNT SMLPHHKDAE

        250        260        270        280        290        300
SVAVVTRGRA TLTLVSQETR ESFNLECGDV IRVPAGATVY VINQDSNERL EMVKLLQPVN

        310        320        330        340        350        360
NPGQFREYYA AGAKSPDQSY LRVFSNDILV AALNTPRDRL ERFFDQQEQR EGVIIRASQE

        370        380        390        400        410        420
KLRALSQHAM SAGQRPWGRR SSGGPISLKS ESPSYSNQFG QFFEACPEEH RQLQEMDVLV

        430        440        450        460        470        480
NYAEIKRGAM MVPHYNSKAT VVVYVVEGTG RYEMACPHVS SQSYEGQGRR EQEEEESTGR

        490        500        510        520        530        540
FQKVTARLAR GDIFVIPAGH PIAITASQNE NLRLLGFDIN GENNQRDFLA GQNNIINQLE

        550        560        570        580        590
REAKELSFNM PREEIEEIFE SQMESYFVPT ERQSRRGQGR DHPLASILDF AFF
```

Figure 20: Sequence of Jug r 2. Peptides identified by mass spectrometry are marked in different colours.

The final batch of 11S globulin of walnut was characterized by 2D-electrophoresis (see Figures 20 and 21). Three spots can be seen on the 2D-gel which correspond to proteins of about 15 kDa with a pI of about 5 to 6.5.

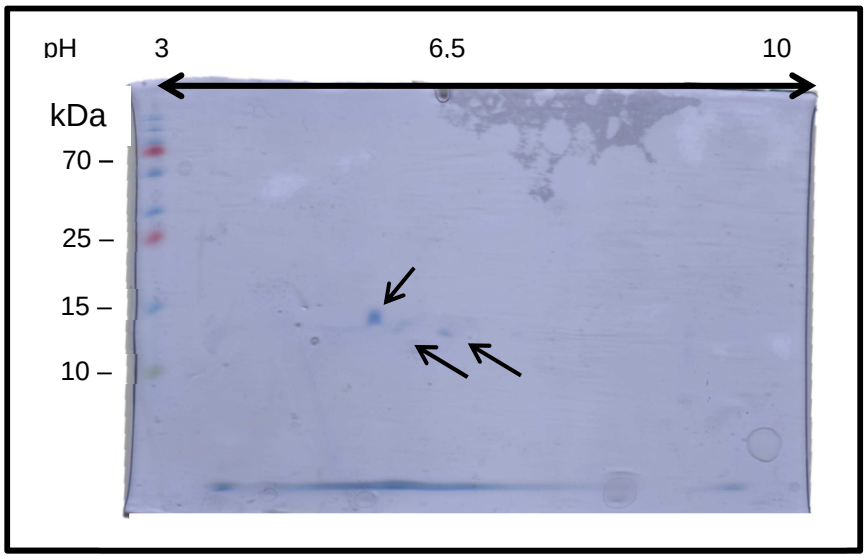

Figure 21: Coomassie stained 2D-electrophoresis of Batch 4. Spots at about 15 kDa with a pI of about 5 to 6,5 can be seen (marked by arrows).

3.3 Immunological characterization of purified 2S albumins from peanut and tree nuts

3.3.1 2S albumin from peanut, Ara h 6

Ara h 6 was tested for recognition of rabbit-anti-Ara h 2 antibody. Ara h 6 and Ara h 2 both belong to 2S albumins and show a weak cross-reactivity [87]. Cor a 14, Ber e 1 and walnut extract were applied to see if there is any cross-reactivity (see Figure 22 lanes 3-5). Ara h 2/6 was used as a positive control. Only the positive control showed a reaction (see Figure 22 lane 6). This means that the antibody specifically recognizes Ara h 2, but not the other proteins.

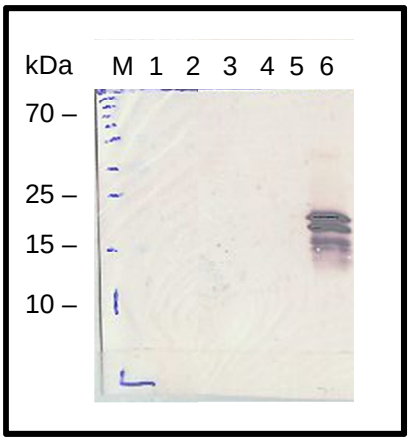

Figure 22: Anti-Ara h 2 Immunoblot. Lane 1 = Peanut Pool A, Lane 2 = Ara h 6, Lane 3 = Cor a 14, Lane 4 = Ber e 1, Lane 5 = walnut protein extract, Lane 6 = Ara h 2/6.

3.3.2 2S albumin from walnut, Jug r 1

First of all sera from walnut allergic patients were tested in IgE-ELISA for their reaction to Jug r 1/2 and Jug r 1/3 (see Table 7). It can be seen that 12 of 19 sera (B190, B191, B194, B200, B202, B203, S230, S233, S235, S280, S281 and S286) show IgE-binding activity to Jug r 1/2. The sera B191, B202 and B203 were known to show a strong reaction to Jug r 1. This positive reaction was confirmed with our experiment.

Seven out of 10 sera tested for IgE reactivity to Jug r 1/3 were positive (S201, S230, S233, S235, S280, S281 and S286).

Serum	IgE-binding capacity	
	Jug r 1/2	Jug r 1/3
B190	+	n.t.
B191	++	n.t.
B194	+	n.t.
B195	-	n.t.
B200	+	n.t.
B201	-	n.t.
B202	++	n.t.
B203	++	n.t.
B206	-	n.t.
S201	-	+
S202	-	-
S203	-	-
S204	-	-
S230	+	++
S233	+	+++
S235	++	+++
S280	+	++
S281	+	++
S286	+	++

Table 7: IgE-binding activity of sera from patients with walnut allergy. – indicates no IgE-binding activity, + indicates OD (405 nm) values of 0.01-0.2, ++ indicates OD (405 nm) values of 0.21-1, +++ indicates OD (405 nm) values higher than 1. n.t. = not tested

3.3.3 11S globulin from walnut, Jug r 4

IgE-reactivity of Jug r 4 was tested using seven sera of walnut allergic patients. Six sera showed IgE-binding capacity to our final pool containing Jug r 4 (data not shown).

3.4 IgE-cross-reactivity among 2S albumins

The sera from 20 walnut allergic patients were tested if they react to Jug r 1/2, Cor a 14, Ber e 1, Jug r 1/3, Ara h 2/6. Four sera (B191, B194, B200 and B203) reacted to all of the tested nut allergens. Also serum CH-P-53 was tested if it reacts to Cor a 14, Ber e 1, Ara h 2/6 and Jug r 1/2. It reacted predominantly to Cor a 14. According to that it reacted to Jug r 1/2. Therefore it was chosen for inhibition experiments investigating the cross-reaction between Cor a 14 and Jug r 1/2 (see Table 8).

To sum it up, 18 of 20 tested sera reacted to Jug r 1/2, 15 of 20 to Cor a 14, 10 of 20 to Ara h 2/6 and Ber e 1 each, and 7 out of 8 tested sera reacted to Jug r 1/3.

Serum	IgE-binding capacity				
	Jug r 1/2	Cor a 14	Ara h 2/6	Ber e 1	Jug r 1/3
B190	+	++	++	-	+
B191	++	++	++	++	++
B194	++	++	++	+	++
B195	+	-	-	-	+
B200	++	+	-	-	n.t.
B201	+	++	-	-	+++
B202	+++	+	-	-	+++
B203	++	++	++	++	++
B206	-	-	-	-	-
S200	+	+	+	+	n.t.
S235	+	-	-	-	n.t.
S239	-	-	-	-	n.t.
S245	+	+	+	+	n.t.
S251	+	+	+	+	n.t.
S252	+	+	+++	+	n.t.
S262	+	+	+	+	n.t.
S284	+	+	-	+	n.t.
S295	+	-	-	-	n.t.
S298	+++	+++	+++	+++	n.t.
CH-P-53	++	+	-	-	n.t.

Table 8: IgE-binding capacity of sera from walnut allergic patients to Jug r 1/2, Cor a 14, Ara h 2/6, Ber e 1 andn Jug r 1/3. – indicates no IgE-binding activity, + indicates OD (405 nm) values of 0.01-0.2, ++ indicates OD (405 nm) values of 0.21-1, +++ indicates OD (405 nm) values higher than 1. n.t. = not tested. Sera used for IgE-inhibition ELISA are marked grey.

Using selected patients' sera (CH-P-53 and B200) IgE cross-inhibition was performed. The plate was coated with Cor a 14 or Jug r 1/2, respectively and Cor a 14 or Jug r 1/2 were used for inhibition (Figure 23 and 24).

Using serum CH-P-53 IgE cross-inhibition was performed (see Figure 23). IgE-binding to Cor a 14 can be inhibited in a dose dependent manner by preincubation of Jug r 1/2 by 20%-28% (0.01 µg/ml-10 µg/ml, see Figure 23A, green line).

As a control self inhibition using Cor a 14 was performed. For 100% inhibition 10 µg/ml Cor a 14 are needed. Using 0.01 µg/ml and 1 µg/ml the self inhibition was 60% and 95%, respectively (see Figure 23A, orange line).

Reversely, IgE-binding to Jug r 1/2 was inhibited by preincubation of Cor a 14 (see Figure 23B, orange line). Within the range of 0.01 µg/ml to 10 µg/ml inhibition of 87% to 100% was obtained.

Preincubation with Jug r 1/2 inhibited IgE-binding to immobilized Jug r 1/2 by 60% to 100% (0.01 µg/ml and 1 µg/ml, see Figure 23B, green line).

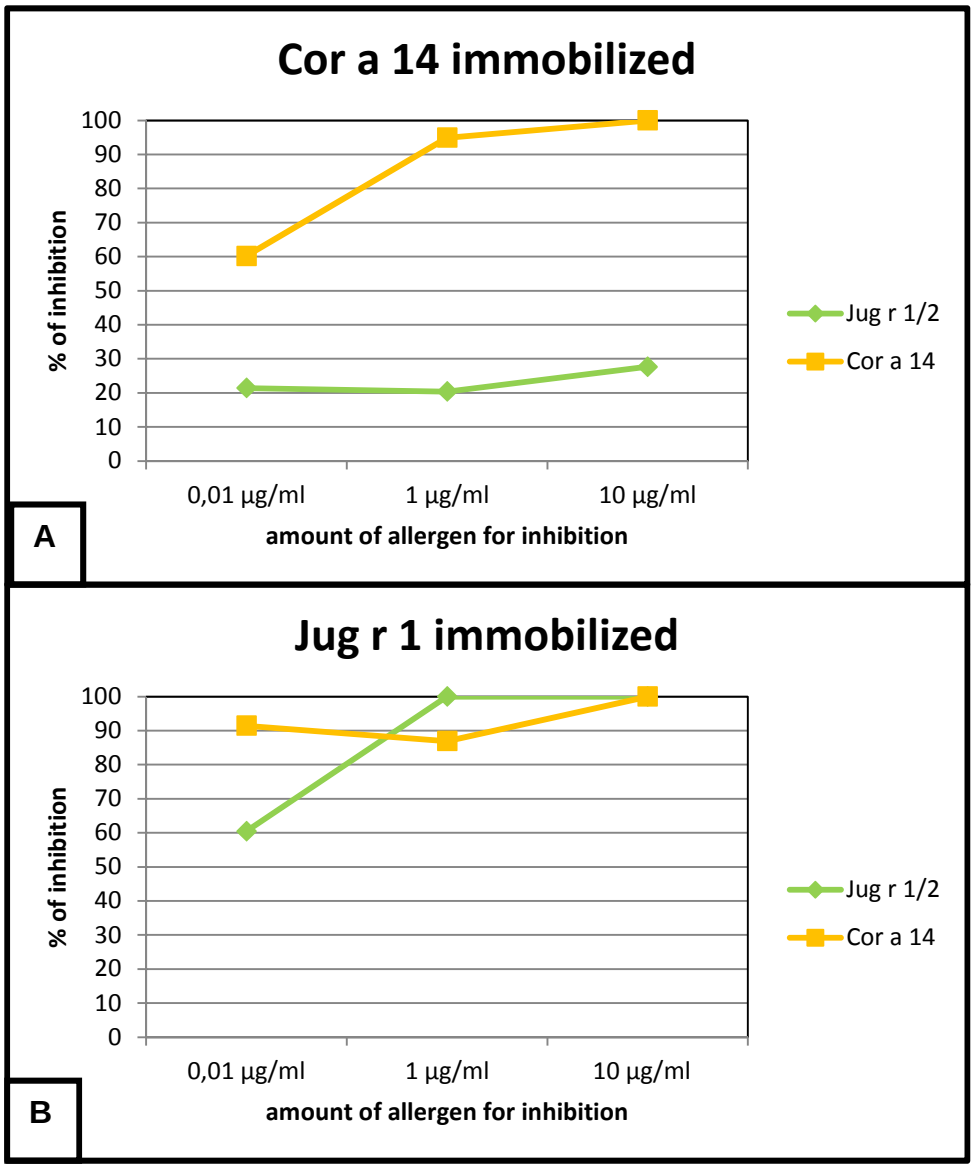

Figure 23: IgE-inhibition ELISA of Jug r 1/2 and Cor a 14 using serum CH-P-53. The wells were coated with Cor a 14 (A) and Jug r 1/2 (B), respectively. Serum CH-P-53 was preincubated in a dose dependent manner (0.01 – 10 µg/ml) of Jug r 1/2 and Cor a 14, respectively.

A second serum (B200) was used to investigate cross-reaction between Jug r 1/2 and Cor a 14 (see Figure 24). In Figure 24A (green line) inhibition of IgE-binding to Cor a 14 after preincubation with Jug r 1/2 is shown. Inhibition ranged from 56% to 100%. Within the range of 0.01 µg/ml to 10 µg/ml Jug r 1/2 inhibition of IgE-binding to Cor a 14 was between 17% and 24% in a dose-dependent way (see Figure 24B, orange line).

Self inhibition was done as a control and it can be shown that a only 1 µg/ml of Jug r 1/2 needed to reach an inhibition of 100%. Using 0.01 µg/ml Jug r 1/2 the self inhibition was 79% (see Figure 24B, green line).

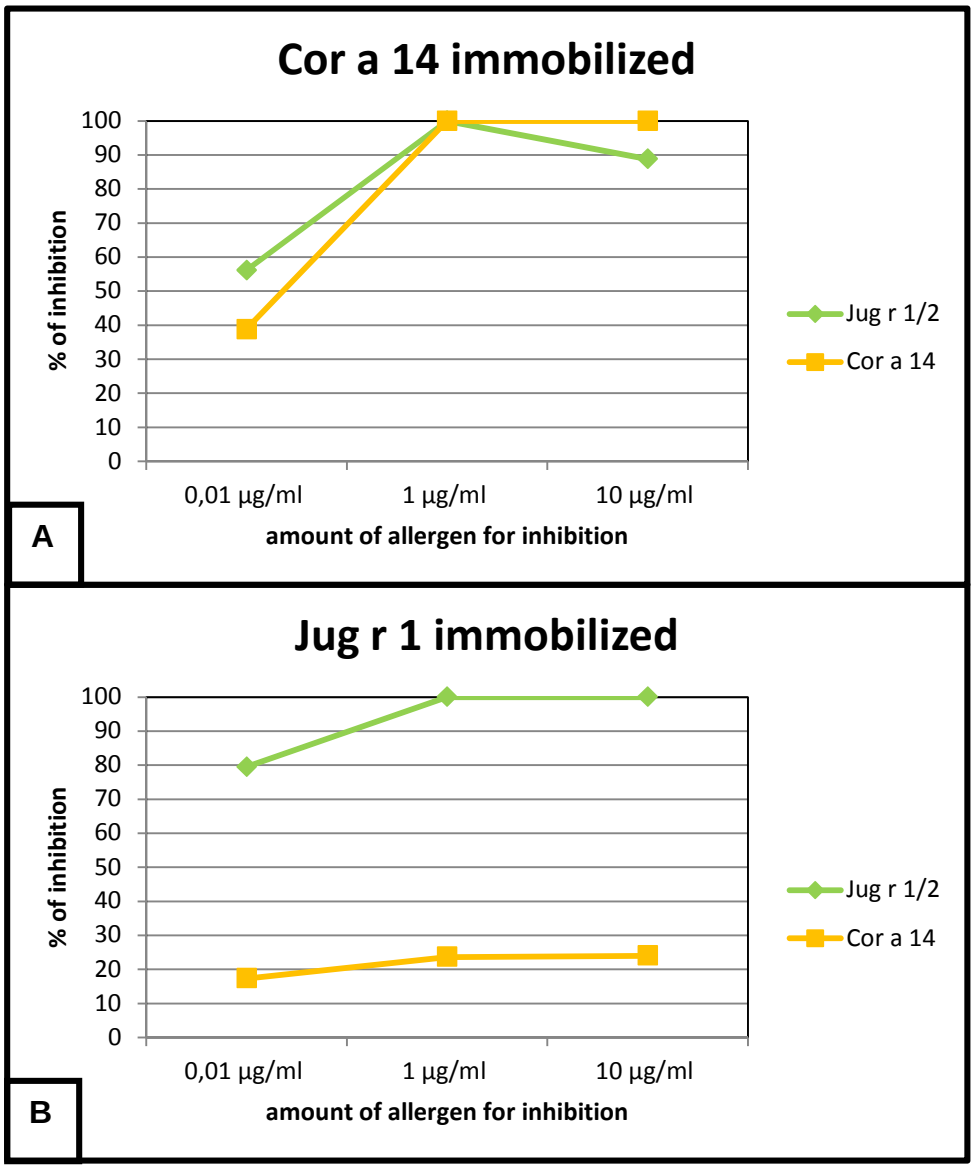

Figure 24: IgE-inhibition ELISA of Cor a 14 and Jug r 1/2 using serum B200. The wells were coated with Jug r 1/2 (A) and Cor a 14 (B), respectively. Serum B200 was preincubated with different amounts of Jug r 1/2 and Cor a 14, respectively.

4 Discussion

2S albumins are seed proteins in plants. Furthermore, 2S albumins from tree nuts, peanut and seeds are known to induce severe food allergic reactions in predisposed individuals.

The aim of this master thesis was to establish and modify purification protocols for 2S albumins from tree nuts and peanut. The allergens were characterized biochemically by SDS-PAGE, 2D-electrophoresis and mass spectrometry and immunologically by immunoblot, IgE-ELISA and IgE-Inhibition ELISA using sera from nut allergic patients.

In 2004, Suhr et al. [87] published a protocol for the purification of Ara h 6, 2 S albumin from peanut including gel filtration and anion exchange chromatography. Furthermore, they studied the stability of Ara h 6 according to heat and pepsin digestion, verifying that the compact 3D structure of the protein is not affected by these treatments. In our protocol we defatted the nut flour using n-hexane before extraction. It is important to remove the whole n-hexane through centrifugation and doing filtering steps because otherwise it would disturb further purification steps.
Next, we did two chromatography steps, cation exchange chromatography and hydrophobic interaction chromatography. Subsquently, we did gel filtration as it is done in the protocol of Suhr et al.

Ara h 2 and Ara h 6 are both members of the 2S albumin protein family. Their amino acid sequence similarity is 56%. However, they share a compact and robust 3D structure formed by four disulfide bridges. While Ara h 2 is not further processed after translation and consists of only one subunit, Ara h 6 is processed into a small and a large subunit, respectively. Both, Ara h 2 and Ara h 6 are expressed in peanuts and their IgE cross reactivity was already demonstrated [42]. Within this master thesis Ara h 6 was purified to near homogeneity from peanuts and confirmed by mass spectrometry.

The 2S albumin, Jug r 1, was isolated from walnuts. In 2009 Sordet et al. [59] published a protocol for purification of Jug r 1. We used a different protein extraction buffer and different columns for chromatography, cation exchange chromatography and hydrophobic interaction chromatography instead of affinity chromatography. In Coomassie stained SDS-PAGE the protein migrates at approximately 13 kDa. In addition, we did 2D-electrophoresis, mass spectrometry and IgE-ELISA. Jug r 1 consists of a number of isoforms present in the nut. That is why in the 2D gel several spots can be seen. We could detect spots at a pH range between 5 and 6 which correspond to our target proteins. The additional spots at basic pH correspond to other proteins still present in our Jug r 1 enriched batch. Using mass spectrometry we showed that next to Jug r 1 also Jug r 2, the 7S globulin (vicillin) is found in our final batch. The sample was therefore designated Jug r 1/2.

Sera from walnut allergic patients were tested for their reaction to Jug r 1/2 and compared to Jug r 1/3 using IgE-ELISA. Twelve out of 19 sera were positive to Jug r 1/2 and 7 out of 10 tested sera were positive to Jug r 1/3. Thus verifying that Jug r 1 and Jug r 2 are major allergens in this patients collective.

Finally, Jug r 4, the 11S globulin was purified from walnut protein extract. Stepwise ammonium sulfate precipitation was performed with 20%, 70% and 90%, respectively. That way proteins were fractionated. Jug r 4 was further purified from the 90% ammonium sulfate fraction using anion exchange chromatography. There are some protocols published how to extract proteins from walnut [63, 123] but in none of them Jug r 4 was purified in further steps.

Identifying the relevant IgE epitopes of seed storage protein, and of 2S albumins in particular is relevant for assessing the risk of cross reactivity of allergens from different species (e.g. among tree nuts and peanuts). There are two forms of IgE binding epitopes, linear and conformational epitopes, respectively. Linear epitopes consist of amino acid residues which are adjacent. Conformational epitopes are formed by amino acids which are further apart in

the primary sequence but in close proximity in the folded protein. In cross-reactivity epitopes shared by two or more different allergens are recognized by specific antibodies. For IgE cross-reactivity Aalberse postulated that overall amino acid similarity of > 50% is relevant and above 70% is related with clinical symptoms of allergy [124]. At present only a limited number of studies are available which investigated the IgE cross reactivity between nut allergens. In general, 2S albumins from nuts share a characteristic 3D structure stabilized by four disulfide bridges. However, they show a high level of polymorphism due to the presence of isoforms and several posttranslational modifications. Moreover, their sequence similarity within and among plant species is rather low [39]. 2S albumins within the botanical order Fagales share quite high sequence similarity. Cor a 14, Jug r 1 and Car i 1 show a sequence similarity of 57% to 88% for instance. As compared to sequence similarity of 30% to 36% to the 2S albumins (Ara h 2, Ara h 6, Ara h 7) from peanut [125]. This means that the probability of cross-reactivity is lower between 2S albumins of the Fagales and peanut than between two 2S albumins from species of the Fagales. One immunodominant IgE epitope has been identified from a number of 2S albumins, the unstructured, flexible and solvent exposed hypervariable region between helix III and helix IV [39, 125, 126]. Moreover, it was shown that the epitope QGL**RGEE**ME**E**MV from amino acid 33 to 44 is an important sequence in IgE-binding in Jug r 1 [60]. As the highlighted sequence also is present in Cor a 14 this might be a reason for cross-reactivity.

In the present master thesis two sera from tree nut allergic patients were used to assess the level of IgE cross reactivity between Jug r 1 and Cor a 14. One serum displayed higher IgE-binding capacity to Cora 14 and less to Jug r 1/2. Consecutively, preincubation of the serum with Cor a 14 totally abolished IgE-binding to Jug r 1/2. The reverse experiment showed only 28% inhibition of IgE-binding to Cora 14 after preincubation with Jug r 1/2. The second patient's serum presented higher IgE-binding activity to Jug r 1/2 as compared to Cor a 14. In parallel, preincubation of the serum with Jug r 1/2 totally inhibited IgE recognition of Cor a 14, while the reverse experiment provided only partial

inhibition of IgE-binding to Jug r 1/2. These two examples point at different ways of sensitization for the patients which turn into different patterns of cross-reactivity. With the use of additional 2S albumins from nuts and seeds the range of IgE cross-reactivity can be assessed in a patient specific manner. This in turn will help to formulate precise dietary recommendations and avoid unnecessary exclusion diets. Moreover, these data contribute to identify the major IgE epitopes of 2S albumins for a certain patients' collective and to design new tools for immunotherapy of nut allergies.

5 Abstract

Food allergy is an IgE-mediated adverse reaction to food proteins leading to symptoms like urticaria, angiocœdema, rhinitis, asthma, emesis, diarrhoea or life-threatening anaphylactic shock.

The main allergen sources in food allergy are milk, eggs, peanut, tree nuts, seafood, shellfish, soy and wheat.

About 2.3% of children and 0.4 to 1.4% of adults are affected by tree nut or peanut allergy.

Important nut allergens are 2S albumins, cupins and non-specific lipid transfer proteins. They are resistant to heat treatment and protease digestion and an allergic reaction elicted by them leads to severe symptoms.

In this master thesis purification protocols for the 2S albumins of walnut (Jug r 1) and peanut (Ara h 6) were established. For purification different chromatography methods were used. The purified allergens were characterized by SDS-PAGE, 2D-electrophoresis, mass spectrometry, immunoblot and IgE-ELISA. Furthermore cross-reactivity between 2S albumins from hazelnut and walnut was investigated by IgE-Inhibition ELISA.

Twenty sera of patients allergic to walnut were tested for their IgE-binding to 2S albumins of walnut, peanut, hazelnut and Brazil nut. Eighteen out of the 20 tested sera reacted to Jug r 1/2, 15 out of 20 to Cor a 14, 10 out of 20 to Ara h 2/6 and Ber e 1 each, and 7 out of 8 tested sera reacted to Jug r 1/3.

Using IgE-Inhibition ELISA cross-reactivity between Cor a 14 and Jug r 1 was tested using selected patients' sera. The range of cross-reactivity was found to be patient specific, overall ranging from 17 to 100%.

The investigation of cross-reactivity between allergens from the same protein family and different allergen sources can contribute to improved allergenic risk management.

6 Zusammenfassung

Nahrungsmittelallergie ist eine IgE-vermittelte Reaktion gegen Nahrungsmittelproteine. Die Symptome reichen von Urtikaria, Angioödem, Rhinitis, Asthma, Erbrechen und Diarrhoe bis zum lebensbedrohlichen anaphylaktischen Schock.

Die Hauptallergenquellen für Nahrungsmittelallergene sind Milch, Eier, Erdnüsse, Baumnüsse, Meeresfrüchte, Krustentiere, Soja und Weizen.

2,3% der Kinder und 0,4 bis 1,4% der Erwachsenen sind von Nussallergien betroffen.

Wichtige Nussallergene sind 2S Albumine, Cupine und nicht-spezifische Lipidtransferproteine. Diese Allergene sind hitzestabil und resistent gegenüber proteolytischem Verdau.

In dieser Masterarbeit wurden Reinigungsprotokolle für die 2S Albumine der Erdnuss (Ara h 6) und der Walnuss (Jug r 1) etabliert. Für die Reinigung wurden unterschiedliche Chromatographiemethoden verwendet.

Die aufgereinigten Allergene wurden mit folgenden Methoden charakterisiert: SDS-PAGE, 2D-Elektrophorese, Massenspektrometrie, Immunblot und IgE-ELISA. Weiters wurde die IgE-Kreuzreaktivität zwischen 2S Albuminen der Haselnuss und der Walnuss untersucht.

20 Patientenseren wurden auf ihre IgE-Bindung an 2S Albumine der Walnuss, Erdnuss, Haselnuss und Paranuss getestet. 18 der 20 getesteten Seren zeigten IgE-Bindung zu Jug r 1/2, 15 reagierten auf Cor a 14, je 10 reagierten auf Ara h 2/6 und Ber e 1, 7 von 8 getesteten Seren reagierten auf Jug r 1/3.

Die Kreuzreaktion zwischen Cor a 14 und Jug r 1 wurde mit ausgewählten Patientenseren getestet. Die IgE-Kreuzreaktivität ist patientenspezifisch und liegt im Bereich von 17 bis 100%.

Die Untersuchung der Kreuzreaktivität zwischen Allergenen einer Proteinfamilie aus verschiedenen Allergenquellen kann zum verbesserten Risikomanagement einer Nahrungsmittelallergie beitragen.

7 References

1. von Pirquet, C., *Allergie.* Munchen Med Wochenschr, 1906. **53**: p. 1457.
2. Kay, A.B., *100 years of 'Allergy': can von Pirquet's word be rescued?* Clin Exp Allergy, 2006. **36**(5): p. 555-9.
3. Gell R, C.P., *The classification of allergic reactions underlying disease.*, in *Clinical Aspects of Immunology*, C.R. Gell P, Lachmann P, Editor 1963, Blackwell Scientific Publications: Oxford.
4. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Cellular and Molecular Immunology*. 6th ed2007.
5. Descotes, J. and G. Choquet-Kastylevsky, *Gell and Coombs's classification: is it still valid?* Toxicology, 2001. **158**(1-2): p. 43-9.
6. Rajan, T.V., *The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation.* Trends Immunol, 2003. **24**(7): p. 376-9.
7. Marc, D., Olson K, *Hypersensitivity Reactions and Methods of Detection.* NeuroScience, 2009.
8. Arthus, M., *Injections répétées de serum de cheval chez le lapin.* CR Soc Biol 1903. **55**: p. 817-820.
9. Nosbaum, A., et al., *Allergic and irritant contact dermatitis.* Eur J Dermatol, 2009. **19**(4): p. 325-32.
10. Rorke, S. and S.T. Holgate, *The atopy phenotype revisited.* Revue Francaise D Allergologie Et D Immunologie Clinique, 2004. **44**(5): p. 436-444.
11. Moulin, D., et al., *Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells.* Cytokine, 2007. **40**(3): p. 216-25.
12. Schmitz, J., et al., *IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines.* Immunity, 2005. **23**(5): p. 479-90.
13. Wang, J. and H.A. Sampson, *Food allergy.* J Clin Invest, 2011. **121**(3): p. 827-35.
14. Maggi, E., *The TH1/TH2 paradigm in allergy.* Immunotechnology, 1998. **3**(4): p. 233-44.
15. Gould, H.J., et al., *The biology of IGE and the basis of allergic disease.* Annu Rev Immunol, 2003. **21**: p. 579-628.
16. Metcalfe, D.D., D. Baram, and Y.A. Mekori, *Mast cells.* Physiol Rev, 1997. **77**(4): p. 1033-79.
17. Asero, R., et al., *IgE-mediated food allergy diagnosis: Current status and new perspectives.* Mol Nutr Food Res, 2007. **51**(1): p. 135-47.
18. Zuidmeer, L., et al., *The prevalence of plant food allergies: a systematic review.* J Allergy Clin Immunol, 2008. **121**(5): p. 1210-1218 e4.
19. Roux, K.H., S.S. Teuber, and S.K. Sathe, *Tree nut allergens.* Int Arch Allergy Immunol, 2003. **131**(4): p. 234-44.
20. Breiteneder, H. and C. Radauer, *A classification of plant food allergens.* J Allergy Clin Immunol, 2004. **113**(5): p. 821-30; quiz 831.
21. Hoffmann-Sommergruber, K. and E.N. Mills, *Food allergen protein families and their structural characteristics and application in component-resolved diagnosis: new data from the EuroPrevall project.* Anal Bioanal Chem, 2009. **395**(1): p. 25-35.

22. Kader, J.C., *Lipid-Transfer Proteins in Plants.* Annu Rev Plant Physiol Plant Mol Biol, 1996. **47**: p. 627-654.
23. Douliez, J.P., et al., *Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels.* Journal of Cereal Science, 2000. **32**(1): p. 1-20.
24. Wang, S.Y., et al., *A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean.* Peptides, 2004. **25**(8): p. 1235-42.
25. Kader, J.C., *Proteins and the intracellular exchange of lipids. I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber.* Biochim Biophys Acta, 1975. **380**(1): p. 31-44.
26. Lleonart, R., et al., *Food allergy: identification of the major IgE-binding component of peach (Prunus persica).* Ann Allergy, 1992. **69**(2): p. 128-30.
27. Fernandez-Rivas, M., et al., *Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods.* J Allergy Clin Immunol, 2006. **118**(2): p. 481-8.
28. Asero, R., *Detection and clinical characterization of patients with oral allergy syndrome caused by stable allergens in Rosaceae and nuts.* Ann Allergy Asthma Immunol, 1999. **83**(5): p. 377-83.
29. Asero, R., et al., *Immunological cross-reactivity between lipid transfer proteins from botanically unrelated plant-derived foods: a clinical study.* Allergy, 2002. **57**(10): p. 900-6.
30. Asero, R., et al., *Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion.* Int Arch Allergy Immunol, 2000. **122**(1): p. 20-32.
31. Duffort, O.A., et al., *Immunoassay to quantify the major peach allergen Pru p 3 in foodstuffs. Differential allergen release and stability under physiological conditions.* J Agric Food Chem, 2002. **50**(26): p. 7738-41.
32. Gaier, S., et al., *Purification and structural stability of the peach allergens Pru p 1 and Pru p 3.* Mol Nutr Food Res, 2008. **52 Suppl 2**: p. S220-9.
33. Brenna, O., et al., *Technological processes to decrease the allergenicity of peach juice and nectar.* J Agric Food Chem, 2000. **48**(2): p. 493-7.
34. Lindorff-Larsen, K. and J.R. Winther, *Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases.* FEBS Lett, 2001. **488**(3): p. 145-8.
35. Scheurer, S., et al., *Strong allergenicity of Pru av 3, the lipid transfer protein from cherry, is related to high stability against thermal processing and digestion.* J Allergy Clin Immunol, 2004. **114**(4): p. 900-7.
36. Youle, R.J. and A.H.C. Huang, *Occurrence of Low-Molecular Weight and High Cysteine Containing Albumin Storage Proteins in Oilseeds of Diverse Species.* American Journal of Botany, 1981. **68**(1): p. 44-48.
37. Aggizio, A., Da, Cunha, M, Carvalho, AO, Oliveira, MA, Ribeiro, SFF, Gomes, V, *The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells* Plant Sci, 2006. **171**: p. 515-522.

38. Pantoja-Uceda, D., et al., *Solution structure of RicC3, a 2S albumin storage protein from Ricinus communis.* Biochemistry, 2003. **42**(47): p. 13839-47.
39. Moreno, F.J. and A. Clemente, *2S Albumin Storage Proteins: What Makes them Food Allergens?* Open Biochem J, 2008. **2**: p. 16-28.
40. Pastorello, E.A., et al., *The major allergen of sesame seeds (Sesamum indicum) is a 2S albumin.* J Chromatogr B Biomed Sci Appl, 2001. **756**(1-2): p. 85-93.
41. Akkerdaas, J.H., et al., *IgE binding to pepsin-digested food extracts.* Int Arch Allergy Immunol, 2005. **138**(3): p. 203-8.
42. Lehmann, K., et al., *Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions.* Biochem J, 2006. **395**(3): p. 463-72.
43. Clemente, A., et al., *Use of the indirect competitive ELISA for the detection of Brazil nut in food products.* Food Control, 2004. **15**(1): p. 65-69.
44. Jenkins, J.A., et al., *Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis.* J Allergy Clin Immunol, 2005. **115**(1): p. 163-70.
45. Monsalve, R., M. Villalba, and R. Rodriguez, *Allergy to Mustard seeds: The importance of 2S albumins as food allergens.* Internet Symp Food Allergens, 2001. **3**: p. 57-69.
46. Ortolani, C., et al., *Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study.* J Allergy Clin Immunol, 2000. **105**(3): p. 577-81.
47. Hansen, K.S., et al., *Component-resolved in vitro diagnosis of hazelnut allergy in Europe.* J Allergy Clin Immunol, 2009. **123**(5): p. 1134-41, 1141 e1-3.
48. Lüttkopf, D., et al., *Cloning, expression and characterisation of profilin as a minor allergen in hazelnut.* Allergy, 2002. **57**: p. 87.
49. Müller, U., et al., *Allergens in raw and roasted hazelnuts (Corylus avellana) and their cross-reactivity to pollen.* Eur Food Res Technol, 2000. **212**: p. 2-12.
50. Skamstrup Hansen, K., et al., *Roasted hazelnuts - allergenic activity evaluated by double-blind, placebo-controlled food challenge.* Allergy, 2003. **58**: p. 132-138.
51. Vieths, S., et al., *Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure.* Eur Food Res Technol, 1999. **209**: p. 379-388.
52. Schocker, F., et al., *IgE binding to unique hazelnut allergens: identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions.* Eur J Nutr, 2000. **39**(4): p. 172-80.
53. Pastorello, E.A., et al., *Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results.* J Allergy Clin Immunol, 2002. **109**(3): p. 563-70.
54. Rigby, N.M., et al., *The purification and characterisation of allergenic hazelnut seed proteins.* Mol Nutr Food Res, 2008. **52 Suppl 2**: p. S251-61.

55. Lauer, I., et al., *Hazelnut (Corylus avellana) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity.* Biochem J, 2004. **383**(Pt 2): p. 327-34.
56. Akkerdaas, J.H., et al., *Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library.* Mol Nutr Food Res, 2006. **50**(1): p. 18-23.
57. Garino, C., et al., *Isolation, cloning, and characterization of the 2S albumin: a new allergen from hazelnut.* Mol Nutr Food Res, 2010. **54**(9): p. 1257-65.
58. Teuber, S.S., et al., *Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (Juglans regia), a major food allergen.* J Allergy Clin Immunol, 1998. **101**(6 Pt 1): p. 807-14.
59. Sordet, C., et al., *Expression of Jug r 1, the 2S albumin allergen from walnut (Juglans regia), as a correctly folded and functional recombinant protein.* Peptides, 2009. **30**(7): p. 1213-21.
60. Robotham, J.M., et al., *Linear IgE epitope mapping of the English walnut (Juglans regia) major food allergen, Jug r 1.* J Allergy Clin Immunol, 2002. **109**(1): p. 143-9.
61. Teuber, S.S., et al., *Identification and cloning of a complementary DNA encoding a vicilin-like proprotein, jug r 2, from english walnut kernel (Juglans regia), a major food allergen.* J Allergy Clin Immunol, 1999. **104**(6): p. 1311-20.
62. Pastorello, E.A., et al., *Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen.* J Allergy Clin Immunol, 2004. **114**(4): p. 908-14.
63. Teuber, S.S. and W.R. Peterson, *Systemic allergic reaction to coconut (Cocos nucifera) in 2 subjects with hypersensitivity to tree nut and demonstration of cross-reactivity to legumin-like seed storage proteins: new coconut and walnut food allergens.* J Allergy Clin Immunol, 1999. **103**(6): p. 1180-5.
64. Wallowitz, M., et al., *Jug r 4, a legumin group food allergen from walnut (Juglans regia Cv. Chandler).* J Agric Food Chem, 2006. **54**(21): p. 8369-75.
65. Badley, R.A., et al., *The structure, physical and chemical properties of the soy bean protein glycinin.* Biochim Biophys Acta, 1975. **412**(2): p. 214-28.
66. Kitamura, K., T. Takagi, and K. Shibasaki, *Subunit structure of soybean 11S globulin.* Agric Biol Chem, 1976. **40**: p. 1837-1844.
67. Alcocer, M., L. Rundqvist, and G. Larsson, *Ber e 1 protein: the versatile major allergen from Brazil nut seeds.* Biotechnol Lett, 2012. **34**(4): p. 597-610.
68. Sun, S.S., S.B. Altenbach, and F.W. Leung, *Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (Bertholletia excelsa H.B.K.).* Eur J Biochem, 1987. **162**(3): p. 477-83.
69. Antunes, A.J. and P. Markakis, *Protein supplementation of Navy beans with Brazil nuts.* J Agric Food Chem, 1977. **25**(5): p. 1096-7.

70. Gander, E.S., et al., *Isolation, characterization and expression of a gene coding for a 2S albumin from Bertholletia excelsa (Brazil nut).* Plant Mol Biol, 1991. **16**(3): p. 437-48.
71. Vincentz, M., et al., *ACGT and vicilin core sequences in a promoter domain required for seed-specific expression of a 2S storage protein gene are recognized by the opaque-2 regulatory protein.* Plant Mol Biol, 1997. **34**(6): p. 879-89.
72. Murtagh, G.J., et al., *In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8.* Clin Exp Allergy, 2003. **33**(8): p. 1147-52.
73. Murtagh, G.J., et al., *Stability of recombinant 2 S albumin allergens in vitro.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 913-5.
74. Koppelman, S.J., et al., *Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin.* J Agric Food Chem, 2005. **53**(1): p. 123-31.
75. Alcocer, M.J., et al., *The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins.* J Mol Biol, 2002. **324**(1): p. 165-75.
76. Moreno, F.J., et al., *Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (Bertholletia excelsa).* Biochim Biophys Acta, 2004. **1698**(2): p. 175-86.
77. Hide, D.W., *Clinical curio: allergy to Brazil nut.* Br Med J, 1983. **287**.
78. Arshad, S.H., et al., *Clinical and immunological characteristics of Brazil nut allergy.* Clin Exp Allergy, 1991. **21**(3): p. 373-6.
79. Nordlee, J.A., et al., *Identification of a Brazil-nut allergen in transgenic soybeans.* N Engl J Med, 1996. **334**(11): p. 688-92.
80. Pastorello, E.A., et al., *Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy.* J Allergy Clin Immunol, 1998. **102**(6 Pt 1): p. 1021-7.
81. Bartolome, B., et al., *Allergens from Brazil nut: immunochemical characterization.* Allergol Immunopathol (Madr), 1997. **25**(3): p. 135-44.
82. Hartig, T., *Über das Klebermehl.* Botanische Zeitung, 1855. **13**: p. 881-882.
83. Kulis, M., et al., *The 2S albumin allergens of Arachis hypogaea, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice.* Clin Exp Allergy, 2012. **42**(2): p. 326-36.
84. Koppelman, S.J., et al., *Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2.* Clin Exp Allergy, 2005. **35**(4): p. 490-7.
85. Koppelman, S.J., et al., *Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides.* Mol Nutr Food Res, 2010. **54**(12): p. 1711-21.
86. Barre, A., et al., *Homology modelling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes.* Immunol Lett, 2005. **100**(2): p. 153-8.

87. Suhr, M., et al., *Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat.* Mol Nutr Food Res, 2004. **48**(5): p. 390-9.
88. Mills, E.N., et al., *Allergens of the cupin superfamily.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 925-9.
89. Viquez, O.M., K.N. Konan, and H.W. Dodo, *Structure and organization of the genomic clone of a major peanut allergen gene, Ara h 1.* Mol Immunol, 2003. **40**(9): p. 565-71.
90. Rabjohn, P., et al., *Molecular cloning and epitope analysis of the peanut allergen Ara h 3.* J Clin Invest, 1999. **103**(4): p. 535-42.
91. Kleber-Janke, T., et al., *Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology.* Int Arch Allergy Immunol, 1999. **119**(4): p. 265-74.
92. Kang, I. and M. Gallo, *Cloning and characterization of a novel peanut allergen Ara h 3 isoform displaying potentially decreased allergenicity.* Plant Science, 2007. **172**: p. 345-353.
93. Kleber-Janke, T., et al., *Patient-tailored cloning of allergens by phage display: peanut (Arachis hypogaea) profilin, a food allergen derived from a rare mRNA.* J Chromatogr B Biomed Sci Appl, 2001. **756**(1-2): p. 295-305.
94. Mittag, D., et al., *Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy.* J Allergy Clin Immunol, 2004. **114**(6): p. 1410-7.
95. Chapman, M.D., et al., *Nomenclature and structural biology of allergens.* J Allergy Clin Immunol, 2007. **119**(2): p. 414-20.
96. Pons, L., et al., *Purification and cloning of two high molecular mass isoforms of peanut seed oleosin encoded by cDNAs of equal sizes.* Plant Physiology and Biochemistry, 2005. **43**: p. 695-668.
97. Boulay, A., et al., *A EuroPrevall review of factors affecting incidence of peanut allergy: priorities for research and policy.* Allergy, 2008. **63**(7): p. 797-809.
98. Flinterman, A.E., et al., *Determination of no-observed-adverse-effect levels and eliciting doses in a representative group of peanut-sensitized children.* J Allergy Clin Immunol, 2006. **117**(2): p. 448-54.
99. Grundy, J., et al., *Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts.* J Allergy Clin Immunol, 2002. **110**(5): p. 784-9.
100. Sicherer, S.H. and H.A. Sampson, *Peanut allergy: emerging concepts and approaches for an apparent epidemic.* J Allergy Clin Immunol, 2007. **120**(3): p. 491-503; quiz 504-5.
101. Skypala I, V.C., *Food Hypersensitivity: Diagnosing and Managing Food Allergies and Intolerance. .* Blackwell Publishing Ltd, 2009.
102. Aalberse, R.C., J. Akkerdaas, and R. van Ree, *Cross-reactivity of IgE antibodies to allergens.* Allergy, 2001. **56**(6): p. 478-90.
103. Robotham, J.M., et al., *Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope.* Mol Immunol, 2010. **47**(9): p. 1830-8.

104. Valenta, R., et al., *Immunotherapy of allergic disease.* Adv Immunol, 2004. **82**: p. 105-53.
105. Niemi, M., et al., *Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen.* Structure, 2007. **15**(11): p. 1413-21.
106. Padavattan, S., et al., *High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography.* J Immunol, 2009. **182**(4): p. 2141-51.
107. Lehrer, S.B., R. Ayuso, and G. Reese, *Current understanding of food allergens.* Ann N Y Acad Sci, 2002. **964**: p. 69-85.
108. Moneret-Vautrin, D.A., et al., *Food allergy to peanuts in France - evaluation of 142 observations.* Clinical and Experimental Allergy, 1998. **28**(9): p. 1113-1119.
109. Teuber, S.S., et al., *Tree nut allergy.* Curr Allergy Asthma Rep, 2003. **3**(1): p. 54-61.
110. Sparham, S.J. and E.R. Huehns, *Separation of Human Globin Chains by Ion-Exchange Chromatography on Cm-Sepharose Cl-6b.* Hemoglobin, 1979. **3**(1): p. 13-20.
111. Queiroz, J.A., C.T. Tomaz, and J.M. Cabral, *Hydrophobic interaction chromatography of proteins.* J Biotechnol, 2001. **87**(2): p. 143-59.
112. Hagel, L., et al., *Properties, in theory and practice, of novel gel filtration media for standard liquid chromatography.* J Chromatogr, 1989. **476**: p. 329-44.
113. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
114. Diezel, W., G. Kopperschlager, and E. Hofmann, *An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie Brilliant Blue.* Anal Biochem, 1972. **48**(2): p. 617-20.
115. Meyer, T.S. and B.L. Lamberts, *Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips.* Biochim Biophys Acta, 1965. **107**(1): p. 144-5.
116. Smith, P.K., et al., *Measurement of protein using bicinchoninic acid.* Anal Biochem, 1985. **150**(1): p. 76-85.
117. Wiechelman, K.J., R.D. Braun, and J.D. Fitzpatrick, *Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation.* Anal Biochem, 1988. **175**(1): p. 231-7.
118. Righetti, P.G., B.C. Brost, and R.S. Snyder, *On the limiting pore size of hydrophilic gels for electrophoresis and isoelectric focusing.* J Biochem Biophys Methods, 1981. **4**(5-6): p. 347-63.
119. Righetti, P.G. and S. Hjerten, *High-molecular-weight carrier ampholytes for isoelectric focusing of peptides.* J Biochem Biophys Methods, 1981. **5**(5): p. 259-72.
120. Grossert, J.S., *A retrospective view of mass spectrometry and natural products - sixty years of progress, with a focus on contributions by R. Graham Cooks.* International Journal of Mass Spectrometry, 2001. **212**(1-3): p. 65-79.

121. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
122. Lequin, R.M., *Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA).* Clin Chem, 2005. **51**(12): p. 2415-8.
123. Dandekar, A.M., et al., *Vector development for the introduction and regulation of novel transgens in walnut plants.* Walnut Research Reports, 1993: p. 42-58.
124. Aalberse, R.C., *Structural biology of allergens.* J Allergy Clin Immunol, 2000. **106**(2): p. 228-38.
125. Sharma, G.M., et al., *Cloning and characterization of 2S albumin, Car i 1, a major allergen in pecan.* J Agric Food Chem, 2011. **59**(8): p. 4130-9.
126. Pantoja-Uceda, D., et al., *Solution structure of a methionine-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties.* Biochemistry, 2004. **43**(22): p. 6976-86.

Curriculum vitae

Name: Christiane Agreiter
e-mail: a0625485@unet.univie.ac.at
Date of birth: March 7th, 1988
Place of birth: Wels (Austria)
Nationality: Austria

Education:

1994 – 1998: Elementary School, Wels
1998 – 2006: Wirtschaftskundliches Realgymnasium, Wels
June 2006: school leaving examination (with distinction)
October 2006 - January 2007: Study of Politics (University of Vienna)
March 2007 - November 2010: Study of Biology (Bachelor: Microbiology/Genetics, University of Vienna)
Since November 2010: Study of Immunology (Master: Molecular Microbiology and Immunobiology, University of Vienna)

Scientific Experience:

September 2006: GEN-AU Summerschool („Institute of Applied Genetics and Cell Biology", University of Natural Resources and Life Sciences, Vienna)

August 2007: Internship at „Institute of Applied Genetics and Cell Biology" (University of Natural Resources and Life Sciences, Vienna)

July/August 2008: Internship at „Gregor Mendel Institute of Molecular Plant Biology"

October 2008 - January 2009: Technicial Assistant at „Gregor Mendel Institute of Molecular Plant Biology"

August/September 2009: Bachelor Thesis "Expansion of virusspecific T-cells" at „St.Anna Children's Cancer Research Institute"

August 2010: Internship at "St.Anna Children's Cancer Research Institute"

August/September 2011: Internship at "Institute of Pathology" (Charité, Berlin)

Since January 2012: Master Thesis "Purification of seed storage proteins from tree nuts and peanut and their range of cross-reactivity – implication for the nut allergic patient" at "Department of Pathophysiology and Allergy Research" (Medical University of Vienna)