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Impact of the food matrix on allergenic properties of purified hazelnut allergens

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Contents

List of abbreviations

AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue R-250
CD	Circular dichroism
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
DX	Dextran
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Foetal calf serum
FPLC	Fast performance liquid chromatography
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
IAA	Iodoacetamide
IEC	Ion exchange chromatography
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton (kg/mol)
LPS	Lipopolysaccharide
MALDI-MS	Matrix assisted laser desorption/ionization-mass spectrometry

MEM	Minimum essential medium
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute
ml	Millilitre (10^{-3} litre)
mM	Millimolar (10^{-3} mol/dm^3)
MR	Molar ratio
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
NBT	4-Nitro blue tetrazodium chloride
NC	Nitrocellulose
NHS	Normal human serum
nsLTP	Non-specific lipid transfer protein
OAS	Oral allergy syndrome
pI	Isoelectric point
rpm	Revolutions per minute (1/min)
PR	Pathogenesis-related
PVPP	Polyvinylpyrrolidone
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sIgE	Specific serum immunoglobulin E
TEMED	N,N,N,N-tetramethylethylenediamine
T_H cell	T helper cell
TF	Transferrin
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylene (20) sorbitan monolaureate
v/v	Volume per volume (ml/ml)
w/v	Weight per volume (g/ml)

1. Introduction

1.1. Hypersensitivity diseases

Hypersensitivity diseases are pathologic reactions that are caused by inappropriate activation of the immune system in response to self antigens or foreign antigens. Hypersensitivity reactions were originally categorized into four subtypes by Philip Gell and Robin Coombs in 1963. Immediate hypersensitivity (Type I) was described as disorder caused by excessive production of IgE antibodies, whereas Type II and Type III were classified as IgG or IgM mediated diseases and Type IV as abnormal reaction provoked by T-lymphocytes (Tab. 1.1).[1, 2]

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 (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 (neutrophils and macrophages) • Abnormalities in cellular functions, e.g. hormone receptor signaling
Immune complex mediated (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 (Type IV)	• $CD4^+$ T-cells (cytokine-mediated inflammation) • $CD8^+$ CTLs (T-cell mediated cytolysis)	• Recruitment and activation of leukocytes • Direct target cell killing, cytokine-mediated inflammation

Table 1.1.: Classification of hypersensitivity diseases according to the type of immune response and the mechanisms of cell and tissue damage. Modified from Abbas et al., Cellular and Molecular Immunology.[1]

In the clinical praxis hypersensitivity diseases are often mixtures of the four types depicted in Tab. 1.1., which are caused by complex interactions of the humoral as well as cell-mediated immune responses that are further influenced by multiple effector mechanisms.[1, 2]

1.1.1. Immediate hypersensitivity (Type I)

In the following we will concentrate on immediate hypersensitivity diseases. Even though everybody is in contact with environmental antigens, strong hypersensitivity reactions only develop in atopic individuals. Atopy has been defined as the genetic predisposition to produce IgE-antibodies against antigens from common environmental sources, e.g. pollen, house dust mites or certain food proteins.[1] Typical symptoms of immediate hypersensitivity include itching and swelling of mouth, tongue, lips and throat (OAS) as well as atopic dermatitis, rhinoconjunctivitis, asthma bronchiale and gastrointestinal disorders. In general, the mechanisms of immediate hypersensitivity, commonly denominated as allergies are divided into three phases: sensitization phase, immediate response and late-phase reaction (Fig. 1.1.).

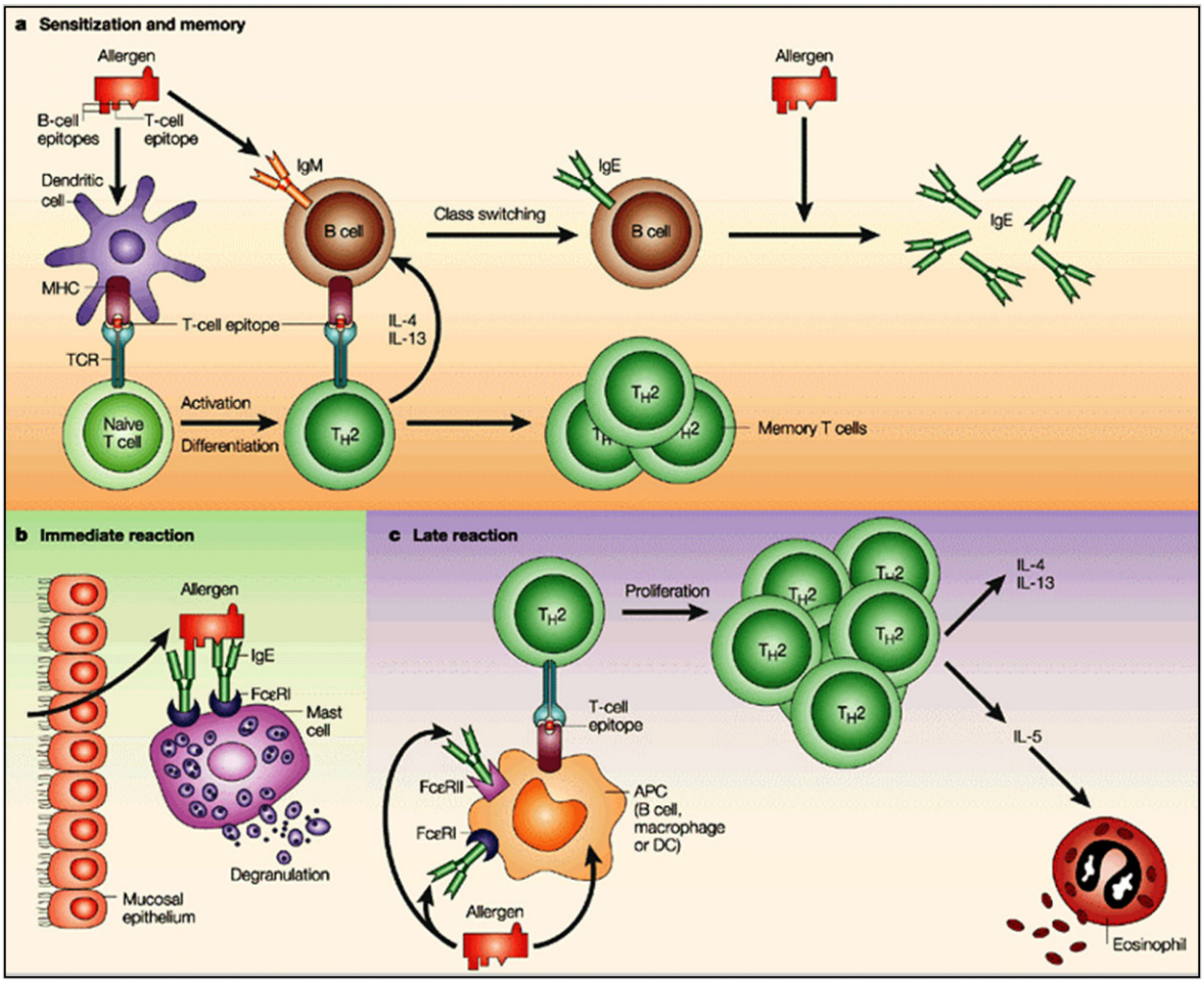


Figure 1.1.: Mechanisms of immediate hypersensitivity. a) Sensitization and memory, b) Immediate reaction, c) Late-phase reaction.[3]

Initial antigen contact occurs at mucosal surfaces of the respiratory tract or the GIT after inhalation or ingestion. Specialized antigen presenting cells (APCs), such as dendritic cells (DCs) are located in epithelia through which allergenic proteins enter. DCs and specific B-cells capture antigens at their immature state. DCs convert the proteins into peptide-MHC class II complexes and migrate to the draining lymph nodes, where they present the processed peptides to naïve $CD4^+$ T-cells. Followingly, the T-cells become activated and differentiate into specific T_H2-cells and follicular helper cells (T_{FH}). T_H2-cells and T_{FH}-cells stimulate B-cells through both, secreted cytokines (IL-4, IL-13) and interaction via the CD40 ligand on the cell surface. As a consequence, the B-cell undergoes heavy isotope switching from membrane bound IgM and IgD to secreted antigen-specific IgE. The hallmarks of allergy are activation of T_H2-cells and production of abnormally high serum IgE levels. Secreted IgE antibodies bind to high-affinity IgE-receptors (FcεRI) on the surface of mast cells and basophils (sensitization phase). The IgE-coated, sensitized mast cells are inactive, but ready to become activated upon repeated antigen stimulus.[1] The sensitization phase is characterized by establishment of IgE^+ memory B-cells and allergen-specific memory T-cells.[3]

Upon repeated contact, the allergen is recognized and hence boosts IgE^+ memory B-cells, which receive help from T-cells. Mast cells become activated by binding of multivalent antigens to IgE molecules that are attached to FcεRI receptors. Therefore, cross-linking of IgE must occur by at least two epitopes on the allergen surface. The following cell degranulation is accompanied by rapid release of inflammatory mediators from cytoplasmic granules into surrounding tissues (immediate response). These mediators include biogenic amines (e.g. histamines), lipid mediators (e.g. leukotrienes, prostaglandins, platelet-activating factor), cytokines (e.g. tumor necrosis factor, IL-1, IL-4, IL-5, IL-6, IL-13) and enzymes (e.g. tryptase), which are responsible for the pathologic reactions in immediate hypersensitivity. These cytokines are required for proliferation, differentiation and activity of mast cells, basophils and eosinophils. Immediate hypersensitivity reactions are induced within seconds or minutes after contact with extrinsic allergens and can trigger inflammation, vasodilatation, vascular leakage, edema, bronchoconstriction and intestinal hypermotility. When a sensitized individual is exposed to the specific antigen via intradermal injection or skin contact, a characteristic “wheal and flare reaction” appears within 5-10 minutes. This immediate hypersensitivity reaction is caused by the release of mediators,

notably histamine, from skin mast cells that cause dilatation of local blood vessels and leakage of plasma. After mast cell degranulation the inflammatory mediators are newly synthesized to allow repeated reaction upon antigen encounter.[1]

The immediate reaction is always followed by a slowly developing late-phase reaction due to the accumulation of inflammatory leukocytes (neutrophils, eosinophils, basophils), T_H2-cells and proinflammatory cytokines secreted by T_H2-cells (IL-4, IL-5, IL-13). IL-5 induces accumulation of tissue eosinophils and release of inflammatory mediators from eosinophils.[3] Symptoms usually appear between 2-24 hours after allergen contact. Repeated or constant allergen exposure often leads to chronic inflammatory processes that cause tissue damage and tissue remodeling.[1]

1.1.2. Aetiology of hypersensitivity reactions

Both, nonatopic and atopic individuals possess different portions of the three allergen-specific T-cell subsets: T_H1, T_H2 and T_{Reg} cells. It is known that the innate immune system of healthy individuals favours T_H1-cell response, which downregulates T_H2-mediated response. In contrast, the immune system of atopic persons induces T_H2-cell activation.[4] T-regulatory (T_{Reg}) cells are the dominant subset specific for common environmental antigens. They are considered as key regulator cells of immune responses as they suppress the undesired activity of effector T_H2-cells and thereby inhibit production of cytokines, such as IL-4, IL-5 and IL-13.[5] As can be seen from Fig. 1.2., T_{Reg} cell derived cytokines IL-10 and TGF-β inhibit the secretion of IgE-antibodies and promote production of non-inflammatory IgG4 and IgA. Furthermore, IL-10 and TGF-β suppress effector cells of allergic inflammation, including mast cells, basophils and eosinophils.[6] Although the aetiology of hypersensitivity reactions is not entirely clear until today, it is believed that an imbalance between allergen-specific T_{Reg} cells and T_H2-cells may contribute to development of allergic diseases.[7]

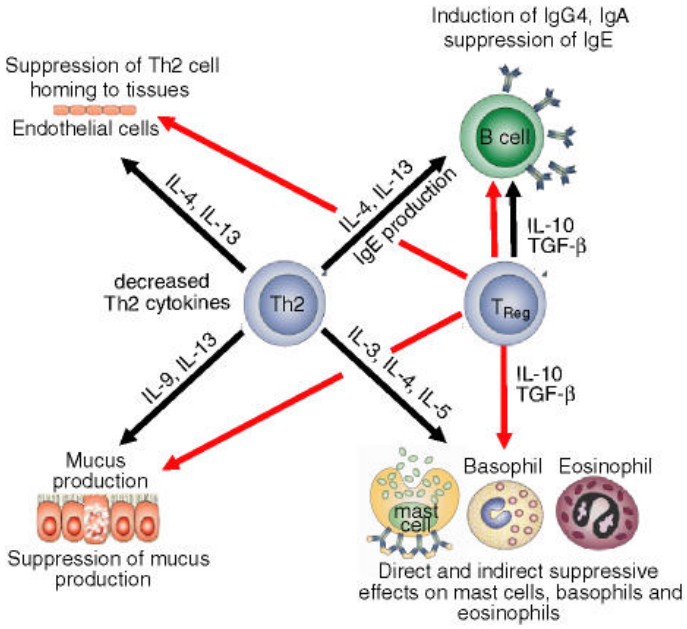


Figure 1.2.: T_{Reg} cells suppress development of allergic T_H2-cell response either via direct cell-cell contact with target cells or indirectly through action of secreted cytokines IL-10, TGF-β.[6]

The susceptibility to develop an allergy is strongly influenced by inherited genes. Genome-wide screens have been identified several chromosomal regions that may contain genes associated with high serum IgE levels, atopy and asthma. Some of these genes are thought to regulate T_H2 responses, others may have tissue-specific effects e.g. airway remodelling in the lung of patients suffering from asthma. Various studies reported that there is significant linkage between atopy and chromosomal loci 13q12-14 and 5q31-33.[8, 9] Another susceptibility locus for severe atopy was discovered on chromosome 11q13.[10] Another, but controversial theory for the development of allergic diseases is the so called “hygiene hypothesis".[11] This theory could provide a possible explanation for the increasing prevalence of allergic diseases in industrialised countries due to lifestyle changes. The “hygiene hypothesis" proposes that exposure to common environmental microorganisms in childhood plays an important role in the establishment of the immune system. In contrast, hygienic conditions and reduced exposure to microbial components are correlated with the risk to develop allergic diseases. Although the hygiene hypothesis could be a possible explanation for the increased incidence of allergies over the past decades, the complex mechanisms of genetic and environmental interactions have not been entirely uncovered until today. It is likely that additional unknown factors also contribute to the development of allergic disorders.[12]

1.2. Food allergy

According to the classification of the European Academy of Allergy and Clinical Immunology (EAACI), adverse reactions to food have been categorized depending on the underlying pathogenic mechanisms (Fig. 1.3.). As shown in the figure below, a distinction is made between toxic and non-toxic reactions. The latter are further divided into non-immune-mediated (food intolerance) and immune-mediated hypersensitivity (food allergy).[13]

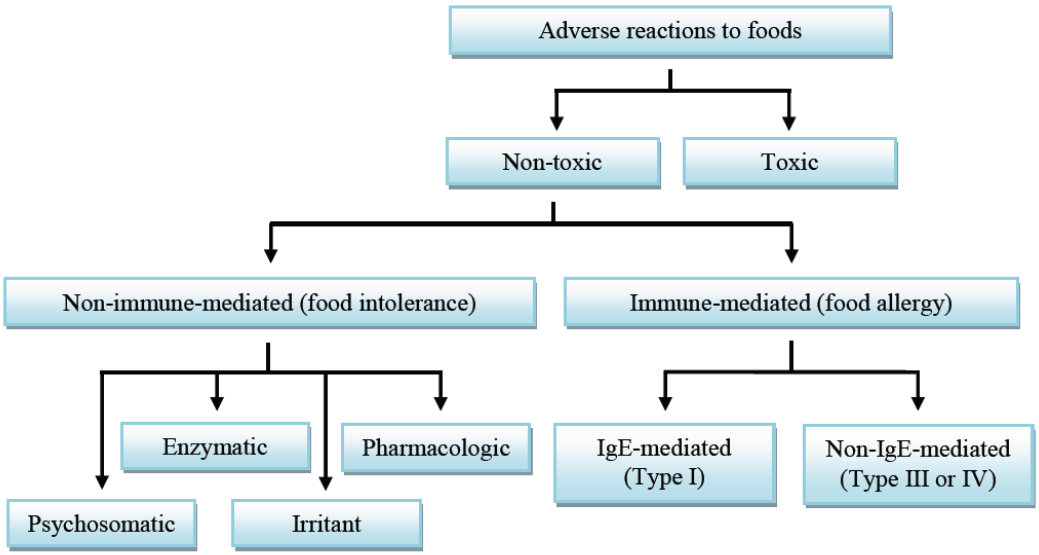


Figure 1.3.: Classification of adverse reactions to food according to the classification of EAACI. Modified from Bruijnzeel-Koomen et al. (1995): Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee.[13]

Non-immune-mediated reactions can be either caused by pharmacological reactions, enzymatic disorders or certain unclear irritant and psychosomatic reactions. On the contrary, immune-mediated hypersensitivity involves responses of the immune system and production of antigen specific IgE-antibodies. The typical IgE-mediated reactions correspond to Type I hypersensitivity, whereas non-IgE mediated constitute Type III or IV.[14] The differentiation between IgE-mediated and non-IgE-mediated disorders may be rather difficult, because symptoms often resemble each other.[15] It is assumed that food induced allergic disorders can either result from IgE-mediated hypersensitivity, non-IgE-mediated hypersensitivity or from a mixture of both types.[16]

The actual prevalence of food allergy is difficult to determine, because existing studies are often inconsistent or the criteria for diagnosis are not uniform. It is estimated that approximately 6-8% of young children and 1,5-2% of the adult population is affected by allergic disorders.[16] A recent study regarding the worldwide epidemiology revealed, that the prevalence of food-challenge confirmed food allergy is about 1% and that it is higher among children than adults.[17]

1.2.1. Food allergens

Various studies demonstrated that only members of a few protein families have the potential to sensitize predisposed individuals and to elicit allergic reactions upon second exposure to the antigen. Food allergies can be caused by a large numbers of foods. Major food allergen sources causing allergic reactions in adults are peanuts, tree nuts, fish and shellfish, whereas in children mainly milk, egg, peanuts, tree nuts and fish account for hypersensitivity reactions.[16] Typical features of allergenic food proteins are: low molecular weight, stability to thermal denaturation, resistance to acidic pH and digestive enzymes of the human gastrointestinal tract (GIT), glycosylation and high solubility in body fluids.[18] Due to their stable structure, food allergens retain their immunological reactive form during passage of the GIT. The GIT is one of the largest immunological organs of the human body, which is constantly exposed to a huge variety of ingested foods. Depending on their physicochemical properties, food allergens have the potential to trigger allergic reactions. When antigens come into contact with the intestinal surface of the GIT, they get absorbed by the mucosa and thus interact with cells of the immune system. At this point, sensitization can occur. The development of oral tolerance is an important protective mechanism in healthy individuals to suppress systemic humoral and cell-mediated immune response to innocuous food antigens. It is assumed, that a loss of oral tolerance, increased intestinal permeability and defects in T-cell activity could lead to the development of allergies.[19]

1.2.2. Classification of plant food allergens

In early studies plant proteins have been classified according to their solubility. The so called “Osborne fractionation”, developed by TB Osborne, is based on the solubility of extracted proteins in water (albumins), dilute salt solutions (globulins), alcohol/water mixtures (prolamins) and dilute alkali

(glutelins).[20] Nowadays, revised classification systems regarding the current knowledge of structural and functional properties of allergenic plant proteins are preferentially used.[21]

Food allergens comprise different food allergens from animals as well as plant food sources. Almost all animal food allergens belong to a restricted number of protein families: caseins, tropomyosins and EF-hand proteins, mainly parvalbumin. It has been shown that the ability of animal derived proteins to elicit allergic reactions depends on their evolutionary distance from the human proteome. There is evidence that proteins with a sequence identity to human homologous proteins higher than 62% are unlikely allergenic.[22] Plant food allergens have also been grouped into a small number of families and superfamilies based on their structural and functional characteristics. The majority of these allergens belong to the cupin superfamily or prolamin superfamily (see 1.2.3.). The cupin superfamily constitutes a number of proteins with conserved β-barrel structure, including vicilins and legumines. Furthermore, the pathogenesis-related (PR) proteins represent a heterogeneous group of 14 protein families to which many plant food allergens are homolog. PR proteins can be constitutively expressed in plants upon pathogen infection and other stress situations, such as wounding or unfavourable environmental conditions.[23, 24]

In general, two types of plant food allergens are known: class I food allergens or also referred to as “true food allergens and class 2 food allergens, which are often called “incomplete food allergens” or “nonsensitizing elicitors”. Class I food allergy develops by primary sensitization through the GIT and is mainly observed in young children. In contrast, in class 2 food allergy allergic reactions are induced as a consequence of sensitization to inhalant allergens, e.g. homologous pollen allergens, and affects predominantly adults.[25, 26] For example, patients that suffer from birch pollen allergy often also develop allergic symptoms after ingestion of certain plant-derived foods, such as apples, celery, carrots and hazelnuts. This can be explained by cross-reactivity. Cross reactivity arises due to the structural similarity between specific IgE-antibodies, e.g. of the major birch pollen allergen (Bet v 1) and homologous proteins which are present in foods. Pollen-related allergens are generally less stable against thermal and proteolytic treatment.[27] Several studies have shown that different phenotypes of food allergy can be distinguished by IgE-epitope mapping. Cross-reactive IgE-antibodies can be either directed to sequential epitopes or conformational epitopes. Sequential epitopes consist of linear arranged amino acids. On the contrary, conformational epitopes are comprised of amino acids that are located in close proximity to

each other in folded proteins. It has been discovered that the presence of specific IgE-antibodies in serum of allergic patients that predominantly bind to conformational epitopes is a predictive marker for transient allergy, whereas high occurrence of antibodies directed against linear epitopes are associated with persistent allergy as has been described for milk and egg allergens. Thus identification of IgE-binding epitopes may be a useful tool for diagnosis and prognosis (severity and persistence) of food allergy in the future.[28]

1.2.3. Food allergens of the prolamin superfamily

The prolamin superfamily is comprised of different allergenic seed storage proteins (2S albumins, non-specific lipid transfer proteins (nsLTPs), cereal α-amylase inhibitors and protease inhibitors) as well as non allergenic proteins (hybrid proline-rich proteins, cereal indulines, α-globulins).[29] 2S albumins, nsLTPs and cereal α-amylase/trypsin inhibitors share similar biochemical and physicochemical properties characteristic for allergenic proteins, such as high thermal stability and resistance to proteolysis. Furthermore, proteins belonging to the prolamin superfamily are low in molecular weight. They contain eight conserved cysteine residues and have a similar structure rich in α-helices.[18] Among those, several members of class 1 food allergens can be found. Particular characteristics of 2S albumins and nsLTPs will be outlined in the following chapters, as this study aims to investigate intrinsic characteristics of hazelnut 2S albumin, Cor a 14 and nsLTP, Cor a 8, as well as the impact of extrinsic factors on their allergenicity.

1.2.3.1. 2S albumins

2S albumin are a major group of seed storage proteins present in diverse dicotyledonus plants.[30] Representative allergens of the 2S albumin family are: Ana o 3 (cashew)[31], Ara h 2 (peanut), Car i 1 (pecan)[32], Ber e 1 (Brazil nut)[33], Cor a 14 (hazelnut)[34], Jug r 1 (English walnut)[35] and Ses i 6 (sesame)[36]. 2S albumins are low molecular weight proteins (12-15 kDa) rich in arginine, asparagine, cysteine and glutamine.[26] Their three dimensional structure consist of a bundle of α-helices, folded in a right-handed superhelix. All 2S albumins share a typical fold of four α-helices that are connected by flexible loops and held together by four disulfide bonds.[37] Within the plant, 2S albumin precursor peptides (18-21 kDa) are synthesized on the rough endoplasmic reticulum. Followingly the precursor is transported into the lumen

of the ER, where four intra-chain disulphide bonds form and the protein gets folded to its typical structure. After that, the protein is transported to the vacuole, where it gets proteolytically processed to a polypeptide of about 12-14 kDa. Usually the resulting peptide is post-translationally further processed into two subunits of approximately 3-4 kDa and 8-10 kDa.[30] The small and the large subunit remain linked via intermolecular bonds between the cysteine residues 1-5 and 2-3. In the large subunit, intramolecular disulfide bonds form between cysteine residues 4-7 and 6-8. As 2S albumins are encoded by a multigene family they show a high level of polymorphism resulting in various isoforms. Although proteins belonging to the 2S albumin family exhibit quite different primary structures, they share a conserved skeleton of eight cysteine residues and a similar pattern of four disulfide bridges. A typical post-translational modification of 2S albumins is the cleavage of C-terminal amino acids of both subunits, also called "C-terminal microheterogeneity". The hypervariable region between α-helix III-IV can adopt different conformations and was identified as an important immunodominant IgE-epitope in 2S albumins.[37] It has been hypothesized that the rare cross-reactivity among 2S albumins could be explained by the low sequence identity of the IgE-binding hypervariable regions.[38] Cross-reactivities have been reported for epitopes from peanuts and tree nuts, such as Car i 1 (pecan), Jug r 1 (English walnut) and Ana o 3 (cashew).[31, 32] Due to their compact structure, 2S albumins are very stable to heat treatment and highly resistant to acidic pH and digestive enzymes which are present in the human GIT. The fact that these proteins retain their immunologically active form during passage through the digestive system makes them potential food allergens.[37, 39]

1.2.3.2. Non-specific lipid transfer proteins (nsLTPs)

The non-specific lipid transfer proteins (nsLTPs) are extracellular proteins, mainly found in the outer epidermal tissues of plant organs.[30] Non-specific lipid transfer proteins were identified in a various number of fruits from the *Rosaceae* family as well as in seeds, vegetables and pollen.[40] Examples for nsLTPs are Ara h 9 (peanut)[41], Pru p 3 (peach)[42], Lac s 1 (lettuce)[43], Mal d 3 (apple)[44] and Zea m 14 (corn)[45]. Originally, nsLTPs were named after their potential to bind and transfer several lipid classes between vesicles and membranes. But the fact that they are accumulated in extracellular plant tissues lead to the assumption that nsLTPs are implicated in defence mechanisms. The presumption that nsLTPs play

a role in plant defence, by inhibiting the growth of bacteria and fungi upon pathogen infection could be confirmed.[40, 46] Therefore, nsLTPs were assigned to the family of PR-14 of pathogenesis related (PR) proteins.[47] Non-specific lipid transfer proteins are monomeric, low molecular weight proteins (7-9 kDa) with eight conserved cysteine residues. Their characteristic three dimensional structures are comprised of four α-helices connected by flexible loops, which are stabilised by four disulfide bonds. The bundle of helices builds up a central, hydrophobic, tunnel which is able to accommodate lipids.[40] Proteins belonging to the nsLTPs family are remarkable stable to thermal denaturation and highly resistant to the gastrointestinal environment. For this reason non-specific LTPs retain their immunological reactivity during digestion and enable them to sensitize predisposed individuals through the GIT.[48] Because of their high structural and sequence similarity, LTPs show a high degree of cross-reactivity with plant food allergens as well as with pollen allergens.[49, 50]

1.2.4. Hazelnut *(Corylus avellana)*

The common hazel (*Corylus avellana*) is native to Europe and western Asia. It is a member of the *Betulaceae* family including nut-bearing trees and shrubs, such as birches, alders and hornbeams. The hazel has been cultivated for its nuts, which can be eaten raw, roasted or otherwise processed in various food products. [51]

1.2.4.1. Hazelnut components

Tree nuts and peanuts are rich in total fat and unsaturated fatty acids. The consumption of nuts is associated with beneficial effects on health, like prevention of coronary heart disease, gallstones and diabetes.[51] The chemical composition of hazelnuts varies depending on geographical and environmental conditions. Hazelnut seeds are rich in proteins and lipids, vitamins (vitamin E, vitamin B1, vitamin B2, vitamin B6), amino acids (glutaminc acid, arginine, aspartic acid) and minerals (potassium, phosphorus, calcium, magnesium, selenium). The content of triaglycerides can be up to 64%, mainly composed of mono- and polyunsaturated fatty acids with oleic acid and linoleic acid as predominant fatty acids.[52, 53]

1.2.4.2. Hazelnut allergy

Peanut and tree nut allergy (e.g. hazelnut, almond, Brazil nut, cashew, pecan) is an increasing cause of IgE-mediated severe reactions among children as well as adults.[54-56] Allergic reactions after ingestion of nuts can be manifested in a wide spectrum of symptoms, varying from mild oral allergy syndrome (OAS), urticaria, angioedema, gastrointestinal anaphylaxis or systemic anaphylaxis. The individual sensitization profile of the patient and geographic characteristics can be associated with the severity of symptoms.[57]

Across Europe, the prevalence of hazelnut allergy is estimated about 0.2% among children and approximately 4.5% among adults living in birch-endemic regions.[58, 59] Different sensitization patterns were reported between hazelnut allergic patients in Northern and Southern Europe. Two kinds of hazelnut allergies, the pollen-related and the pollen-unrelated hazelnut allergy have been described.[57] The pollen-related hazelnut allergy is caused by primary sensitization to a homologous birch (*Betula verrucosa*) pollen allergen and thus is mostly found in birch-endemic regions of Northern Europe. It is usually associated with mild OAS after consumption of hazelnuts due to the IgE-cross-reactivity of Cor a 1.04 (Tab. 1.2.) with the birch pollen allergen Bet v 1.[57, 60] Pollen-unrelated hazelnut allergy arises from sensitization to allergenic food proteins through the GIT and often elicits severe, life-threatening systemic reactions. This type of hazelnut allergy is predominantly observed in patients residing in the Mediterranean area. Allergens associated with primary allergy to hazelnut are Cor a 8 (nsLTP), Cor a 9 (globulin), Cor a 11 (vicilin) and Cor a 14 (2S albumin).[57, 61, 62]

1.2.4.3. Hazelnut allergens

So far, the major hazel pollen allergen Cor a 1.01 (Tab. 1.2.) and four different variants of hazelnuts allergen Cor a 1.04 (Cor a 1.0401, Cor a 1.0402, Cor a 1.0403, Cor a 1.0404) have been identified. Cor a 1 is also assigned to the family of pathogenesis-related proteins PR-10.[63, 64] The allergenicity of Cor a 1 can be reduced by autoclaving and roasting[65, 66], as well as by simulated gastrointestinal digestion with pepsin and trypsin.[67] Another heat-labile and protease-labile allergen from hazelnut is the profilin Cor a 2 (Tab. 1.2.), which is associated with mild OAS. Cor a 2 is homologous to the birch pollen profilin,

Bet v 2, and has been described as a minor allergen in Central and North Europe.[65, 68] The hazelnut nsLTP, Cor a 8, (Tab. 1.2.) belongs to the prolamin superfamily that represents a widespread class of

panallergens (see 1.2.3.2.).[69] As aforementioned, nsLTPs are correlated with the pollen-unrelated hazelnut allergy and is frequently observed in patients living in the Mediterranean area.[62, 70] Due to the compact protein structure of four tightly packed α-helices that are stabilised by four disulfide bonds, Cor a 8 is highly resistant to heat treatment and proteolytic digestion. Thus, Cor a 8 retains its immunological active fold in the gastrointestinal environment and has the potential to induce life-threatening, systemic reactions in allergic patients.[71, 72] Allergenic nsLTPs have been identified in many fruits of the *Rosaceae* family (e.g. peach, apricot, apple, cherry) as well as in other plant derived foods, such as nut kernels. Patients allergic to the nsLTP in peach are often also affected by allergy to hazelnuts. *In vitro* experiments showed that the nsLTP from peach Pru p 3, initiates sensitization to the homologous nsLTP in hazelnut Cor a 8. It was shown that the T-cell reactivity to the hazelnut nsLTP is based on the cross-reactivity with Pru p 3, as Cor a 8 is rapidly degraded during allergen processing and lacks major T-cell activating regions.[73] Other allergenic proteins identified in hazelnuts are the 11S globulin, Cor a 9[74] and the 7S vicilin, Cor a 11, which exhibits a glycosylation pattern common for major allergens (Tab. 1.2.).[75] Moreover hazelnuts contain different isoforms of oleosins, including Cor a 12 and Cor a 13.[76] A further important protein involved in severe allergic reactions is Cor a 14 the 2S albumin from hazelnut (see 1.2.3.1.).[34] It is assumed that the compact protein structure of Cor a 14, comprised of four α-helices that are connected via flexible loops and held together by four disulfide bridges, contribute to its allergenicity. It has been demonstrated that the structure of Cor a 14 could not be degraded by heat treatment or proteolysis. There is evidence that sensitization to both allergens, Cor a 14 and Cor a 9, is associated with severe hazelnut allergy.[61] Cor a 14 shares common IgE-binding epitopes with allergens from peanuts and other tree nuts, which may account for the cross-reactivity among them.[31, 32, 77]

Table 1.2.: Hazelnut (Corylus avellana) allergens according to the International Union of Immunological Societies (www.allergen.org).

Allergen	Biochemical name	MW (kDa)	pI	Accession No. (UniProt)
Cor a 1.04	Pathogenesis-related protein, PR-10	17	6.2	Q9SWR4
Cor a 2	Profilin	14	4.9	Q9AXH5
Cor a 8	Non-specific lipid transfer protein 1 (nsLTP)	9	9.3	Q9ATH2
Cor a 9	11S globulin	59	6.5	Q8W1C2
Cor a 11	7S vicilin	48	6.1	Q8S4P9
Cor a 12	Oleosin	17	10.5	Q84T21
Cor a 13	Oleosin	14-16	9.9	Q84T91
Cor a 14	2S albumin	15-16	5.6	D0PWG2

1.3. Diagnosis and therapy of food allergy

Diagnosis of food allergy is based on a detailed anamnesis in conjunction with different *in vivo* and *in vitro* tests. Firstly, investigation of the individual family history and personal case history is obligatory to give an overall picture of the patient's state of health. It must be considered that multiple factors, including the genetic predisposition (atopy), physiological condition, age, type of suspicious food allergen and environment, have an influence on the severity of allergic reactions.[78] Followingly, different diagnostic *in vitro* and *in vivo* screening tests are applied to provide evidence for specific serum IgE-antibodies (sIgE).[79] Skin prick testing (SPT) is usually the first recommended *in vivo* method when an IgE-mediated food allergy is suspected, as it only provokes an immediate hypersensitive reaction confined to the skin. For SPT, the skin is pricked with a lancet before drops of allergen extract are applied. The result is considered positive when a wheal and flare reaction develops within 15 minutes at locations where a specific extract was introduced into the skin. The outcome is always checked against the positive control (histamine) and negative control (NaCl solution). It must be considered that a positive outcome in SPT only indicates sensitization to the suspected food, but does not confirm clinical allergy.[80] Diagnostic methods further include the detection and quantification of food-specific IgE-antibodies by *in vitro* blood tests, such as ImmunoCAP®[81] and ImmunoCAP®-ISAC (Immuno-solid allergen CHIP). However, standardized skin tests and sIgE tests have the disadvantage that they could give false-positive

results due to cross-reactivity between homologous allergens.[82] Another disadvantage of *in vitro* tests and SPT is that the used allergen extracts often show distinct variability in their composition and thus often lead to either false-negative or false-positive results.[83] None of the above mentioned methods can distinguish between food allergy and simply sensitization. A novel diagnostic *in vitro* test is component-resolved diagnosis (CRD).[84, 85] CRD is used to determine individual sensitization patterns of patients to purified or recombinant allergens and correlation with the severity of clinical symptoms.[86, 87] Although component-resolved diagnosis helps to overcome the problem of batch to batch differences between total extracts, the problem of cross-reactivity persist. Moreover, this diagnostic test is incapable of distinguishing between sensitization and allergy. Thus careful interpretation of the test results is mandatory. The insufficient diagnostic performance of available tests requires confirmation of a suspected diagnosis by double-blind placebo-controlled food challenge (DBPCFC).[88] Although this *in vivo* method is the most specific, it is a rather risky diagnostic tool. During DBPCFC, increasing doses of the food or masked placebo are given to the patient in emergency units under medical supervision. The challenge is considered positive when symptoms appear in association with the suspected food.

Patients with documented IgE-mediated allergy need to avoid ingestion of the allergenic food as well as eating any kind of processed food products containing these allergens. Furthermore it is important that people who develop life threatening reactions must be educated in recognition of early symptoms and adequate training in intramuscular adrenaline (epinephrine) injection by using an autoinjector. Persons who experienced an allergic reaction should immediately receive additional medical treatment in the hospital.[89]

There is no approved therapy to cure food allergies, but various allergen-nonspecific and allergen-specific strategies are currently under investigation.[90] The latter one involves continuous exposure to increasing allergen doses in order to induce desensitization or tolerance induction. Allergen-specific treatments include subcutaneous immunotherapy (SCIT), oral immunotherapy (OIT) and sublingual immunotherapy (SLIT). Several studies confirmed a significant increase in tolerance induction due to immunotherapy. However, clinical trials are needed to evaluate safety, efficacy and long-term effects of these novel therapies.[91, 92]

1.4. Protein-food matrix interactions

The presence of different food components may have a crucial effect on the allergenicity of food proteins. It is not known why some foods sensitizing via the gastrointestinal tract are prevalent allergenic foods and others are not. The surface of the human intestinal mucosa constitutes a selectively permeable barrier that prevents penetration of pathogens and foreign antigens and allows nutrient absorption of ingested foods as well as waste secretion.[93] An important function of the single layered intestinal epithelium is the separation of the external environment from the mucosal immune system located in the underlying gut-associated lymphoid tissue (GALT). Loss of the mucosal barrier function can lead to the development of several diseases.[93]

Class I food allergens are thought to sensitize via the oral route due to their high resistance to acidic pH and digestive enzymes in the human gastrointestinal tract. Allergens retain their immunoreactive protein structure during passage of the GIT and reach the intestinal mucosa in an intact form where they get absorbed. Hence, the proteins come in contact with components of the gut immune system in the GALT and may sensitize predisposed individuals or elicit an allergic reaction.[94] A number of food allergens were shown to possess highly stable protein structures, for example Pru p 3, the lipid transfer protein in peach, which has been described as primary sensitizer for the LTP family.[73] An i*n vitro* study demonstrated that Pru p 3 mostly retained its three-dimensional structure after simulated gastrointestinal digestion and was even able to induce production of T_H2 cytokines, typical for allergic reactions. For this reason, the stability of food allergens is brought into connection with their high allergenic capacity.[95] Many proteins belonging to the 2S albumin family are also highly resistant to the harsh conditions within the human GIT. An *in vitro* simulated gastrointestinal digestion study using Ber e 1, the 2S albumin from Brazil nut, revealed that the core protein structure was hardly affected even after extensive proteolysis. B-cell and T-cell binding epitopes on the protein surface remained unaffected, allowing the protein to maintain its allergenic activity.[39] Furthermore, the transport of purified native and digested allergens from the apical to the basolateral side of human intestinal epithelial Caco-2 cell monolayers was studied. The results demonstrated that both proteins Ber e 1 and Ses i 1, were transported to the basolateral side of Caco-2 cell monolayers in a similar way before and after *in vitro* gastrointestinal digestion, indicating their adverse effect for food allergic patients.[96]

However, little is known about the role of protein-food matrix interactions and how dietary components influence the immune reaction in response to a certain allergen. In addition to intrinsic factors determining the allergenicity of potential allergens, also extrinsic factors may contribute decisively to the development of allergic responses. In the last years there was increasing interest in defining the effect of dietary components present within the food matrix, including lipids, carbohydrates and other proteins on allergic response to a certain allergen.

Dietary lipids are present in the food matrix of diverse foods, for example as oily texture in nuts. In mammals the digestion of fats, starts when lingual and gastric enzymes are mixed together with ingested food components in the stomach. Lipids become mechanically emulsified by peristaltic movements of the stomach. Pancreatic enzymes and bile, which is produced by the liver, are added to the food pulp when it enters the duodenum. These digestive enzymes additionally help to break down dietary lipids making them water-soluble.

Besides dietary fats that are ingested with various foods, also bile provides lipids, e.g. phospholipids, unesterfied cholesterol and bile acids as mentioned above. At this point, protein-lipid complexes can be generated. Fatty acids are absorbed by membrane-associated fatty-acid binding proteins located on the apical site of enterocytes in the small intestine. These fatty acid transporter proteins enable the transfer of nutrients across the intestinal cell epithelium to the blood.[97] Several studies indicated a protective effect of gastric phosphatidylcholine (PC) against proteolysis of food allergens in the GIT. An *in vitro* simulated digestion assay with grape nsLTPs revealed that the protein was highly resistant to simulated gastric as well as duodenal enzymatic digestion due to the presence of PC.[98] The protective effect of phosphatidylcholine for proteins has been further confirmed by other *in vitro* gastroduodenal digestion assays, for example with mammalian milk allergens α-lactalbumin and β-lactoglobulin.[99, 100]

High allergenic foods such as nuts and tree nuts are rich in nutrients, but they also contain a large amount of fatty compounds.[53] These may protect allergenic food proteins from the proteolytic attack in the gastrointestinal tract. It has been demonstrated that the presence of lipids prevents digestion of proteins and thus leads to enhanced protein stability which is associated with high allergenic activity. It has been speculated that certain lipids may have an immunomodulatory effect because they activate the immune system towards an enhanced T_H2-cell response. For instance, it has been reported that purified peanut

allergens had only low immune stimulating capacity in mice, whereas the presence of whole peanut extract lead to enhanced immune responses. These findings lead to the assumption that there must be an effect of the food matrix on immune responses that alters the allergenic activity of food proteins.[101] Another investigation demonstrated that the presence of Brazil nut lipids was obligatory to induce production of allergen-specific IgE and IgG antibodies in mice. In contrast, intradermal injection of Ber e 1 alone was not sufficient to elicit an immune response to Ber e 1.[102] A further study regarding the development of Brazil nut allergy also depicted the importance of lipids for allergic sensitization. One specific lipid fraction, containing neutral and common Brazil nut derived phospholipids, was able to induce Ber e 1 specific immunoglobulin E, when it was mixed with Ber e 1. These findings suggested that the lipid fraction isolated from Brazil nut contributes an adjuvant activity to Ber e 1 sensitization.[103] In addition, also bacterial lipopolysaccharide (LPS) and other microbial products, which can be often found as contaminants in foods, are known to have a significant influence on immune responses elicited by proteins.[104]

Many plant protein families, like the prolamin superfamily (e.g. nsLTPs, 2S albumins) include important lipid binding proteins. This raises the question how proteins and lipids interact. It has been shown that protein-lipid complexes can occur naturally, for example in oil bodies of seeds, but they can also form during storage and food processing as a result of heating, mixing or homogenization.[105, 106]

It is possible that protein-lipid complexes are generated by electrostatic interactions, hydrophobic binding sites for lipid ligands near the protein surface and via hydrophobic cavities. Non-specific lipid transfer proteins (nsLTPs) possess a characteristic tunnel-like hydrophobic cavity that is able to bind lipid ligands.[40, 107, 108] Because the structure of 2S albumins is similar to that of nsLTPs, it is possible that they may associate with lipids in a similar manner. It has been shown that Ber e 1 binds lipids in a stoichiometry of 1:1, which lead to the hypothesis that Ber e 1 could accommodate lipids in its central hydrophobic cavity.[103] One example for a lipid binding protein belonging to the family of 2S albumins, is SFA8 from sunflower, a protein with good emulsifying properties. A study on SFA8 revealed that it is able to adsorb to the oil/water interface and thus stabilizes oil-in-water emulsions. It has been presumed that the emulsifying properties of SFA8 are due to hydrophobic patches on the protein surface, which may bind lipids. Furthermore, it could be shown that a single tryptophan residue of the protein becomes

located in the oil-phase, suggesting a protein-lipid interaction.[109] Another example for a lipid-binding protein is Sin a 1, the major allergen in mustard seeds, which forms protein-lipid complexes with acid phospholipid vesicles.[110]

1.5. Objective of the study

The respective food matrix must be taken into account when the allergenic activity of a specific allergen is determined. The objective of the present study is to investigate the allergenicity of purified hazelnut allergens with and without nut derived lipids. In the first set of experiments two important hazelnut allergens, the non-specific lipid transfer protein, Cor a 8, and the 2S albumin, Cor a 14, are obtained by means of protein purification from raw nuts. Final batches of purified proteins are characterized by standardized physicochemical and immunological methods. Therefore various parameters, including purity, authenticity, correct folding, IgE-reactivity and allergenic activity are assessed. After having established these methods, the protective effect of hazelnut derived lipids should be evaluated by *in vitro* cellular assays, including rat basophil histamine release assays and protein uptake experiments with dendritic cells. In this study we intend to provide insights on the impact of lipids on the allergic activity of purified food proteins. Results obtained by this study should contribute to a better understanding of protein-food matrix interactions.

2. Material and methods

2.1. Material

2.1.1. Chemicals and reagents

Table 2.1.: List of chemicals and reagents

Chemicals and Reagents	Supplier
4-Nitro blue tetrazodium chloride (NBT) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) Cyclohexylaminopropanesulfonic acid (CAPS) Dithiothreitol (DTT) Tris(hydroxymethyl)aminomethane (TRIS)	Biomol GmbH, Hamburg, Germany
Acrylamide (C_3H_5NO) Bis-Acrylamide ($C_7H_{10}N_2O_2$) Bovine Serum Albumin (BSA) Coomassie Brilliant Blue R-250 (CBB) Ethylene diamine tetraacetic acid (EDTA) Glycerol ($C_3H_8O_3$) Glycine ($C_2H_5NO_2$) Milk powder N,N,N,N-tetramethylethylenediamine (TEMED) Sodium acetate ($C_2H_3NaO_2$) Sodiumdodecylsulfate (SDS)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
C_Y3 Mono-Reactive dye Percoll	GE Healthcare, Little Chalfont, UK
EndoZyme® recombinant Factor C (rFC) Assay	Hyglos GmbH, Bernried, Germany
Hygromycin B	Invitrogen, Carlsbad, CA, USA
AlexaFluor®488 TFP Dye Ammonium persulfate ($(NH_4)_2S_2O_8$) Dulbecco's phosphate-buffered saline (1x) (DPBS) Foetal Calf Serum (FCS) GlutaMAX-I L-glutamine Minimum essential medium (1x) (MEM) Trypan Blue	Life Technologies, Carlsbad, CA, USA
2-(N-morpholino)ethanesulfonic acid (MES) Acetone (C_3H_6O)	Merck, KGaA, Darmstadt, Germany

Bromophenolblue Dimethylsulfoxide (DMSO) Disodim hydrogenphosphate dihydrate (Na_2HPO_4 x $2H_2O$) Geneticin sulphate (G-418) Iodoacetamide (IAA) Isopropanol (C_3H_8O) Magnesium chloride hexahydrate ($MgCl_2$ x $6H_2O$) Methanol (CH_3OH) n-Hexane (C_6H_{14}) Potassium chloride (KCl) Potassium dihydrogen phosphate (KH_2PO_4) Sodium bicarbonate ($NaHCO_3$) Sodium dihydrogenphosphate monohydrate (NaH_2PO_4 x H_2O) Sodium chloride (NaCl) Sodium hydrogen carbonate ($NaHCO_3$) Sodium hydroxide (NaOH) Sodium azide (NaN_3) Thiourea (CH_4N_2S) Urea (CH_4N_2O)	
4-Methylumbelliferyl-beta-D-glucuronide (4-MUG) Citric acid ($C_6H_8O_7$) Deuterium Oxide (D_2O) Hydrochloric acid (HCl) Penicillin-Streptomycin Polyvinylpyrrolidone (PVPP) Ponceau S SIGMAFAST™ p-Nitrophenyl phosphate tablets Triton X-100 Polyoxyethylene (20) sorbitan monolaureate (Tween-20) Tyrode's salt	Sigma-Aldrich, St. Louis, MO, USA
Page Ruler™ Plus Prestained Protein Ladder	Thermo Fisher Scientific, Waltham, MA, USA
Acetic acid (CH_3COOH) Ethanol (C_2H_6O)	VWR International, Darmstadt, Germany

2.1.2. Patient's sera

Serum samples were obtained from fourteen allergic patients with reported symptoms to hazelnut (Tab. 2.2.). Out of these, eleven were also allergic to peanut, seven to almond, four to walnut, three to soy, one to fish, one to orange and one to peach. In addition all persons had symptoms to different inhalant-allergen sources, including pollen from birch, grass and ambrosia, mould, house dust mites, animal dander, such as cat and dog. Allergen-specific IgE values for Cor a 1.0402, Cor a 8 and Cor a 9 were determined by ImmunoCAP®-ISAC microarray (Thermo Fisher Scientific, Waltham, MA, USA). Eight patients showed positive IgE values for Cor a 1.0401, nine for Cor a 8 and two for Cor a 9. Cor a 14 was not tested by the ISAC array. Cor a 14 specific IgE was determined by ELISA as described in chapter 2.2.8.1. In addition, sera from non-allergic subjects (NHS) were used for control experiments.

Table 2.2.: List of human sera (n=14) from hazelnut allergic patients indicating age, sex, total IgE values, allergen-specific IgE values, as well as detailed information about allergies to foods and symptoms to inhalant allergens.

Patient	Serum	Total IgE (kU/l)	Food allergies, e.g nuts, fruits	Allergies to inhalant allergen sources	Allergen-specific IgE (kU/l)		
					Co r a 1.0401	Co r a 8	Co r a 9
1	CR	4,357	hazelnut, walnut, peanut, almond	grass, house dust mites	0	0.4	0
2	CE-P-2	669	hazelnut, almond, peach, peanut	grass, birch	0	11.9	0
3	CE-P-9	234	hazelnut, peanut	grass, birch, ambrosia	0	0.3	0
4	EMV KM	340	hazelnut, peanut	house dust mites, animal dander	0.3	3.1	2
5	EMV DT	>5,000	hazelnut, walnut, peanut, almond	birch, grass	127.2	0	0.4
6	CH-P-43	640	hazelnut, peanut, almond	cat	0	1.3	0
7	CH-P-52	694	hazelnut, peanut	birch, ambrosia	3.4	0	0
8	CH-P-53	67	Hazelnut, walnut, almond	grass	0	0	0
9	CH-P-54	-	hazelnut, peanut	grass, cat, dog	1.3	0	0
10	CH-P-55	164	hazelnut, peanut, soy	grass, birch	30.4	48.7	0
11	CH-P-56	205	hazelnut, walnut, almond, orange	birch, cat, house dust mites	2.1	1.1	0
12	CH-P-57	669	hazelnut, peanut soy, almond, fish	grass, moulds, cat, house dust mites	1.1	3.9	0
13	CH-P-58	277	hazelnut, peanut, soy	house dust mites, grass, birch	26.7	0	0
14	CH-P-59	143	hazelnut	moulds, house dust mites	0	0.4	0

2.2. Methods

2.2.1. Purification of proteins and oil from hazelnuts

2.2.1.1. Preparation of total protein extract from hazelnuts

Shelled, raw hazelnuts (100 g, Beach Flower Back Mix, Turkey) were frozen and ground in a blender. Nuts were defatted twice by stirring them in four volumes (w/v) of n-hexane for 45 min at RT. After centrifugation (4,000 rpm, 15 min, RT) the defatted nuts were air dried over night. Dried hazelnut flour was weight (72 g) and extracted with ten volumes of 20 mM sodium acetate, pH 4.5, containing 0.5 M NaCl and 3% PVPP by stirring it for 3 hours at RT. The mixture was centrifuged at 14,000 rpm for 45 min at 4 °C and the supernatant was cleared by filtration. The protein concentration of the total extract was determined by BCA assay as described in chapter 2.2.6.1. Aliquots were stored at -20 °C

2.2.1.2. Extraction of hazelnut oil

Hazelnut oil was extracted with n-hexane as described in chapter 2.2.1.1. The obtained extract was air dried at RT in order to evaporate the organic solvent. The weight of hazelnut oil was gravimetrically determined and aliquots were stored at -20 °C.

2.2.1.3. Isolation of hazelnut prolamins from total protein extract

The total protein extract from section 2.2.1.1. was cooled down to 0 °C, before globulins were precipitated with cold methanol (60%, v/v). After centrifugation (14,000 rpm, 45 min, 4 °C), the pellet containing globulins was discarded and three volumes of cold acetone were added to the supernatant. The resulting mixture was incubated at -20 °C for 10 hours to precipitate prolamins. Precipitated prolamins were collected by centrifugation (14,000 rpm, 30 min, 4 °C) and the pellet was dissolved in 50 ml MilliQ water. Dialysis was performed with 1,000 Da MWCO dialysis tubes (Spectra/Por®, CA, USA) against water over night at 4 °C. After each precipitation step (methanol, acetone), as well as before and after dialysis, aliquots were taken and analysed by 15% SDS-PAGE. The extract containing prolamins was lyophilised and dissolved in 50 ml of 20 mM Tris/HCl, pH 7.5. The protein content of the prolamin extract was obtained by BCA assay (see 2.2.6.1.).

2.2.2. Ion exchange chromatography

Ion exchange chromatography (IEC) is a method for separation and purification of biomolecules, according to their net surface charge. A distinction is made between anion exchange and cation exchange chromatography. In anion exchange chromatography, negatively charged proteins bind to the positively charged column matrix, while in cation exchange chromatography, positively charged proteins bind to the negatively charged column matrix. Is the surrounding environment above the pI of the target protein, it will bind to an anion exchanger and if it is below the pI of the protein, it will bind to a cation exchanger. It must be considered that also the pH of used buffers influences the net surface charge of proteins. In general IEC is comprised of three steps: equilibration of the column, binding of the target protein to the column matrix and elution of the protein by increasing salt gradients.[111]

2.2.2.1. Anion exchange chromatography

Chromatographic separations were performed with a fast performance liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, UK). Protein extracts and buffers were cleared by filtration through a 0.2 µm membrane before application. Aliquots of the sample were loaded in compliance with individual binding capacities of the column matrix.

In a first step the prolamin extract from chapter 2.2.1.3., was separated by a Q Sepharose column (GE Healthcare, Little Chalfont, UK). The Q Sepharose column matrix is a strong anion exchanger, consisting of highly cross-linked agarose with attached quaternary amines as ion exchange group. The binding capacity of the column is 120 mg human serum albumin (HSA) per ml gel. The column was equilibrated with 20 mM Tris/HCl, pH 7.5 (buffer A), before aliquots of the sample were loaded at a flow rate of 1 ml/min. Unbound proteins were removed by washing with buffer A. The flow through, which was expected to contain Cor a 8, was kept for further protein purification (see 2.2.2.2.). Proteins bound to the column were eluted with linear, increasing salt concentrations (0-50%) of 1 M NaCl in buffer A (buffer B). Fractions of 1 ml were collected and absorbance of the protein elution was monitored at 280 nm. Loaded sample, flow through and fractions were analysed by 15% SDS-PAGE and Coomassie staining (see 2.2.7.1. and 2.2.7.3.). Protein enriched fractions, expected to contain Cor a 14, were pooled

according to their protein pattern and stored at 4 °C. Pools were denominated as Pool 1 and Pool 2 and were dialysed (MWCO 1,000, Spectra/Por®, CA USA) at 4 °C over night.

In a second purification step, Pool 1 and 2, which were expected to contain the 2S albumin Cor a 14, were further separated by a prepacked Mono Q column (GE Healthcare, Little Chalfont, UK). The column matrix is a strong anion exchanger consisting of a polystyrene/divinyl benzene matrix coupled with positively charged $-CH_2-N^+(CH_3)_3$ ion exchange groups and a binding capacity of 50 mg protein per ml column material. Pool 1, which contained proteins eluted with lower salt concentration (9-12%) of buffer B, was dialysed against 20 mM Tris/HCl, pH 8.0. Pool 2, which contained proteins eluted with higher salt concentration (12-20%) of buffer B, was dialysed against 20 mM Tris/HCl, pH 6.8. The separation of Pool 1 was carried out with buffer A, pH 8.0, whereas for Pool 2 the pH of buffer A was set to pH 6.8. All other steps were the same. The column was equilibrated with 20 mM Tris/HCl, pH 6.8/pH 8.0 and aliquots of the pools were applied with 1 ml/min. Unbound proteins were washed out with buffer A and proteins bound to the column were eluted with 1 M NaCl in buffer A (buffer B). Fractions were collected at a flow rate of 1 ml/min. Protein elution was carried out with linear gradients from 0-20% or 0-50% to test optimal separation conditions. Selected fractions were analysed by 15% SDS-PAGE and Coomassie staining and were pooled according to their protein patterns.

2.2.2.2. Cation exchange chromatography

The flow through from anion exchange chromatography (see 2.2.2.1.), which was expected to contain the non-specific lipid transfer protein Cor a 8, was purified by a prepacked Mono S column (GE Healthcare, Little Chalfont, UK). The column matrix is a strong cation exchanger consisting of a polystyrene/divinyl benzene matrix coupled with a negatively charged $-CH_2-SO_3^-$ ion exchange group and a binding capacity of 50 mg protein per ml column material. The pI of Cor a 8 is 9,3, therefore the sample was dialysed against 20 mM sodium acetate, pH 7.6. The column was equilibrated with 20 mM sodium acetate, pH 7.6 (buffer A) and the sample (70 ml) was applied in 10 ml aliquots at a flow rate of 1 ml/min. Unbound proteins were removed by washing the column with buffer A. Bound proteins were eluted with a linear gradient (0-20%) of 1 M NaCl in buffer A (buffer B) and 1ml fractions were collected. Fractions were analysed by 15% SDS-PAGE and Coomassie staining.

2.2.3. Gel filtration chromatography

Gel filtration chromatography is a widely used method to separate proteins isocratically, according to their size. As the column matrix consists of porous beads, proteins that are too large to diffuse into the pores migrate quickly through the gel and elute first. Molecules that are small enough to diffuse further into the porous particles are retarded and move through slower. Advantages of gel filtration are that proteins can be separated under mild conditions and that small molecules, like salts and other contaminants of low molecular weight, are removed from the sample.[111]

Gel filtration chromatography was performed with a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, Little Chalfont, UK). Pool 1.2, pH 8.0 and Pool 2.2, pH 6.8, which contained fractions obtained by Mono Q anion exchange chromatography (see 2.2.2.1.) were further separated by gel filtration. Previously, both samples were concentrated to the required column volume of 2 ml. Therefore Pool 2, pH 6.8 was dialysed against MilliQ water, lyophilised and dissolved in 2 ml running buffer containing 20 mM Tris/HCl, pH 8.0, 0.5 M NaCl. Pool 1, pH 8.0 was concentrated to 2 ml by centrifugation with 3 kDa MWCO filter units (Centricon®, Merck Millipore, Billerica, MA) at 2,000 rpm and 4 °C. The gel filtration column was equilibrated with running buffer, samples were applied at a flow rate of 1,3 ml/min and 2 ml fractions were collected. Selected fractions were analysed by 15% SDS-PAGE and Coomassie staining. Fractions containing proteins of lower molecular weight (10-20 kDa) were pooled, dialysed and the protein concentration was determined by the BCA method as described in 2.2.6.1.

2.2.4. Dialysis

Dialysis is an easy method for concentration of protein solutions, for changing the buffer or to remove salts from the sample. Molecules of different sizes are separated by selective diffusion through a semipermeable membrane with pores of a known size. The retention performance of the dialysis membrane is described by the molecular weight cut off (MWCO). By using a 1,000 MWCO membrane, molecules with molecular weights less than 1,000 Da and salts diffuse through the pores, while larger molecules are retained inside.[111]

The sample was transferred into 1,000 or 3,000 MWCO dialysis tubes (Spectra/Por®, Spectrum, Rancho Dominguez, CA). Tubes were sealed with closures and dialysed against selected buffer over night at 4 °C with constant stirring. In general, the ratio of sample to exchange buffer was 1:100 (v/v).

2.2.5. Lyophilisation

As proteins in aqueous solutions can suffer from precipitation, aggregation or other biological reactions over time, lyophilisation provides a useful technique to preserve proteins for long-time storage. During lyophilisation, also known as freeze-drying, the sample is frozen and aqueous solution is removed by sublimation under vacuum. The native structure and the biological activity of proteins are preserved.[112]
Aliquots of samples were transferred into falcon tubes, frozen in liquid nitrogen and stored at -80 °C over night until they were completely frozen. Afterwards the falcons were placed in the freeze dryer ALPHA 1-4 LSC (Christ, Osterode, Germany). Dehydrated protein preparations were resolved in water or buffer of choice.

2.2.6. Determination of protein concentrations

2.2.6.1. Bicinchoninic acid assay

The bicinchoninic acid assay (BCA assay) is a fast colorimetric method used to determine the unknown protein concentration of solutions. This technique is based on the reduction of Cu^{+2} to Cu^{+1} in an alkaline medium, combined with a colorimetric detection of the product. Upon addition of a reaction reagent, containing bicinchoninic acid, complexes of two molecules bicinchoninic acid and one cuprous ion form. These purple-coloured reaction products exhibit strong absorbance at 562 nm and can be measured.[113]
In order to determine the concentrations of purified proteins, the BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used. BSA standards (Tab. 2.3.) and different concentrations of the sample were prepared according to manufacturer's instructions. Standards and samples were pipetted in duplicates of 25 µl into a microplate (Greiner Bio-One GmbH, Germany). Working reagent was prepared with a ratio of 50:1 (v/v) of reagent A to reagent B and 200 µl were added to each well, respectively. The plate was incubated for 30 min at 37 °C and absorbance was measured in a microplate reader

(SpectraMax Plus, Molecular Devices, Sunnyvale, CA). Unknown protein concentrations were calculated by calibration against BSA standards.

Table 2.3.: BSA standards.

Standard	BSA [µl]	Buffer [µl]	Final concentration [µg/ml]
A	10	70	250
B	8	72	200
C	12	148	150
D	8	152	100
E	4	156	50
0	0	160	0

2.2.6.2. Nanodrop spectrophotometry

Another possibility to determine the concentration of a purified protein sample is with spectrophotometric measurement at 280 nm by using the NanoDrop reader (ND-1000, Thermo Fisher Scientific, Waltham, MA). The protein concentration is calculated with the measured absorbance value in combination with the molar extinction coefficient of the protein. A major advantage of this method is that no sample preparation is necessary and therefore protein concentrations can be obtained quickly. But it must be considered that Nandrop spectrophotometry is only applicable for proteins with known extinction coefficients.[114]

The spectrophotometer was cleaned and blanked with ddH_2O before 1 µl of protein sample was applied between the two fiber optic cables. Absorbance at 280 nm was measured and the protein concentration was calculated.

2.2.7. Physicochemical characterisation

2.2.7.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most frequently used methods to separate DNA, RNA and proteins according to their molecular weights. Proteins are denatured by the strong ionic detergent sodium dodecyl sulfate (SDS) and become negatively charged in proportion to their molecular weight. When denatured protein samples are loaded into the wells of a polyacrylamide gel and electric current is applied, the negatively charged proteins move toward the anode and are separated depending on their size. Glycine, which is also part of the sample buffer, is a

zwitterion that gets protonated at pH 6.8 in the stacking gel. Thus glycine molecules additionally push proteins towards the positively charged electrode. When glycine molecules reach the resolving gel with pH 8.8, they become negatively charged and stop. The proteins are compressed on top of the resolving gel and subsequently move further into the porous matrix. Small proteins migrate easily and faster through the cross-linked polymers of the resolving gel than larger ones. The pore size of the gel matrix is determined by the ratio between acrylamide and bis-acrylamide and is chosen based on the size of the target molecule. While higher percentage gels are suitable to separate smaller proteins, lower percentage gels are used for larger proteins.[111, 115]

The resolving gel was prepared by mixing Reagent C, Lower buffer, Upper buffer and ddH_2O according to Tab. 2.4. Polymerisation was started by adding APS and TEMED and the mixture was quickly poured between the 0.75 mm spacer plates of an assembled casting frame. It was covered with isopropanol to achieve a plain surface. Once polymerised, the isopropanol was removed, the stacking gel was added and a comb was inserted to make wells in the gel. Protein samples were prepared by adding 4 x sample buffer (SB) followed by heating at 95 °C for 5 min. For analysis of native proteins, nonreducing SB was used. Reduced protein samples were prepared with SB, containing dithiothreitol (DTT), in order to reduce inter- and intra- molecular disulphide bonds. Polymerised gels were placed in the electrophoresis chamber and equilibrated with 1x electrophoresis buffer. The comb was removed and samples were loaded according to a defined scheme. Prestained protein marker was applied to assess the progress of electrophoresis and to estimate protein sizes. The electrophoresis chamber was filled up with buffer and gels were run at 160 V for about 1 hour. Proteins were either visualised by Coomassie staining (see 2.2.7.3.) and transferred to polyvinylidene difluoride membrane (see 2.2.7.5.) or to nitrocellulose (see 2.2.8.2.). Not immediately used gels were stored at 4 °C.

Reagent C	**Lower buffer**	**Upper buffer**
29.2% acrylamide	1.5 M Tris/HCl, pH 8.8	0.5 M Tris/HCl, pH 6.8
0.8% bis-acrylamide	0.4% SDS	0.4% SDS
dissolved in ddH_2O	dissolved in ddH_2O	dissolved in ddH_2O

4x sample buffer	**10x electrophoresis buffer**
200 mM Tris/HCl, pH 6.8	250 mM Tris pure
300 mM DTT	192 mM glycine
4% SDS	1% SDS
40% glycerol	dissolved in ddH_2O
0.025% Bromophenolblue	
dissolved in ddH_2O	

Table 2.4.: Composition of resolving gels and stacking gels.

SDS-PAGE gels				**2D-SDS-PAGE gel**
Components	**Resolving gel**		**Stacking gel**	**Resolving gel**
	15 %	**18 %**	**4.5%**	**15%**
Reagent C	2.50 ml	3.00 ml	300 µl	6.75 ml
Lower buffer	1.25 ml	1.25 ml	-	3.38 ml
Upper buffer	-	-	500 µl	-
ddH_2O	1.25 ml	0.75 ml	1.2 ml	2.25 ml
10% APS	25 µl	25 µl	20 µl	67.5 µl
TEMED	2.5 µl	2.5 µl	1 µl	6.75 µl

2.2.7.2. 2D-Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-SDS-PAGE) is a sensitive method to analyse complex protein mixtures. Further it is a useful method to identify different isoforms of a protein in a sample. In the first dimension, proteins are separated according to their isoelectric point by isoelectric focusing on IPG polyacrylamide gel strips. In the second dimension the strips are transferred into slots of a polyacrylamide gel and the proteins are separated according to their weight by SDS-PAGE.[111]

Sample preparation: One volume protein (4 µg), was mixed with three volumes of cold 100% acetone. Proteins were precipitated at -20 °C for 1 h and collected by centrifugation (15,000 rpm, 30 min, 4 °C). The supernatant was discarded and the pellet was washed with 100 µl of 90% acetone without resuspending. Samples were centrifuged for 10 min, the supernatant was removed and the pellet was air dried at RT before it was resuspended in 125 µl IEF sample buffer and agitated for 10 min at RT. Proteins

were reduced and alkylated by adding 8 µl of 1 M DTT. For IEF polyacrylamide gel strips with immobilized pH gradients (pH 3-10 Immobiline™ DryStrip, linear pH gradient, GE Healthcare, Little Chalfont, UK) were used. The sample was pipetted into the middle of a strip holder and the strip was placed onto it. A layer of 400 µl mineral oil (Plus One, Dry Strip Cover Fluid, Amersham Biosciences, Uppsala, Sweden) was applied to avoid evaporation. The strip holder was placed in the apparatus (Ettan™ IPGphor™ II IEF System (Amersham Biosciences, Uppsala, Sweden) and rehydration was carried out for 16 hours, followed by 3 hours isoelectric focusing, up to 11,000 Vh at RT. After IEF, the mineral oil layer was removed and the strip was agitated for 15 min in 5 ml equilibration solution I, containing 325 µl of 1 M DTT to reduce disulfide bridges of the proteins. After that the strip was equilibrated for 15 min in 5 ml solution II, containing iodoacetamide for alkylation. Equilibrated strips and 3x3 mm pieces of Whatman paper, soaked with 3 µl protein marker were placed in the slot of a 1.5 mm, 15% SDS-polyacrylamide gel. The slot was sealed with a layer of melted agarose solution, containing 0.5% agarose and 0.002% bromophenolblue, dissolved in 1x electrophoresis buffer. Electrophoresis was started by applying low voltage (60 V) and was increased (160 V) until a blue front had formed. Gels were stained in Coomassie solution as described in 2.3.3.

IEF sample buffer	**Equilibration solution I**	**Equilibration solution II**
7 M Urea	50 mM Tris/HCl, pH 8.8	50 mM Tris/HCl, pH 8.8
2 M Thiourea	6 M Urea	6 M Urea
2% CHAPS	2% SDS	2% SDS
0.5% IPG buffer pH 3-10	30% glycerol (98%)	30% glycerol (98%)
0.002% Bromophenolblue	0.002% Bromophenolblue	0.002% Bromophenolblue
add H_2O up to 10 ml	add 65 mM DTT before use	25 mg/ml Iodoacetamide

2.2.7.3. Coomassie staining and destaining of SDS-PAGE gels

Coomassie dyes are used to visualize separated proteins after gel electrophoresis. This technique relies on the non-specific binding of Coomassie Brilliant Blue R-250 (CBB) to proteins in the gel. During staining, CBB forms complexes with aromatic amino groups of the proteins through ionic interactions between sulfonic groups of the dye and Van der Waals forces. Protein-dye complexes are fixed by acetic acid in the staining solution. As Coomassie Blue R-250 is a non-polar reagent, excess dye can be removed with destaining solution containing acetic acid and methanol.[111]

In order to visualize protein bands after gel electrophoresis, the polyacrylamide gel (0.75 mm) was transferred into a plastic dish. The gel was incubated in Coomassie staining solution for 30-45 min at RT and under constant shaking until it had a uniform blue colour. Staining times differ, depending on the thickness and acrylamide concentration of the gel. Afterwards the staining solution was removed and the gel was shortly rinsed in water. Destaining solution was added and the gel was incubated under shaking until the background was clear and protein bands were visible. Gels were stored in water. Staining and destaining of thicker gels (1.5 mm), was accelerated by application of heat (60 °C) in the water bath.

CBB staining solution	**Destaining solution**
0.125% Coomassie Blue R-250	50% methanol
50% methanol	15% acetic acid
10% acetic acid	dissolved in ddH_2O
dissolved in ddH_2O	

2.2.7.4. Mass spectrometry

Mass spectrometry provides a highly sensitive method to determine the molecular mass of proteins. It is a useful technique for protein identification as well as for characterisation of post-translational modifications. In mass spectrometry the sample is ionized and charged molecules are accelerated in an electric field. Proteins are separated according to their mass-to-charge ratio before they hit a detector. The recorded mass spectrum is unique for each protein and allows identification by correlation to theoretical masses or characteristic fragmentation patterns.[111, 116]

To confirm the identity of purified proteins, native as well as reduced and alkylated proteins were analysed by mass spectrometry. Purified Cor a 8 was reduced with 100 mM DTT for 10 min at 60 °C and alkylated with 200 mM IAA over night at RT. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using HCCA (α-Cyano-4-hydroxycinnamic acid) as matrix. The prepared protein sample was applied on the matrix plate in 0.5 µl spots and introduced into the mass spectrometer (Medical University of Vienna, Department of Pathophysiolgy and Allergy Research). Different batches of native Cor a 14 (Cor a 14.1, Cor a 14.2, Cor a 14.4, Cor a 14.5) were analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) peptide analysis at the VetCORE - Facility for Research lab, University of Veterinary Medicine, Vienna. MALDI-TOF MS are high sensitivity and a large mass range. Additionally, native proteins were digested with trypsin and resulting peptides were analysed. Obtained mass spectra were compared with known masses of the proteins and subunits.

2.2.7.5. N-terminal sequencing

N-terminal sequencing by Edman degradation is a method for identification of proteins by determination of their amino acid sequence. Moreover it can be useful to evaluate the N-terminal processing of a given protein. During Edman degradation, a series of automated chemical reactions proceeds. It starts with the modification of the amino acid at the free N-terminal end of the protein and cleavage of the modified group, followed by identification of the removed amino acid by high performance liquid chromatography (HPLC).[111]

Purified Cor a 8 was prepared for sequencing by using a ProSorb sample preparation cartridge (Applied Biosystems, Foster City, CA, USA). The Prosorb membrane was wet with 10 µl methanol before the sample was diluted to 100 µl with 0.1% trifluoroacetic acid (TFA) directly in the Prosorb vial. Afterwards the absorbent was inserted into the Prosorb device and the fluid was let pass through the membrane completely, followed by 100 µl of 0.1% TFA. The sample vial was removed from the device and the membrane with the adsorbed protein was air dried. The membrane was punched out, put into a microcentrifuge tube and subjected to sequencing. In order to sequence Cor a 14, the protein was separated by 15% SDS-PAGE under reducing conditions. Proteins were blotted to a polyvinylidene

difluoride (PVDF) membrane (see 2.2.8.2.) and stained with Coomassie Brilliant Blue. Protein bands were excised and N-terminal sequencing of the purified protein was performed with a Procise 491 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence information was compared with known sequences in the protein database (BLAST).

2.2.7.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a method to assess secondary structure, conformation and binding properties of a protein. It is a useful method to evaluate structural alterations resulting from changes in pH, temperature or other environmental conditions quickly. During CD spectroscopy left-handed as well as right-handed circularly polarized light is passed through optically active molecules. Depending on the structural characteristics of the molecule, polarized light is differently absorbed and scattered. The recorded circular dichroism spectrum is used to estimate the α-helix, β-sheet and random coil content of the protein.[111]

The secondary structure of purified Cor a 8 and different Cor a 14 batches (Cor a 14.1, Cor a 14.2, Cor a 14.4, Cor a 14.5) was assessed by circular dichroism. Samples were prepared in ddH_2O (2 mg/ml) and measured in quarz cuvettes with a path length of 1 mm at RT. Circular dichroism spectra were recorded on a J-810 spectropolarimeter (Jasco International Co., Hachioji, Tokyo) in a range of 190-260 nm. In order to obtain mean spectra, the data of three measurements were averaged. CD spectra were measured in millidegrees (mdeg) and were converted to mean molar ellipticity [θ].

2.2.8. Immunological characterisation

2.2.8.1. IgE-Enzyme-Linked Immmunosorbent Assay

The Enzyme-Linked Immunosorbent Assay (ELISA) is a biochemical method to detect antibodies or antigens in a sample. In direct ELISA, the antigen is immobilized on a solid phase and subsequently incubated with specific monoclonal or polyclonal primary antibodies. Stable antigen-antibody complexes can be detected by binding of a labelled secondary antibody. As secondary antibodies are coupled with enzyme, such as alkaline phosphatase (AP), the presence of antigen-specific antibodies can be determined

by addition of specific substrate solution. The coloured reaction product is proportional to the amount of antigen-antibody-complexes on the plate and is determined by photometric measurement.[117]

Purified proteins were diluted with 50 mM $NaHCO_3$ buffer, pH 9.6 and a 96 well microtitre plate (MaxiSorp Immunoplate, Roskilde, Denmark) was coated with 5 µg/ml protein by application of 100 µl per well. The plate was incubated at 37 °C for 1 hour or at 4 °C over night. The coating solution was discarded and the plate was washed with Tris-buffered saline (TBS, 0.5% (v/v) Tween-20 (TBST). Free, unspecific binding sites were blocked with 200 µl per well TBST, 3% BSA and incubation for 2 hours at RT. The blocking solution was removed and the plate was washed with TBST. Serum samples were diluted with TBST, 1% BSA and as secondary antibody AP-conjugated mouse anti-human IgE diluted 1:1000 (BD Biosciences, Franklin Lakes, NY, USA). Sera of non-allergic patients were used as controls. Finally, 100 µl serum was added to relevant wells and the plate was incubated at 4 °C over night. After washing the plate for ten times with TBST, 100 µl per well of prepared disodium p-nitrophenyl phosphate solution (Sigma-Aldrich, Steinheim, Germany) was applied. Absorption was measured at 405 nm at different time points.

2.2.8.2. Western blotting

Western blotting allows to detect specific proteins in a sample and to determine relative protein amounts. After separation of proteins by SDS-PAGE, proteins are transferred to a carrier membrane by electroblotting. The membrane is then incubated with primary antibodies e.g. from serum of allergic patients. To prevent unspecific binding, membranes are blocked and a secondary detection antibody specific for the primary antibody is added. The location of protein-antibody complexes are revealed by incubation with substrate solution.[111]

Native and reduced/alkylated proteins (30 µg) were separated by 15% SDS-PAGE on 1.5 mm gels. Nitrocellulose (NC) membrane, Whatman papers and the gel were equilibrated in 1x transfer buffer for 5 min. The Western blot sandwich was assembled in order from the bottom to the top (from anode to cathode) as follows: 2 pieces Whatman paper, NC membrane, gel, 2 pieces Whatman paper. Blotting was performed with the Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell (BioRad, Hercules, CA) for 45-60 min at 160 V. The protein ladder on the gel was used to monitor transfer efficiency. After protein

blotting the nitrocellulose membrane was stained in Ponceau S solution (0.1% Ponceau S, 5% acetic acid) to assess the blotting efficiency. The membrane was air dried and cut into 0.5 mm strips. To prevent unspecific binding, the membrane was blocked with 1x goldbuffer, 3% BSA for 3 hours. The strips were incubated in sera, diluted 1:10 or 1:20 with 1x goldbuffer, 1% BSA, over night at 4 °C under constant shaking. Sera were recovered and NC strips were 4 x 20 min washed in 1x goldbuffer. NC strips were then incubated with 1:20 diluted ^{125}I-labelled α-human IgE antibody (Demeditec Diagnostics Gmbh, Kiel, Germany) over night. Radioactive labelled strips were washed and air dried, before they were fixed on a sheet of paper and stored into an autoradiography cassette (Hypercassette, GE Healthcare, Little Chalfont, UK) together with an X-ray film. Films were exposed to radiation for 1-10 days at -80 °C.

1x Gold buffer

42 mM Na_2HPO_4 x $12H_2O$

8 mM NaH_2PO_4 x $2H_2O$

adjust to pH 7.5

0.05% NaN_3

0.5% Tween-20

10x Transfer buffer

250 mM Tris pure

192 mM glycine

20% methanol

Proteins can also be blotted to polyvinylidene difluoride (PVDF) membranes for N-terminal sequencing. Therefore 15% SDS-PAGE was performed as usual and the gel was incubated in CAPS 1x transfer buffer for 5 min. The PVDF membrane was shortly wet in methanol before it was equilibrated in transfer buffer for 5 min. Blotting was performed as described above. After blotting the membrane was 3x5 min rinsed in MilliQ water and incubated with CBB staining solution for 30 seconds. Destaining was carried out with several changes of destaining solution and rinsed in MilliQ water. The PVDF membrane was dried and bands were cut out.

CAPS transfer buffer 10x stock (1l)

22.1 g CAPS (100 mM)
adjust pH to 11.0 with NaOH

CAPS 1x transfer buffer (1l)

100 ml CAPS transfer buffer 10x stock (10%)

100 ml methanol (10%)

CBB staining solution for PVDF membrane	**Destaining solution**
0.1% Coomassie Blue R-250	50% methanol
40% methanol	1% acetic acid
1% acetic acid	dissolved in ddH_2O
dissolved in ddH_2O	

2.2.8.3. Rat basophil histamine release assay

IgE antibodies are produced in response to a specific antigen during type 1 hypersensitivity. These antibodies bind to high-affinity receptors (FcεRI) on the surface of mast cells and basophils. Upon repeated exposure to the same allergen, receptor bound IgE is cross-linked and degranulation of sensitized mast cells is induced. As a consequence inflammatory mediators like histamine, cytokines, leukotrienes and others are released. The mediator release assay is an in vitro model based on the cellular mechanisms of allergies. It is a useful tool to assess the cross-linking capacity of allergens and to determine their allergenic activity. In 1973 basophil leukemia cells were developed by inducing leukemia with β-chlorethylamine in rats. This cell line expressed the FcεRI receptor and displayed functions of mast cells, but was not suitable for degranulation studies using human IgE. Different sub-clones were developed until the histamine secreting RBL-2H3 clone was isolated. Nowadays, various RBL-2H3 derived cell lines are available.[118]

Mediator release assays were performed with RBL cells, RS-ATL8 expressing α, β and γ-chains of the human high-affinity IgE receptor (FcεRI). The biological activity of allergens mixed with hazelnut oil and allergens alone was investigated. Hazelnut oil-protein mixtures were prepared by adding hazelnut oil (10 mg/ml) at a ratio of 1:2 and sonication for 3x5 min. Cells sensitized with human IgE (1 mg/ml, Diatec AAS, Oslo, Norway) and cross-linked by using α-human IgE (10 mg/ml, Source BioScience, Nottingham, UK) served as positive control. Experiments were performed with a RS-ATL8 cell line, obtained from Ryosuke Nakamura, National Institute of Health Sciences, Tokyo, was used.[119, 120] Cells were cultured in MEM containing 10% heat-inactivated fetal bovine serum, 2 mM glutamax, 1% penicillin-streptomycin, 1 mg/ml geneticin and 0.2 mg/ml hygromycin B. RS-ATL8 cells in the stationary growth phase were harvested with a cell scraper in 10 ml PBS. Cells were counted by using a Bürker-Türk hemocytometer

and the cell number was adjusted to $5x10^4$ cells/ml, before 50 µl/well cell suspension was divided over a sterile Costar® 96-well, flat-bottom plate (Sigma Aldrich, St. Louis, Missouri, USA). RBL cells were sensitized with 50µl/well of diluted sera from hazelnut allergic patients over night at 37 °C, 5% CO_2. The cells were washed three times with Tyrode's wash buffer, before cell degranulation was induced by adding 50 µl/well of allergens diluted with allergen challenge buffer. Cells were incubated at 37 °C for 1 hour. RBL cells for total release control were lysed with 10 µl 10% Triton X-100 in ddH_2O. The plate was centrifuged with 1,200 rpm for 5 min at RT, 50 µl of supernatants was transferred into a new microtitre plate and mixed with 50 µl of 4-MUG-citrate buffer substrate solution. Incubation at 37 °C for 1 hour followed and the reaction was stopped by pipetting 100 µl of glycine buffer into each well. The concentration of β-hexosaminidase, which is also present in the granules of mast cells and is released during degranulation, was quantified by photometric measurements (Infinite® 200 PRO, Tecan) at 405 nm. The specific release was expressed as percentage of specific release, calculated by using the formula: $(OD_{\text{experimental release}} - OD_{\text{spontaneous release}}) / (OD_{\text{total release}} - OD_{\text{spontaneous release}}) \times 100$.

2x Tyrode's buffer

9.6 g Tyrode's salt

1 g $NaHCO_3$

500 ml ddH_2O

adjust pH to 7.2 with NaOH

add ddH_2O up to 1,000 ml

Tyrode's wash buffer

35 ml 2x Tyrode's buffer

35 ml ddH_2O

0.07 g BSA

Allergen challenge buffer

7.5 ml 2x Tyrode's buffer

7.5 ml D_2O

0.15 g BSA

Glycine buffer (stop solution)

15 g glycine

11.7 g NaCl

adjust pH to 10.7 with NaOH

add ddH_2O up to 1,000 ml

Citrate buffer (substrate solution)

1.05 g citric acid (0.1 M)

adjust pH to 4.5 with NaOH

add ddH_2O up to 50 ml

2.2.8.4. Protein uptake and surface marker expression experiments

Dendritic cells (DCs) are specialised antigen presenting cells. They initiate and regulate adaptive immune responses against pathogens and play a crucial role in the development of the immunologic memory. Dendritic cells capture antigens at their immature state and convert them into peptide-MHC class II complexes. These complexes are then presented on the cell surface in combination with costimulatory factors (e.g. CD80, CD86). Through interaction with naïve T-cells, immune responses are initiated and DCs produce various pathogen-specific cytokines, e.g. IL-12 or tumor-necrosis factor (TNF). In cell culture, DCs can be derived from human blood monocytes by incubation with maturation factors, such as granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4).[121]

2.2.8.4.1. Endotoxin detection assay

Maturation of dendritic cells could be activated by microbial or viral contamination with LPS, dsRNA or CpG DNA.[122] For this reason endotoxin concentrations of purified allergens as well as of allergens mixed with lipids were determined by using the EndoZyme® recombinant Factor C assay (Hyglos GmbH, Bernried, Germany) according to the manufacturer's instructions.

2.2.8.4.2. Protein labelling

Purified proteins (100 µg) were labelled with Alexa Fluor®488 TFP and Cy3 Mono-Reactive dye (BD, Franklin Lakes, NJ, USA). Therefore Cor a 8 and Cor a 14 were diluted in PBS up to a volume of 100 µl, mixed with 1/10 volume of 1 M sodium bicarbonate. After that the appropriate volume of dye solution, calculated by the formula ([(µg protein / protein MW) x 1,000] x MR) / (11.3), was added. Protein-dye mixtures were incubated for 1 hour at RT under constant agitation. Unbound dye was removed by extensive dialysis against PBS over night. The degree of labelling was determined by measuring the absorbance of conjugate solutions at 280 nm and A_{max} of the used dye (Alexa Fluor®488 TFP: A_{max} = 495 nm, Cy3 Mono-Reactive: A_{max} = 552 nm), respectively. Protein concentrations were calculated in mg/ml:

Alexa Fluor®488 TFP: [A_{280} - 0.11(A_{495})] x dilution factor) / (A_{280} of protein at 1 mg/ml)

Cy3 Mono-Reactive: ([A_{280} - 0.11(A_{552})] x dilution factor) / (A_{280} of protein at 1 mg/ml)

2.2.8.4.3. Isolation of peripheral blood mononuclear cells

In order to isolate peripheral blood mononuclear cells (PBMCs), 80-90 ml blood from non-allergic donors was collected in heparinised tubes. The blood was transferred into a sterile cell culture flask and diluted with cold HBSS to a total volume of 150 ml. Six falcon tubes, each containing 15 ml Ficoll were prepared and carefully overlayed with 25 ml of diluted blood. In order to separate the different cell types, density centrifugation (30 min, 1,000xg, no break, RT) was carried out. The interphase ring containing PBMCs was collected and transferred to three fresh falcon tubes. HBSS/2% FCS was added to a final volume of 40 ml, respectively. The cells were washed three times (10 min, 600xg, RT, no break), supernatants were decanted and PBMCs were resuspended in 30 ml HBSS/2% FCS. Subsequently monocytes and peripheral blood lymphocytes were isolated (see 2.2.8.4.4.).

2.2.8.4.4. Separation of monocytes and blood lymphocytes

Peripheral blood mononuclear cells (PBMCs), which were prepared as described in 2.2.8.4.3. were centrifuged (10 min, 600xg, RT, 2x) to wash the cells. Then PBMCs were resuspended in 40 ml IMDM/1% FCS and repeatedly centrifuged (10 min, 600xg, RT, 3x). Cells were carefully resuspended in 8 ml SIP (60%) and transferred to percoll tubes, 2 ml respectively. The cell suspension was overlayed with 4,5 ml SIP (47%) and 2 ml SIP (34%), respectively, before tubes were centrifuged for 45 min at 2100xg without break. Cells accumulated between the gradient layers. Monocytes were located in the upper interphase between layers of 34% and 47%, whereas peripheral blood lymphocytes (PBLs) were concentrated in the lower interphase between the 47% and 60% layers. Both cell types were collected and transferred to fresh tubes. Monocytes were mixed with 30 ml IMDM/1% FCS and PBLs with 30 ml HBSS/2% FCS. After centrifugation (10 min, 600xg, RT), supernatants were discarded and cell pellets were resuspended in 10 ml IMDM/10% FCS, respectively. Peripheral blood lymphocytes were stored at 4 °C until naïve $CD4^+$ T-cells were isolated (see 2.2.8.4.6.). Monocytes were subsequently used for the *in vitro* generation of dendritic cells (see 2.2.8.4.5.).

2.2.8.4.5. Generation of dendritic cells (DCs) from monocytes

Monocytes were counted, collected by centrifugation (10 min, 600xg, RT) and resuspended in the required amount of IMDM/1% FCS to achieve a cell number of $0.4x10^6$ cells per ml. One ml of the cell suspension was transferred into wells of a sterile Costar® 24-well, flat-bottom plate (Sigma Aldrich, St. Louis, Missouri, USA), respectively. After incubation for 45 min at 37 °C most of the cells were attached to the bottom. Monocytes were washed with 1 ml prewarmed IMDM/1% FCS (2x) before 1 ml IMDM/10% FCS containing 500 U/ml GM-CSF and 250 U/ml IL-4 was added to each well and the plate was incubated at 37 °C. After three days, 500 µl medium was removed from each well and the same amount of medium containing the double concentration of maturation factors (1,000 U/ml GM-CSF, 500 U/ml IL-4) was added. Cells were incubated for another three days at 37 °C to become dendritic cells. Half of the obtained naïve dendritic cells were further used for protein uptake experiments (chapter 2.2.8.4.7.) and half for coculture with naïve $CD4^+$ T-cells (chapter 2.2.8.4.8.).

2.2.8.4.6. Isolation of naïve $CD4^+$ T-cells from blood lymphocytes

Peripheral blood lymphocytes were isolated as described in 2.2.8.4.4. and stored at 4 °C. The cell suspension was diluted to a total volume of 30 ml IMDM/1% FCS and centrifuged (10 min, 600xg, RT). The supernatant was removed, cells were resuspended in 40 ml IMDM/1% FCS and washed by centrifugation (10 min, 400xg, RT). PBLs were mixed with 10 ml IMDM/1% FCS and the cell number was determined by using a Bürker-Türk hemocytometer. Per $1x10^7$ cells, 40 µl MACS buffer containing 50 ml 10x PBS, 2.5 g BSA and 100 mM EDTA was added. PBLs were carefully resuspended. Subsequently a Naïve $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to isolet naïve $CD4^+$ T-cells. Therefore 10 µl biotin antibody cocktail was added per $1x10^7$ cells, followed by incubation for 10 min at 4 °C under shaking. Thereafter, 2 ml of MACS buffer was added per $1x10^7$ cells. The cells were washed by centrifugation (10 min, 400xg, RT) and were resuspended in 80 µl MACS buffer per $1x10^7$ cells. Then 20 µl anti-biotin microbeads per $1x10^7$ cells were added and tubes were incubated for 15 min at 4 °C under constant shaking. Cells were washed by adding 2 ml MACS buffer per $1x10^7$ cells and centrifugation (10 min, 400xg, RT). The supernatant was removed and the pellet was resuspended in 500 µl MACS buffer ($1x10^8$ cells). The magnetic column supplied with the

isolation kit, was washed before the cell suspension was loaded. The column was washed three times with MACS buffer and unlabelled $CD4^+$ T-cells were collected in a tube. The cell suspension was brought to a volume of 5 ml and centrifuged for 10 min at 400xg and RT. Resulting pellets were resuspended in IMDM/50% FCS containing 10% DMSO ($2x10^6$ cells/ml). Finally, the isolated naïve $CD4^+$ T-cells were transferred into cryotubes and stored at -80 °C.

2.2.8.4.7. Stimulation of monocyte-derived DCs with allergens

For protein uptake experiments, allergen solutions containing 10 µg allergen, GM-CSF (500 U/ml), TNF-α (100 U/ml) and IL-1β (200 U/ml) were prepared. Positive controls containing 500 U/ml GM-CSF and 1 µg/ml LPS respectively were included. The used naïve dendritic cells were generated from peripheral blood mononuclear cells after six days of incubation with maturation factors (see 2.2.8.4.5.). From each well 850 µl medium was removed and 1 ml allergen solution or control solution was added. Immature monocyte-derived dendritic cells were stimulated for 2 days at 37 °C. Followingly, cells were analysed by flow cytometry (see 2.2.8.5.1.).

2.2.8.4.8. Coculture of DCs and autologous naïve $CD4^+$ T-cells

Dendritic cells were generated by incubation with GM-CSF and IL-4 as described above (see 2.2.8.4.5.). DCs were placed on ice for 30 min, before they were harvested by pipetting the medium up and down 10 times. Detached cells were transferred to a falcon and were washed by centrifugation (10 min, 600xg, RT). DCs were resuspended in 10 ml IMDM/1% FCS and repeatedly washed, before they were mixed with 175 µl IMDM/10% FCS and counted using a Bürker-Türk hemocytometer. Cells were diluted (10,000 cells/50 µl) and transferred into the wells of a sterile 96-well, flat-bottom plate (Costar®, Sigma Aldrich, St. Louis, Missouri, USA) in triplicates of 50 µl. The plate was incubated at 37 °C. In the meantime naïve $CD4^+$ T-cells (see 2.2.8.4.6.) stored at -80 °C were thawn in a water bath and immediately diluted in 10 ml of prewarmed IMDM/10% FCS. Naïve $CD4^+$ T-cells were centrifuged (10 min, 600xg, RT), counted and diluted (10,000 cells/100 µl). Finally, 50 µl of the cell suspension was added to the DCs and the plate was incubated for 5 days at 37 °C. Flow cytometry was performed as described in 2.2.8.5.2.

2.2.8.5. Flow cytometry

Flow cytometry is a method employed for multiparametric analysis of cells. Physical and chemical characteristics of different cell types can be analysed up to thousand particles per second. This method allows distinguishing and quantifying various cell types by labelling with different fluorescent dyes. When labelled cells are introduced to the cytometer they get suspended in a stream of fluid by hydrodynamic focussing and pass a laser beam emitting monochromatic light. Consequently, fluorophores attached to the cell scatter light with a specific wavelength, which is recorded and filtered by a detector.[111]

2.2.8.5.1. Flow cytometry of DCs stimulated with allergens

In order to study the effect of different parameters, immature DCs were stimulated with various allergen concentrations and at different incubation times. Allergen dilutions were prepared with serum-free medium. Hazelnut oil-protein mixtures were produced by adding hazelnut oil (10 mg/ml) at a ratio of 1:2 and sonication for 3x5 min. Cells were prepared as described in 2.2.8.4.7. The medium was removed from the plate, fresh serum-free medium (IMDM/1% FCS) was added and DCs were incubated for 30-60 min at 37 °C. Followingly DCs were harvested by pipetting the medium up and down. The cell suspension was transferred to a falcon and washed by centrifugation (10 min, 600xg, RT). Cells were resuspended in 10 ml IMDM/10% FCS, counted and $1x10^5$ cells were seeded to FACS tubes. Tubes were centrifuged (5 min, 600xg, RT) and 300 µl of prepared allergen solutions or oil-protein mixtures was added per tube. DCs were stimulated for 30 or 60 minutes at 37 °C. To examine binding of proteins to cell surface receptors, part of the cells was incubated at 4 °C for 60 min. Controls for receptor mediated protein uptake (transferrin) and non-receptor mediated protein uptake (dextran) were included. Tubes incubated at 37 °C were placed on ice to stop endocytosis and kept in the dark. Cells were washed two times with cold PBS by centrifugation (5 min, 600xg) and pellets were dissolved in 1 ml FACS buffer I, respectively. Cells were repeatedly washed and pellets were dissolved in 200 µl FACS buffer II. Samples were briefly mixed by vortexing and put on the tube holder of the cytometer (FACSCanto™ II system, BD, Franklin Lakes, NJ, USA). Uptake of fluorescent labelled proteins by dendritic cells was analysed using the BD FACSDiva software.

2.2.8.5.2. Flow cytometry of surface marker expression

Co-cultures of stimulated DCs with naïve $CD4^+$ T-cells were prepared as described in 2.2.8.4.8. Flow cytometry was performed after 5 days of incubation at 37 °C. The plate was placed on ice for 30 min, before DCs were harvested in prewarmed IMDM/1% FCS. Cells were washed by centrifugation (10 min, 600xg, RT, 2x), resuspended in 10 ml IMDM/10% FCS and counted. Then 1 x 10^5 cells were seeded in FACS tubes followed by centrifugation for 5 min at 600xg and RT. Supernatants were discarded, cells were washed with FACS buffer I and resuspended in 50 µl FACS buffer II. Antibody solution I and antibody solution II were prepared and added to the cells. Antibody solution I contained PE-CD86 (1:20), FITC-CD80 (1:10) and APC-Cy7-CD11c (1:100). Antibody solution II was comprised of PE-CD83 (1:20) and APC-HLA-DR (1:20). The staining was carried out for 45 min at 4 °C in the dark. Stained cells were washed 2x with 1 ml FACS buffer II by centrifugation (5 min, 1,800 rpm, RT), resuspended in 200 ml FACS buffer II and kept in the dark until analysis. Flow cytometry was performed with the FACSCanto™ II system (BD, Franklin Lakes, NJ, USA).

FACS buffer 1	**FACS staining buffer 2**
1xPBS	1xPBS
1% BSA	1% BSA
0.1% sodium acid	0.1% sodium acid
2% FCS	2% FCS
1% human serum	10% human serum

3. Results

3.1. Extraction of proteins and oil from hazelnuts

From 100 g hazelnuts, we obtained 72 g defatted hazelnut flour. To enhance extraction of alkaline nsLTP, proteins from dried hazelnut flour were extracted at pH 4.5 by using 20 mM sodium acetate, containing 0.5 M NaCl and 3% PVPP. The concentration of the total hazelnut protein extract was determined by BCA assay as shown in Tab. 3.1. The yield from 72 g nut flour was 7224 mg of proteins. A useful method to remove globulins from the total protein extract is by methanol precipitation according to the Osborne fractionation.[20] Prolamins are soluble in alcohol-water mixtures, whereas globulins are not. Therefore globulins were precipitated upon addition of methanol, while prolamins stayed in solution. After removing the globulins by centrifugation, the prolamin content of the supernatant was determined by the BCA method. For extracted prolamins a concentration of 9.4 mg/ml in a volume of 50 ml was measured, resulting in a total yield of approximately 470 mg (Tab. 3.1.). This amount corresponds to 7% of the total protein extract. The overall oil content from 100 g hazelnuts was gravimetrically determined and showed a weight of 2.6 g.

Table 3.1.: Concentrations of total protein extract and prolamin extract obtained by 100 g hazelnuts.

	Concentration	Volume	Total amount	Percentage
Total protein extract	16.8 mg/ml	430 ml	7224 mg	10% of 100 g hazelnuts
Prolamin extract	9.4 mg/ml	50 ml	470 mg	7% of total protein extracted

SDS-PAGE analysis was performed under denaturing conditions to evaluate the presence of allergens in the total hazelnut protein extract as well as in the prolamin extract. The total hazelnut extract showed different protein bands with high molecular weight, corresponding to hazelnut 7S vicilin, Cor a 11 at about 68 kDa, 11S globulin, Cor a 9 at approximately 36 kDa and 20 kDa and lower molecular weight proteins from 10-15 kDa (Fig. 3.1., lane 1). In contrast, for the prolamin extract, only proteins with lower molecular weight, about 10-15 kDa were observed (Fig. 3.1., lanes 2 and 3).

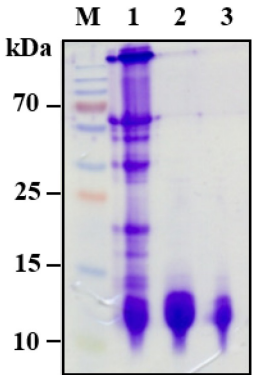


Figure 3.1.: Coomassie stained 15% SDS-PAGE gel of crude hazelnut protein extract and prolamin extract. (M) Prestained molecular weight marker, (1) total hazelnut extract, (2) prolamin extract after acetone precipitation, (3) dialysed prolamin extract.

3.2. Purification of hazelnut prolamins Cor a 14 and Cor a 8

An overview of the purification steps (see chapters 3.2.1, 3.2.2. and 3.2.3) is shown in Fig. 3.2.

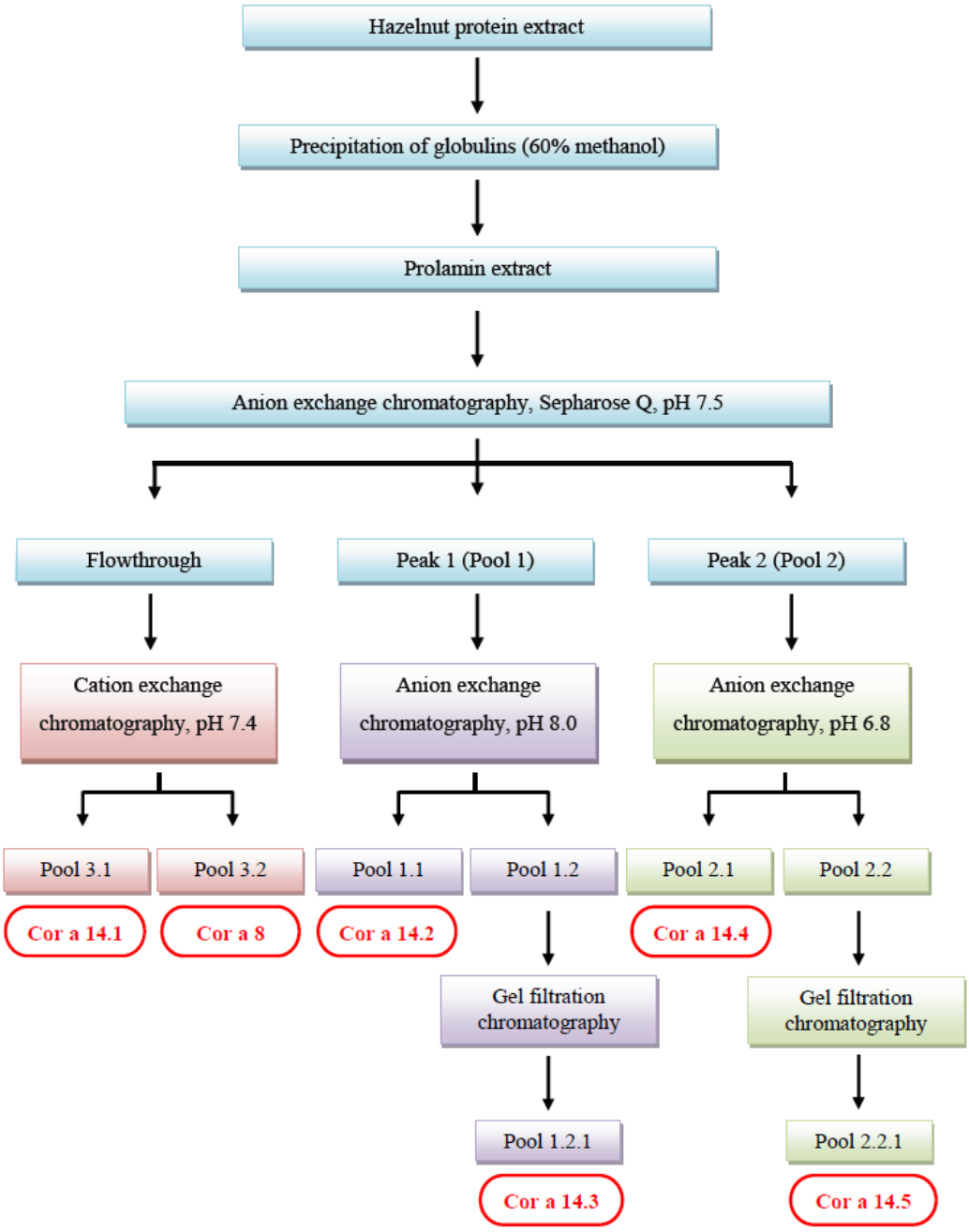


Figure 3.2.: Individual purification steps of hazelnut prolamins Cor a 14 and Cor a 8.

3.2.1. Purification of hazelnut 2S albumin, Cor a 14

The hazelnut prolamin extract was lyophilised and resolved in 20 mM Tris/HCl, pH 7.5, before it was separated by a Q Sepharose anion exchange column. The theoretical pI of Cor a 14 is 5.6. and therefore

the pH of binding buffer (pH 7.5) was set above the pI of the protein. Under these conditions we expected that the target protein Cor a 14 would bind to the column matrix. Proteins bound to the column matrix were eluted by a linear gradient from 0-50% of elution buffer. The chromatogram showed two major peaks, corresponding to proteins eluted at different concentrations of 1 M NaCl (9-12%, 12-20%) (Fig. 3.3.). SDS-PAGE analysis of the fractions was performed under denaturing conditions and revealed that the two peaks contained different protein patterns (Fig. 3.4.). Fractions eluted with lower NaCl concentrations (9-12%), showed two bands at 12-14 kDa and a lower band of 10 kDa. In contrast, fractions eluted with higher salt concentrations (12-20%) contained only proteins at about 12-14 kDa. Therefore, pooled fractions 17-20 were named Pool 1 and pooled fractions 21-27 were named Pool 2. Moreover, Sepharose Q flow through fractions 2-6 (Pool 3) assumed to contain Cor a 8 (Tab. 3.2.), which was not expected to bind to the column because it has a basic pI of 9.3 were stored for further analysis (Fig. 3.3., Tab. 3.2.).

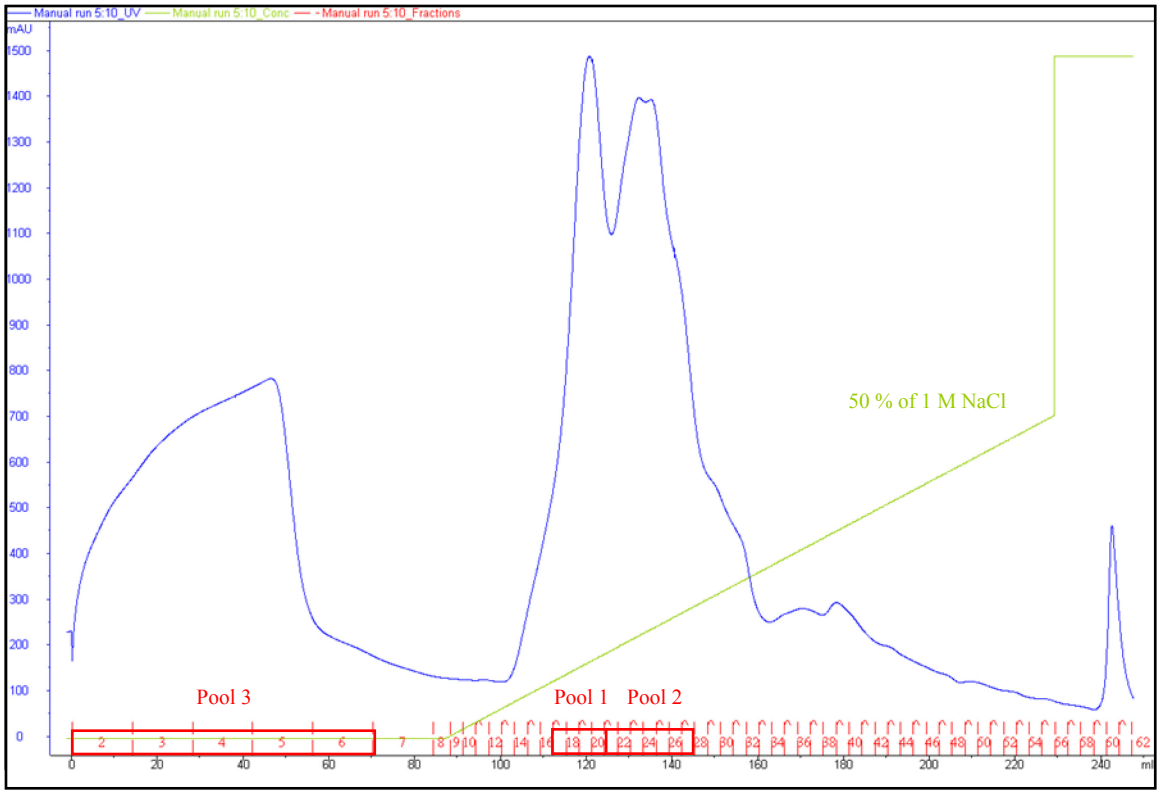


Figure 3.3.: Separation of the hazelnut prolamin extract by Q Sepharose anion exchange chromatography. The absorption at 280 nm is represented by the blue curve, the green line corresponds to the NaCl gradient and fractions are labeled in red. Fractions marked with red rectangles were pooled.

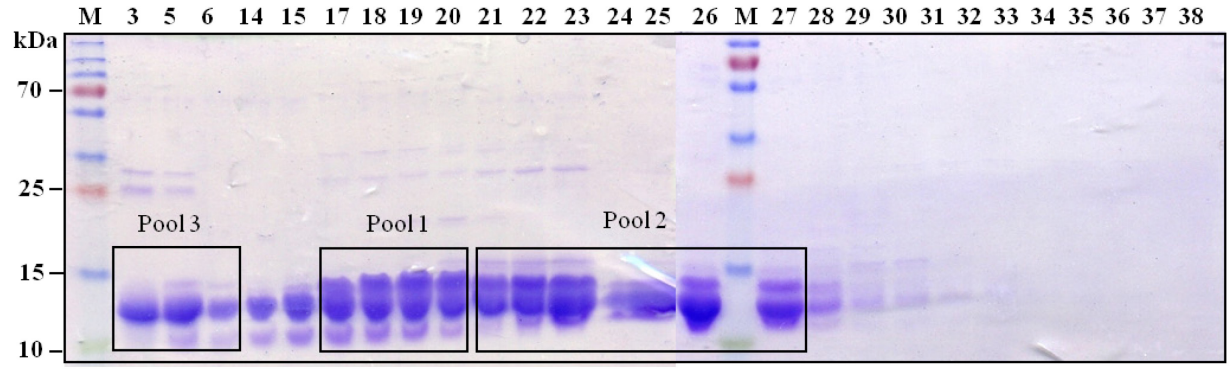


Figure 3.4.: Coomassie stained 15% SDS-PAGE gel of fractions obtained by Q Sepharose anion exchange chromatography. Lanes marked M show molecular weight protein marker, lanes marked with numbers correspond to the number of individual fractions. Fractions were pooled as listed in Tab. 3.2.

Table 3.2.: Pooled fractions (fr) obtained by Q Sepharose anion exchange chromatography.

Pools	**Next purification step**
Pool 1: fr 17-20	Mono Q anion exchange chromatography, pH 8.0
Pool 2: fr 21-27	Mono Q anion exchange chromatography, pH 6.8
Pool 3: fr 2-6	Mono S cation exchange chromatography, pH 7.6 (see chapter 3.2.2.)

Pool 1 was further separated by using a Mono Q anion exchange column and binding buffer of pH 8.0 (Fig. 3.5.). Protein elution was performed with different concentrations of 1 M NaCl, 0-20% (Fig. 3.5., A) and 0-50% (Fig. 3.5., B) to test optimal separation conditions. We found that elution with linear gradients of 20% achieved better resolution. Fractions were checked by 15% SDS-PAGE and Coomassie staining under reducing as well as under nonreducing conditions (Fig. 3.6.). Fractions obtained by elution with 20% gradients were pooled as follows: fractions eluted at lower salt concentrations (0-7%) showed bands at 12-14 kDa, therefore fractions 3-9 were pooled to Pool 1.1. Fractions eluted with higher salt concentrations (7-10%) contained proteins of the same molecular weight, but also high molecular weight proteins. Therefore fractions 10-11 were pooled to Pool 1.2. Fractions obtained by elution with 50% gradients showed a similar protein pattern and were pooled as follows: fractions 3-8, which were eluted at lower salt concentrations (0-12%) and fractions 9-12, which were eluted with higher salt concentrations (12-21%). Tab. 3.3. gives an overview of the pooled fractions.

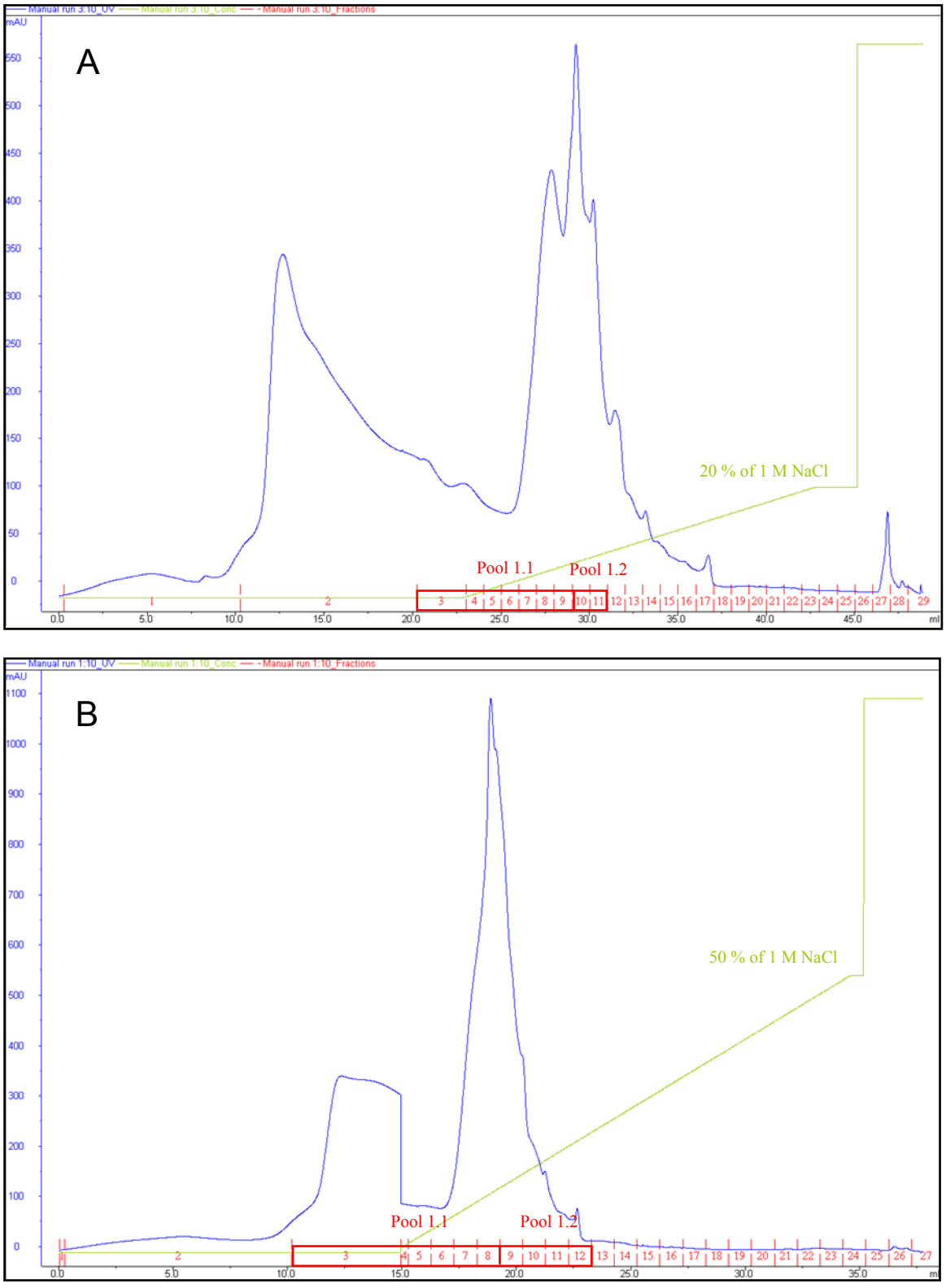


Figure 3.5.: Separation of proteins from Pool 1, pH 8.0 by Mono Q anion exchange chromatography. Proteins bound to the column were eluted by linear gradients of (A) 20% and (B) 50%. The absorption at 280 nm is represented by the blue curve, the green line corresponds to the NaCl gradient and fractions are labeled in red. Fractions marked with red rectangles were pooled.

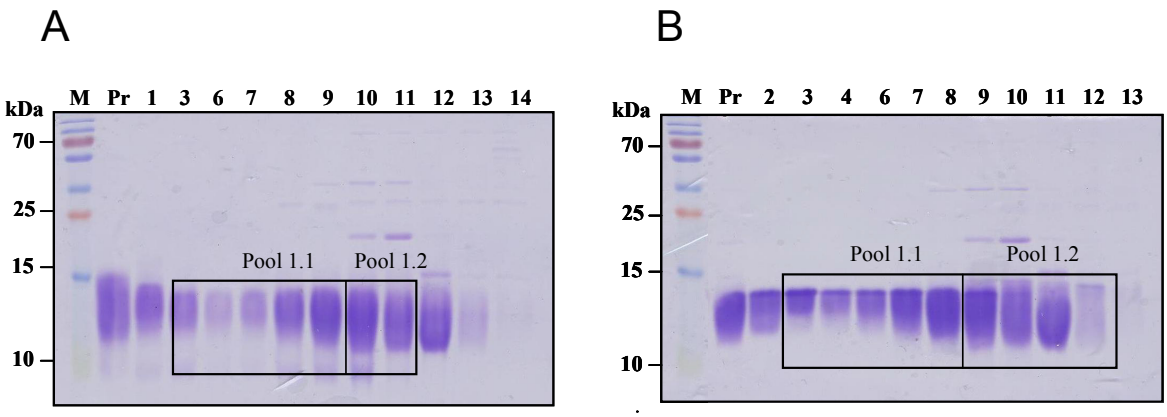


Figure 3.6.: Coomassie stained 15% SDS-PAGE gel of fractions obtained by Mono Q anion exchange chromatography of Pool 1, pH 8.0. Lanes marked M show molecular weight protein marker, Pr corresponds to the applied sample and numbers correspond to the number of individual fractions. (A) Fractions eluted by a linear gradient of 20%, analysed under reducing conditions, (B) Fractions eluted by a gradient of 50%, analysed under nonreducing conditions. Marked fractions were pooled, for details see Tab. 3.3.

Table 3.3.: Pooled fractions (fr) obtained by Mono Q anion exchange chromatography of Pool 1, pH 8.0. Total Pool 1.2 was subjected to gel filtration. Total Pool 1.1 was stored for further analysis.

Protein	Separation conditions	Gradient	Pools	Next purification step
Cor a 14	pH 8.0	20 %	Pool 1.1: fr 3-9	Pools 1.2: Gel filtration
			Pool 1.2: fr 10-11	
		50 %	Pool 1.1: fr 3-8	
			Pool 1.2: fr 9-12	

Pool 2 obtained by Q Sepharose anion exchange chromatography, pH 7.5 (Fig. 3.3.), was also purified by anion exchange chromatography on Mono Q. In contrast to separation of Pool 1, binding buffer with pH 6.8 was used (Fig. 3.7.). Proteins bound to the column matrix were eluted with different concentrations of 1 M NaCl, 0-20% (Fig. 3.7., A) and 0-50% (Fig. 3.7., B). Again, elution with 20% gradients showed better resolution. Fractions were analysed by SDS-PAGE as described for Pool 1 (Fig. 3.8.). Fractions obtained by elution with a 20% gradient were pooled as follows: fractions 3-4 (Pool 2.1), which were eluted at lower salt concentrations (0-5%) and fractions 5-11 (Pool 2.2), which were eluted with higher salt concentrations (5-10%). Fractions obtained by elution with a linear gradient of 50% were pooled as follows: fractions 3-6, which were eluted at lower salt concentrations (0-10%) and fractions 7-12, which were eluted with higher salt concentrations (10-22%). Tab. 3.4. gives an overview of the pooled fractions.

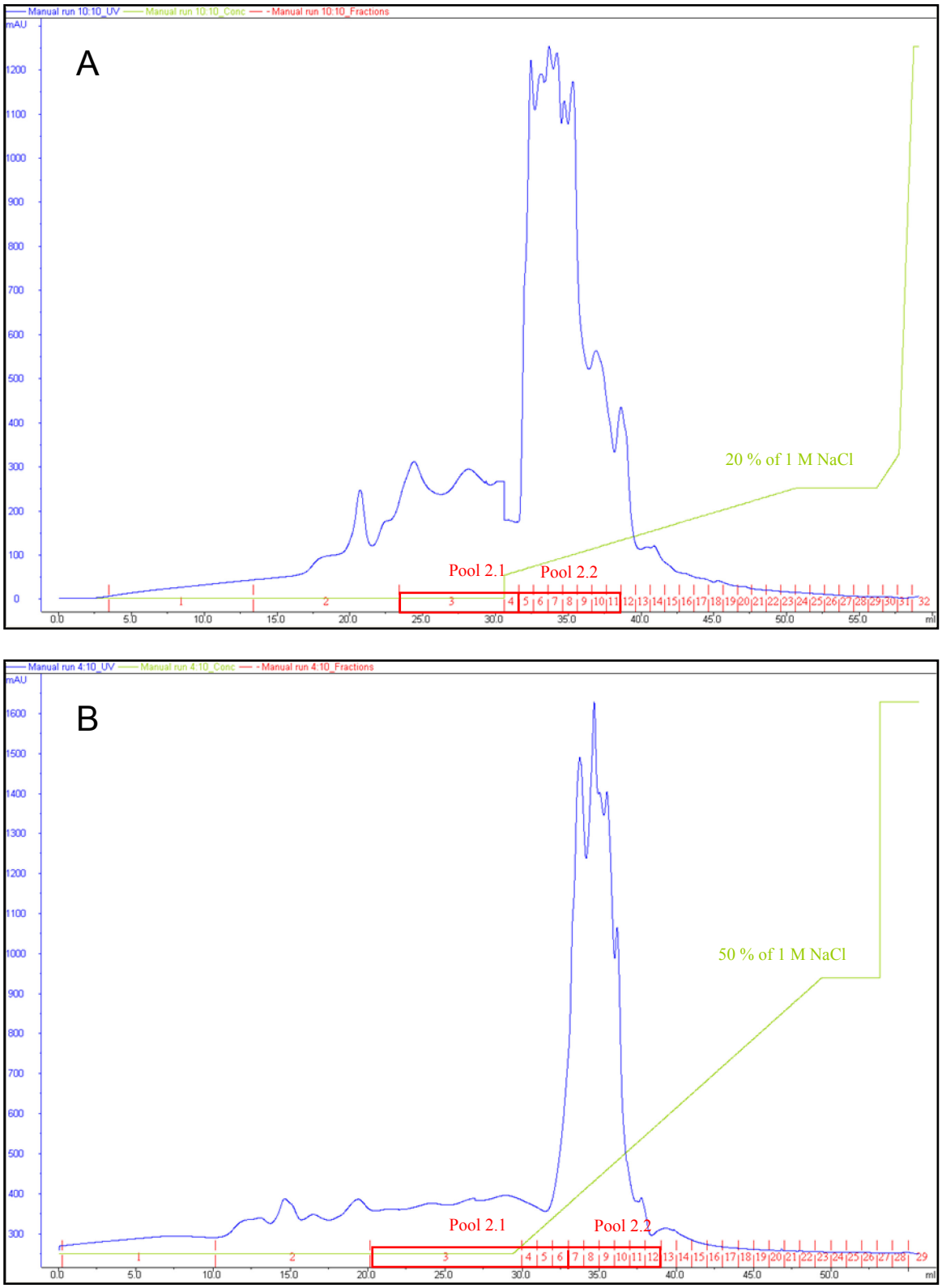


Figure 3.7.: Separation of proteins from Pool 2, pH 6.8 by Mono Q anion exchange chromatography. Proteins bound to the column were eluted by linear gradients of (A) 20% and (B) 50%. The absorption at 280 nm is represented by the blue curve, the green line corresponds to the NaCl gradient and fractions are labeled in red. Fractions marked with red rectangles were pooled.

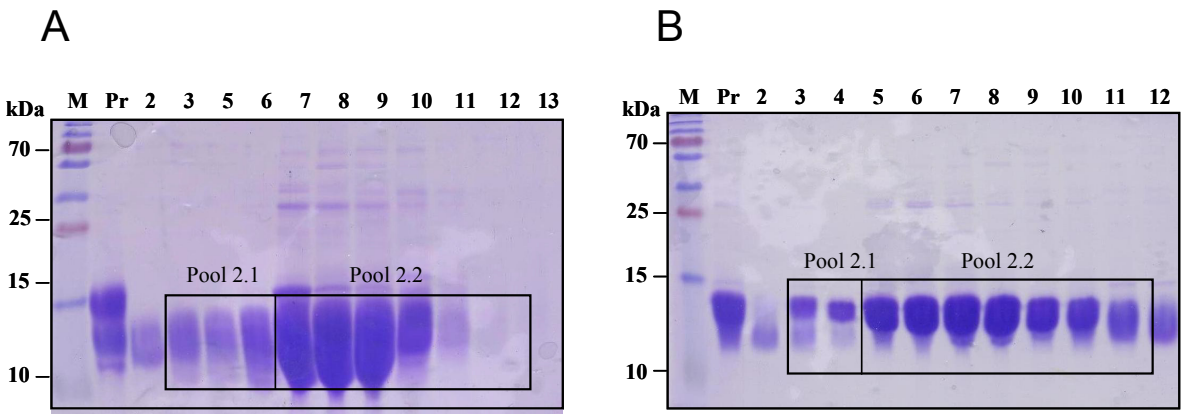


Figure 3.8.: Coomassie stained 15% SDS-PAGE gel of fractions obtained by Mono Q anion exchange chromatography of Pool 2, pH 6.8. Lanes marked M show molecular weight protein marker, Pr corresponds to the applied sample and numbers correspond to the number of individual fractions. (A) Fractions eluted by a linear gradient of 50%, analysed under reducing conditions, (B) Fractions eluted by a gradient of 20%, analysed under nonreducing conditions. Marked fractions were pooled, for details see Tab. 3.4.

Table 3.4.: Pooled fractions (fr) obtained by Mono Q anion exchange chromatography of Pool 2, pH 6.8. Total Pool 2.2 was subjected to gel filtration. Total Pool 2.1 was stored for further analysis.

Protein	Separation conditions	Gradient	Pools	Next purification step
Cor a 14	pH 6.8	20 %	Pool 2.1: fr 3-4	Pools 2.2: Gel filtration
			Pool 2.2: fr 5-11	
		50 %	Pool 2.1: fr 3- 6	
			Pool 2.2: fr 7-12	

For further analysis of purified proteins obtained anion exchange chromatography on Q Sepharose and Mono Q, total pools were dialysed and lyophilised. SDS-PAGE analysis and Coomassie staining under reducing and nonreducing conditions revealed that the pools were highly enriched in proteins with a molecular weight of 10-15 kDa (Fig. 3.9., lane 1-3), giving a first indication for the identity of Cor a 14. However, contaminating high molecular weight proteins were visible on the gel, which were subsequently removed by gel filtration. In lane 4, the flow through obtained by the first anion exchange chromatography, pH 7.5 and assumed to contain Cor a 8 is shown (for details see 3.2.2.).

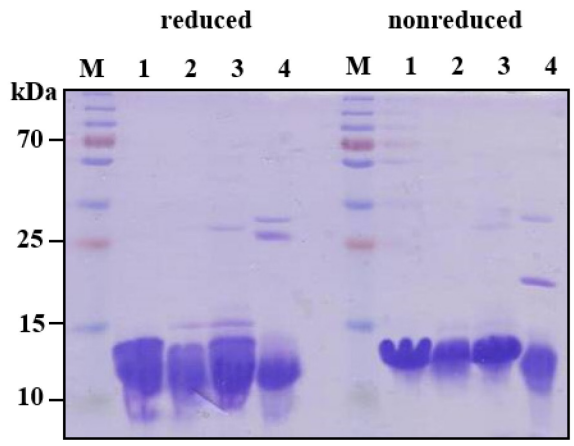


Figure 3.9.: Coomassie stained 15% SDS-PAGE gel of different pools obtained by anion exchange chromatography. Proteins were separated under reducing (left panel) and nonreducing conditions (right panel). (M) prestained protein marker, (1) Pool 1.1, pH 8.0, Mono Q, (2) Pool 2.1, pH 6.8, Mono Q, (3) Pool 2.2, pH 6.8, Mono Q, (4) Sepharose Q flow through fractions (Pool 3), pH 7.5 (for details about Cor a 8 purification see 3.2.2.).

We aimed to remove contaminating high molecular weight proteins visible on SDS-PAGE in Fig. 3.9., lanes 1-3, A and B. In order to obtain pure Cor a 14, Pool 1.2, pH 8.0 and Pool 2.2, pH 6.8 were subjected to gel filtration chromatography. Pools were concentrated and separated by using a HiLoad 16/60 Superdex 200 (Fig. 3.10.). Elution profiles revealed a major peak, probably corresponding to the target protein Cor a 14. SDS-PAGE, conducted under reducing conditions on 15% polyacrylamide gels revealed protein enriched fractions (Fig. 3.11.). Analysis of eluted fractions showed that high molecular weight proteins were eluted at the beginning (Fig. 3.11., A: fractions 14-17 and B: fractions 15-20). The target protein had lower molecular weight and thus eluted later. SDS-PAGE analysis of corresponding fractions showed proteins migrating at 10-20 kDa. In order to obtain pure Cor a 14, only fractions 21-27 were pooled and named Pool 2.2.1, pH 6.8 (Fig. 3.11., A). From Pool 1.2. fractions 21-25 were collected and named Pool 1.2.1, pH 8.0 (Fig. 3.11., B).

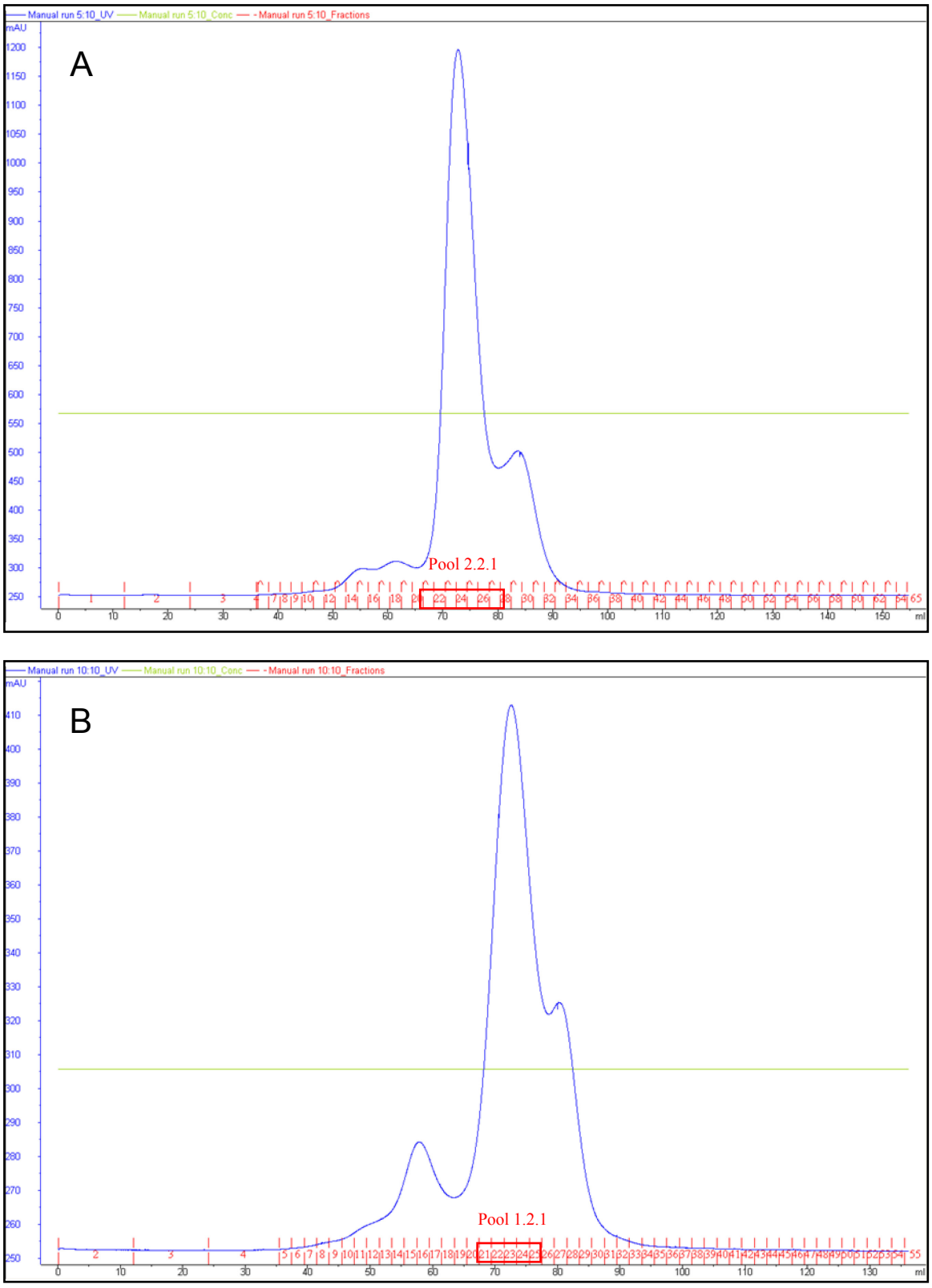


Figure 3.10.: Gel filtration chromatography of (A) Pool 2.2, pH 6.8 and (B) Pool 1.2, pH 8.0. Proteins were eluted isocratically. The absorption at 280 nm is represented by the blue curve and fractions are labeled in red. Fractions marked with red rectangles were pooled.

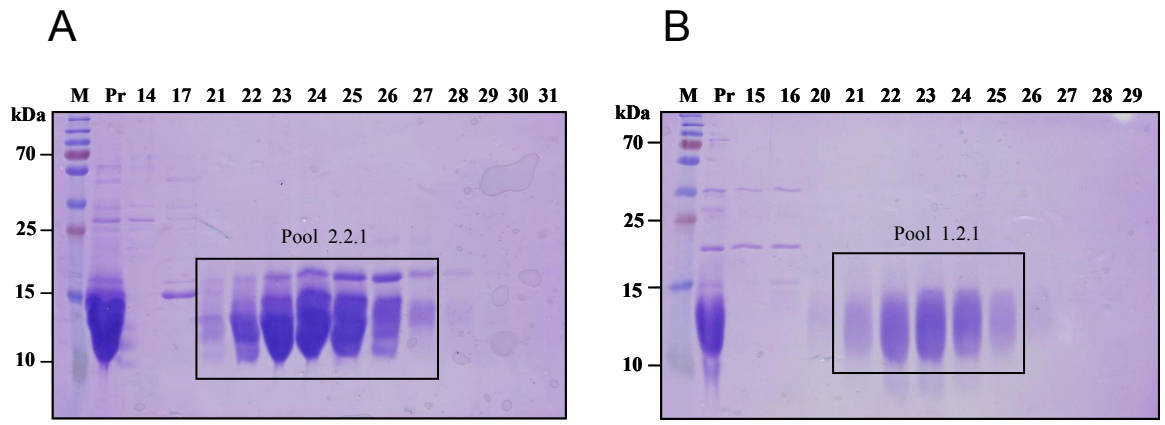


Figure 3.11.: Fractions obtained by gel filtration chromatography analysed by SDS-PAGE under reducing conditions and Coomassie staining. Lanes marked M show molecular weight protein marker and lanes marked with numbers correspond to fractions. (A) Pool 2.2, pH 6.8, (B) Pool 1.2, pH 8.0. Marked fractions were pooled.

Subsequently performed SDS-PAGE analysis under reducing and nonreducing conditions gave a good indication for the identity of protein Cor a 14 (see chapter 3.3., Fig. 3.14).

3.2.2. Purification of hazelnut nsLTP, Cor a 8

The hazelnut prolamin extract was separated by Q Sepharose anion exchange column (see 3.2.1.). Unlike Cor a 14, the nsLTP Cor a 8 is a basic protein with a theoretical pI of 9.3 and should not bind to the Q Sepharose matrix under the described conditions. To change buffer, Sepharose Q flow through fractions (Pool 3, Tab. 3.2.) expected to contain Cor a 8, were dialysed against binding buffer. After dialysis the total volume of the sample was about 70 ml. For further separation, 10 ml aliquots of the sample were applied onto Mono S cation exchange column. In order to achieve binding of Cor a 8 (pI 9.3) to the chromatographic column matrix, the pH of the binding buffer used for cation exchange chromatography was set to pH 7.6, which is below the pI of the target protein. Protein elution was performed with a linear gradient from 0-20% 1 M NaCl of elution buffer. Two representative chromatograms with various protein peaks are shown below (Fig. 3.12.). SDS-PAGE analysis of peak fractions eluted with salt concentrations between 15-20% (Fig. 3.12.,A and B) showed a high amount of purified proteins. Protein with a molecular mass about 12 kDa was visible in the later fractions (Fig. 3.13.). On the basis of these results the fractions were likely enriched in Cor a 8. Corresponding fractions were pooled to Pool 3.2 as can be seen from Tab. 3.5. In addition, also the obtained flow through fractions were pooled as they probably contained residual amounts of Cor a 14. Flow through fractions were named Pool 3.1, respectively.

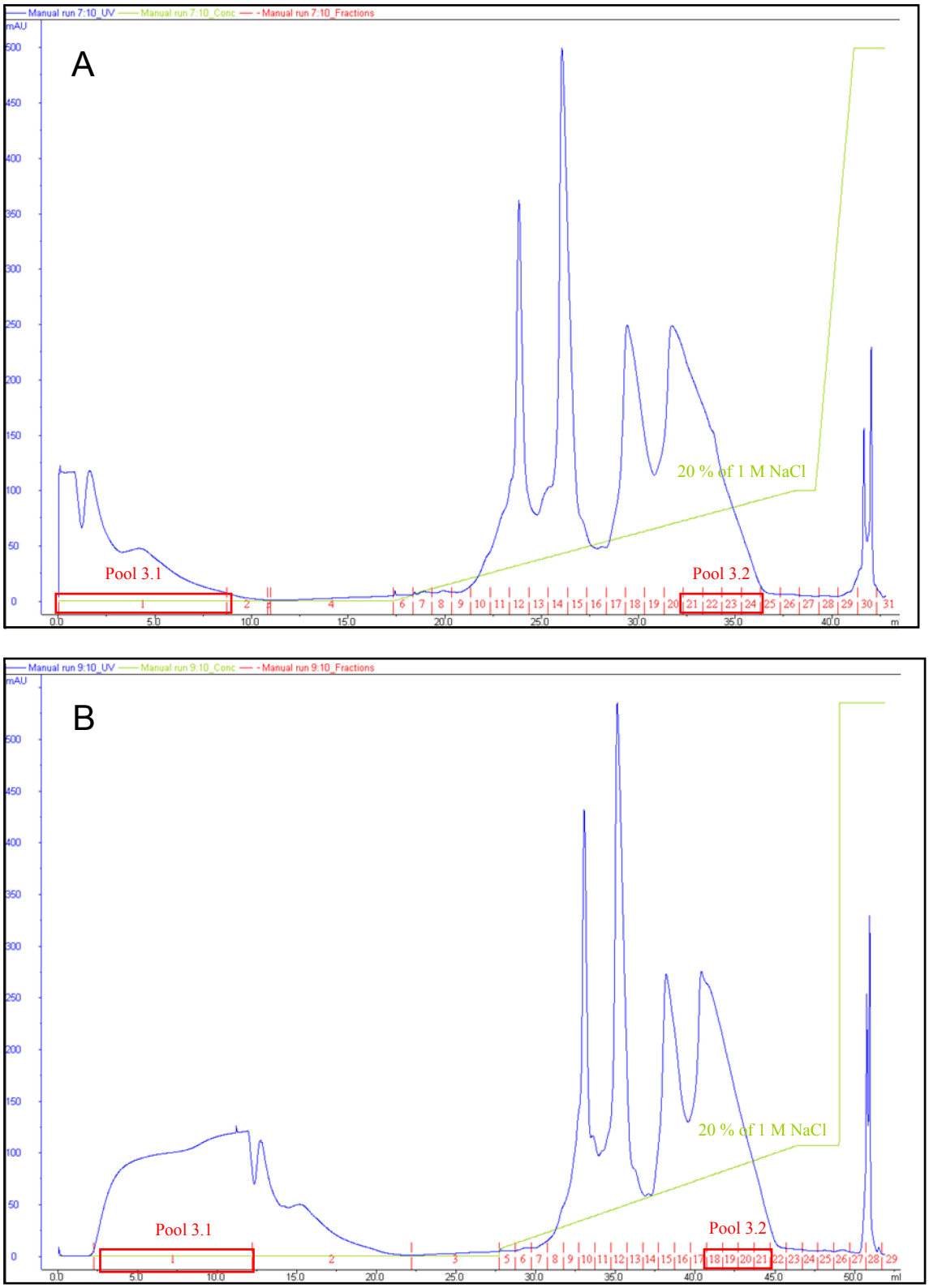


Figure 3.12.: Purification of Cor a 8, pH 7.6, by Mono S cation exchange chromatography. Two representative elution profiles are shown (A and B). Proteins bound to the column were eluted by linear gradients of 20% 1 M NaCl. The absorption at 280 nm is represented by the blue curve, the green line corresponds to the NaCl gradient and fractions are labeled in red. Fractions marked with red rectangles were pooled.

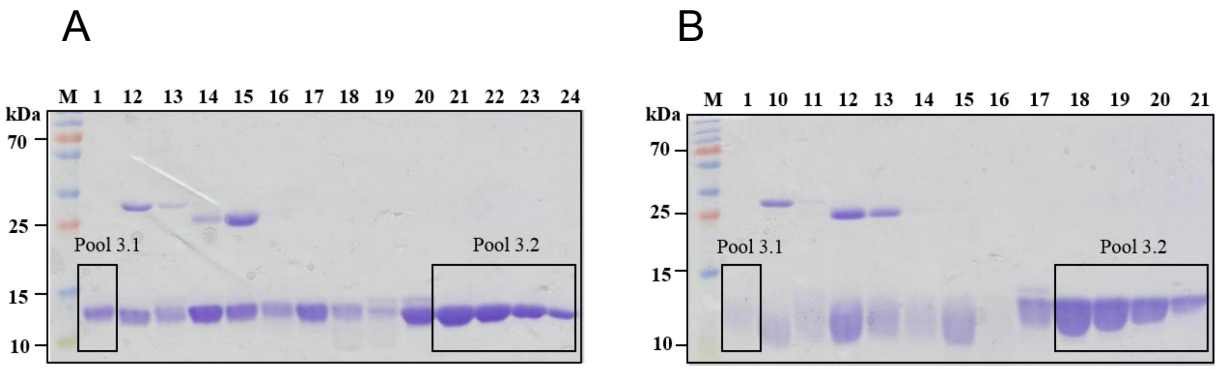


Figure 3.13.: Coomassie stained 15% SDS-PAGE gel of fractions obtained by Mono S cation exchange chromatography. Lanes marked M show molecular weight protein marker and lanes marked with numbers correspond to fractions. Mono S fractions correspond to the chromatogram 3.12, A (A) and 3.12, B (B). Marked fractions were pooled, for details see Tab. 3.5.

Table 3.5.: Overview of pooled flow through fractions (ft) and fractions (fr) obtained by Mono S cation exchange.

Protein	Run	Pool 3.1	Pool 3.2
Cor a 8	1	ft	fr 19-22
	2	ft	fr 21-24
	3	ft	fr 18-21
	4	ft	fr 21-24
	5	ft	fr 19-23
	6	ft	fr 19-22

SDS-PAGE analysis performed under reducing and nonreducing conditions gave a good indication for the identity of protein Cor a 8 (see chapter 3.4., Fig. 3.18).

3.2.3. Determination of protein concentrations

For further analysis all purified pools (Tab. 3.5.) were dialysed and lyophilised. After that, protein concentrations were determined by using the BCA method and the total yield of individual batches was calculated (Tab. 3.6.). The identity of purified Cor a 8 and Cor a 14 was confirmed by mass spectrometry and N-terminal sequencing as described in chapters 3.3. and 3.4.

Table 3.6.: Measured protein concentrations and total amount of hazelnut prolamins Cor a 8 and Cor a 14.

Batches	Pool	Concentration	Volume	Total amount
Cor a 14.1	Pool 3.1	1.9 mg/ml	14.5 ml	27.5 mg
Cor a 14.2	Pool 2.2.1	9.6 mg/ml	2.3 ml	22.0 mg
Cor a 14.3	Pool 1.2.1	3.8 mg/ml	1.0 ml	3.8 mg
Cor a 14.4	Pool 2.1	3.3 mg/ml	9.5 ml	31.3 mg
Cor a 14.5	Pool 1.1	4.4 mg/ml	3.5 ml	14.5 mg
Cor a 8	Pool 3.1	2.1 mg/ml	8.0 ml	16.8 mg

3.3. Physicochemical characterisation of hazelnut 2S albumin, Cor a 14

The purity of produced Cor a 14 batches was assessed under reducing and nonreducing conditions by 15% SDS-PAGE and Coomassie staining. Native proteins migrated under nonreducing conditions as single band at approximately 15 kDa, which corresponds well to the theoretical molecular mass of Cor a 14 (Uniprot accession no.: D0PWG2) (Fig. 3.14., A, Lanes 1-5). In contrast, under reducing conditions a double band about 12-14 kDa and a smaller band at 10 kDa became apparent (Fig. 3.14., B, Lanes 1-5). This protein pattern is typical for heterodimeric 2S albumins, which are comprised of 2 polypeptide chains linked together via disulphide bonds.[37] This result suggests that upon addition of DTT interchain disulphide bonds between cysteine residues were reduced, splitting the protein up into a larger and a small subunit. Furthermore, SDS-PAGE analysis revealed that Cor a 14.2 and Cor a 14.4 as well as Cor a 14.3 and Cor a 14.5 showed more similar protein patterns to each other. Cor a14.1 gave a clear band under nonreducing conditions, whereas after reduction with DTT and alkylation only a weak protein band was visible.

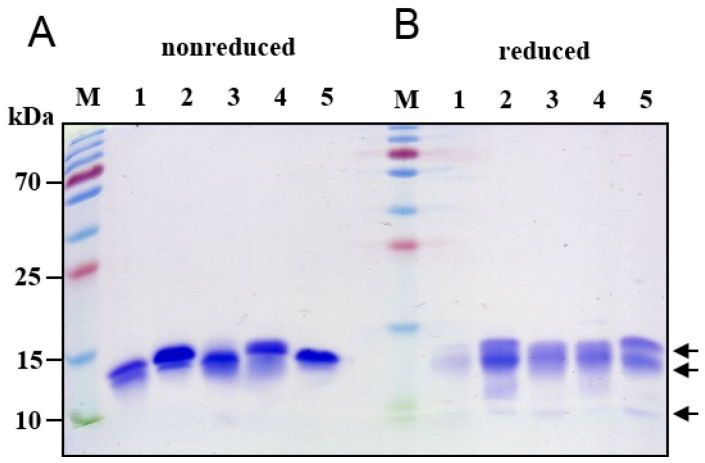
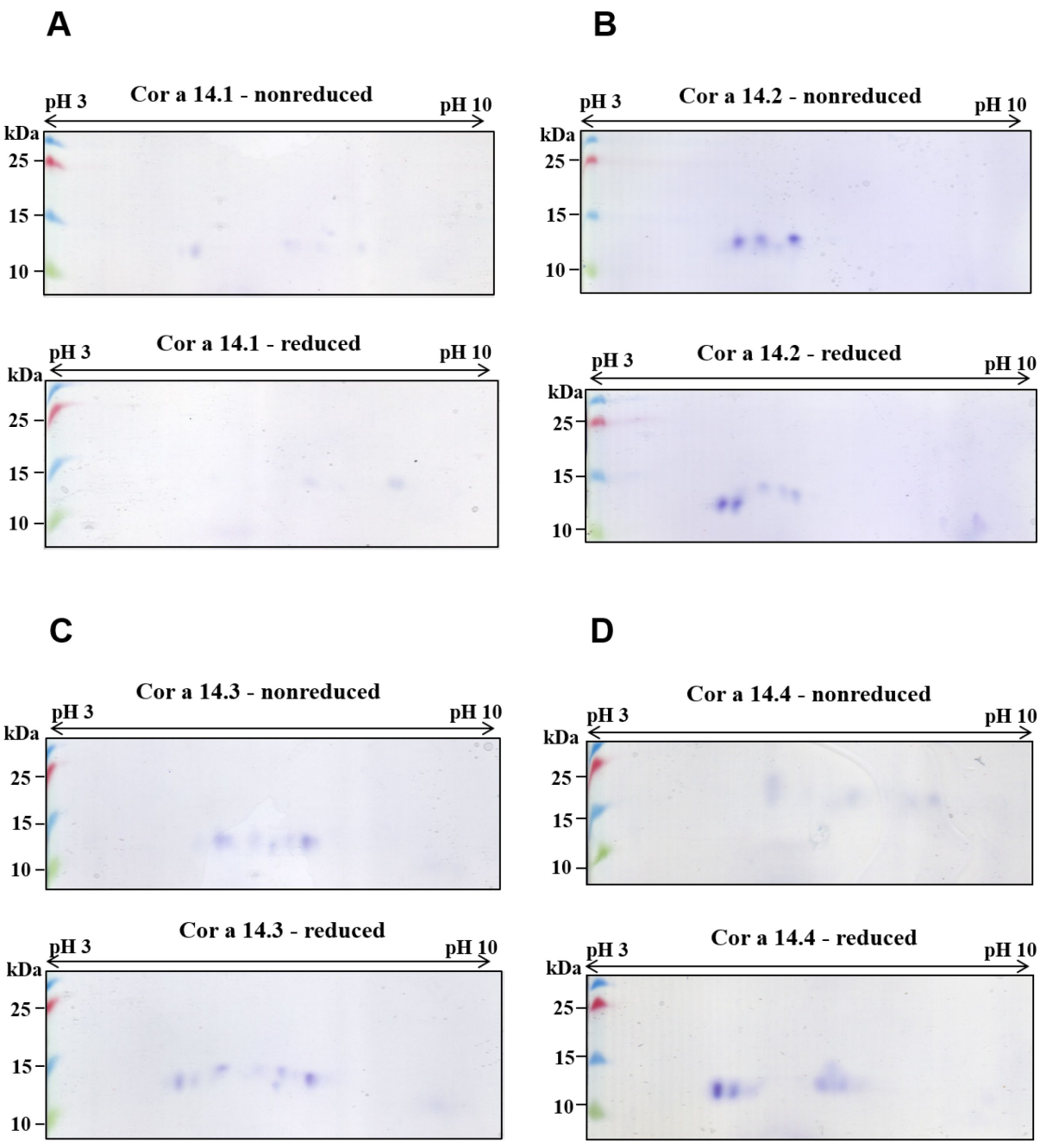


Figure 3.14.: Coomassie stained 15% SDS-PAGE gel of purified Cor a 14 batches. Proteins (2 µg/lane) were separated (A) under nonreducing conditions, (B) under reducing conditions. Proteins were reduced and alkylated by DTT and IAA. (M) prestained protein marker, (1) Cor a 14.1, (2) Cor a 14.2, (3) Cor a 14.3, (4) Cor a 14.4, (5) Cor a 14.5. The subunits of Cor a 14 visible under reducing conditions are indicated by arrows.

In order to evaluate isoforms present in differently produced batches of Cor a 14 (Cor a 14.1, Cor a 14.2, Cor a 14.4, Cor a 14.5), 2D-SDS-PAGE was performed. In the first dimension, native allergens as well as reduced proteins were separated according to their pI. IEF was performed on strips with a wide pH gradient (pH 3-10) to achieve sufficient separation of acidic and basic peptides. In the second dimension,

proteins were resolved according to their molecular weight by SDS-PAGE (Fig. 3.15). Analysis of Cor a 14.1 under nonreducing conditions gave two weak protein spots at approximately 14 kDa and a pI of 5.0-6.5. Under reducing conditions two spots of the same molecular weight and a pI around 7-9 can be seen (Fig. 3.15., A). The 2D gel profiles of nonreduced Cor a 14.2 showed three distinct protein spots with molecular weights about 14 kDa and pI values of pI 5.5-7.0. Compared to analysis under nonreducing conditions, five protein spots with the same masses and a pI between 4.5-7.0 were seen. Under reducing conditions an additional spot with an estimated molecular mass of 10 kDa and a pI of 9.0 was observed (Fig. 3.15., B). Analysis of nonreduced Cor a 14.3 showed five protein spots at approximately 14 kDa and varying pI values between 5.0-7.0. In contrast, under reducing conditions five protein spots with a pI about pI 4.5-7.0 and one additional spot of about 10 kDa and a pI of 9.0 were observed (Fig. 3.15., C). The 2D gel profile revealed for Cor a 14.4 (Fig. 3.15., D) was similar to that of Cor a 14.2 and the gel profile found for Cor a 14.5 was similar to that of Cor a 14.3 (Fig. 3.15., E). In the literature Cor a 14 is indicated with a theoretical molecular mass of 15 kDa and the pI with 5.6 (Uniprot accession no.: D0PWG2). Therefore spots observed under nonreducing conditions with values corresponding to theoretical mass and pI, represent the hazelnut 2S albumin Cor a 14. Additional spots corresponded to different isoforms of the 2S albumin. The 2D gel profiles of reduced proteins showed protein spots at acidic pI 5.0-7.0, which represent the larger subunits of Cor a 14 as well as spots of basic pI 9.0 corresponding to the small subunit of the 2S albumin.


A
Cor a 14.1 - nonreduced
pH 3
pH 10
kDa
25
15
10
Cor a 14.1 - reduced
pH 3
pH 10
kDa
25
15
10
B
Cor a 14.2 - nonreduced
pH 3
pH 10
kDa
25
15
10
Cor a 14.2 - reduced
pH 3
pH 10
kDa
25
15
10
C
Cor a 14.3 - nonreduced
pH 3
pH 10
kDa
25
15
10
Cor a 14.3 - reduced
pH 3
pH 10
kDa
25
15
10
D
Cor a 14.4 - nonreduced
pH 3
pH 10
kDa
25
15
10
Cor a 14.4 - reduced
pH 3
pH 10
kDa
25
15
10

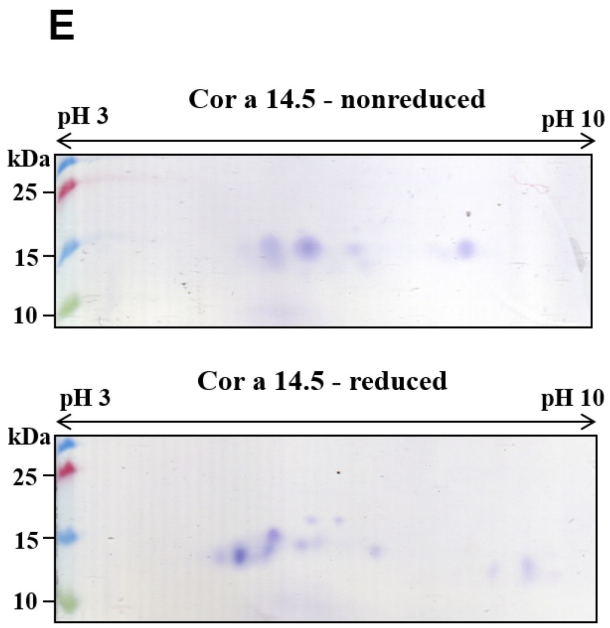


Figure 3.15.: Coomassie stained 2D-SDS-PAGE gels of purified Cor a 14 (4 µg/gel) prepared under reducing and under nonreducing conditions. (A) Cor a 14.1, (B) Cor a 14.2, (C) Cor a 14.3, (D) Cor a 14.4, (E) Cor a 14.5.

Secondary structures of purified Cor a 14 batches (Cor a 14.1, Cor a 14.2, Cor a 14.4, Cor a 14.5) were analysed by CD spectroscopy. All recorded spectra indicated a positive maximum around 195 nm and intense negative minima at 208 nm and 222 nm, suggesting that the allergens were in a native folded state (Fig. 3.16., A). The negative double minimum is characteristic for proteins rich in α-helices, including 2S albumins.[123] The exact amino acid sequence of Cor a 14.2 had been determined by mass spectrometry, therefore the molar ellipticity was calculated. The molar ellipticity spectrum was similar to the CD spectrum, indicating a largely α-helical protein (Fig. 3.16., B).

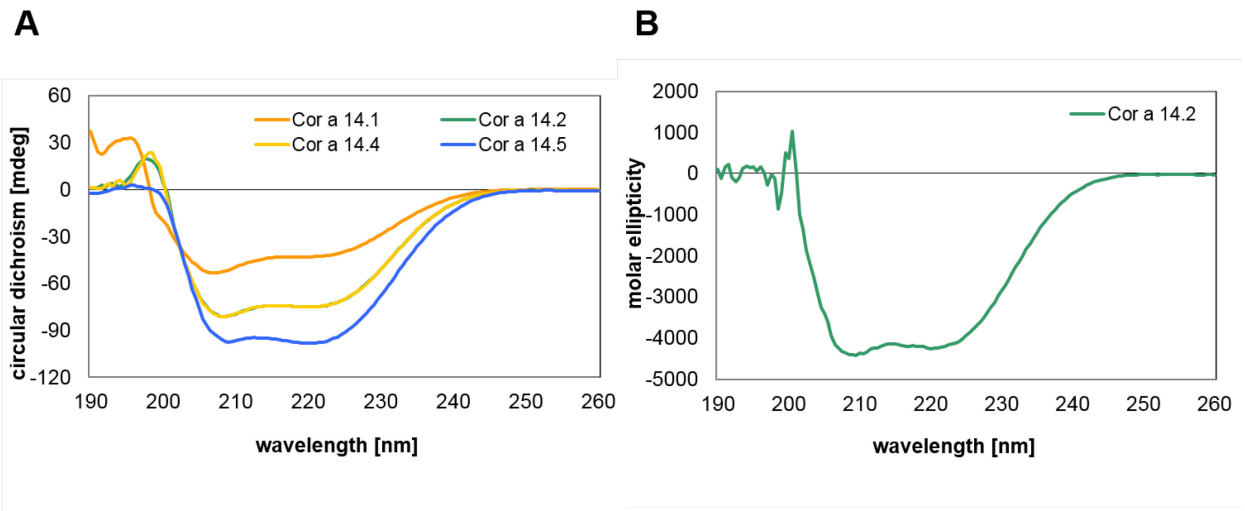


Figure 3.16.: Far UV-spectra of purified 2S albumin, Cor a 14. (A) CD spectra of Cor a 14.1, Cor a 14.2, Cor a 14.4, Cor a 14.5 recorded between 190-260 nm at RT, (B) molar ellipticity of Cor a 14.2.

Finally, purified 2S albumins, Cor a 14.2 and Cor a 14.5 were analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) peptide analysis at the VetCORE - Facility for Research lab, University of Veterinary Medicine, Vienna. Native Cor a 14.2 was digested with trypsin and the resulting peptides were analysed by MALDI-TOF mass spectrometry (Fig. 3.17). The tryptically digested protein could be identified as 2S albumin from *Corylus avellana* with nine peptides and sequence coverage of 42%. Then the theoretical sequence was modified according to post-translational modification by removing the signal peptide and cleaving the protein into two separate subunits by reduction of disulfide bridges.[34] Thereafter the large subunit with ten peptides and sequence coverage of 94% and the small subunit with three peptides giving sequence coverage of 57% were identified by MALDI-TOF mass spectrometry. The result also demonstrated that the C-terminal peptides were missing in both, the large subunit (ARF) and small subunit (GSN), indicating that cleavage by carboxypeptidases had occurred. This phenomenon is typical for 2S albumins and was described as C-terminal microheterogenity in the literature.[37] Therefore repeated modification of the theoretical sequence by removing three C-terminal amino acids in both subunits was performed. Consequently, the large subunit with 11 peptides and sequence coverage of 100% and the small subunit with five peptides giving also 100% sequence coverage were identified. The molecular weight of Cor a 14.2 was about 12,000 Da which is in accordance with the theoretical mass of 11997 Da. MS spectra of the reduced and alkylated

sample confirmed that the protein was cleaved into two subunits with masses of 8,000 Da and 4,000 Da, which are in agreement with the masses of the two subunits of the 2S albumin. MALDI-TOF mass spectrometry analysis of Cor a 14.5 showed that the molecular mass was similar to that of Cor a 14.2. Unlike Cor a 14.2, the MS spectrum of Cor a 14.5 showed additional peaks around 6,000 Da. These sequence parts could not be assigned to the sequence of the 2S albumin Cor a 14.

Cor a 14 (D0PWG2):

```
        10         20         30         40         50         60
MARLATLAAL FAALLLVAHA AAFRTTITTV DVDEDIVNQQ GRRGESCREQ AQRQQNLNQC

        70         80         90        100        110        120
QRYMRQQSQY GSYDGSNQQQ QQELEQCCQQ LRQMDERCRC EGLRQAVMQQ QGEMRGEEMR

       130        140
EVMETARDLP NQCRLSPQRC EIRSARF
```

Figure 3.17.: Theoretical amino acid sequence of Cor a14 (Uniprot accession no.: D0PWG2, aa 1-147). The signal sequence, which is post-translationally cleaved off is highlighted in grey. Purified Cor a 14.2 was identified by MALDI-TOF analysis: the obtained amino acid sequence corresponding to the small subunit is underlined in blue and the amino acid sequence corresponding to the large subunit is underlined in green. The amino acid residues 23-42 are not present in the mature protein.

3.4. Physicochemical characterisation of hazelnut nsLTP, Cor a 8

The purity of hazelnut nsLTP, Cor a 8 was assessed by 15% SDS-PAGE and Coomassie staining. Under nonreducing conditions a single protein band at about 14 kDa was visible. The protein migrated slightly faster upon addition of DTT and the reduction of disulfide bonds (Fig. 3.18.). The revealed molecular weight seemed too high for Cor a 8 compared with the theoretical molecular weight of 9.5 kDa (Uniprot accession no.: Q9ATH2).

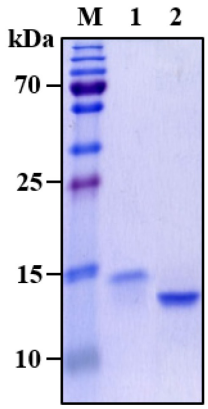


Figure 3.18.: Coomassie stained 15% SDS-PAGE gel of purified Cor a 8 (2 µg/lane). (M) prestained protein marker, (1) analysis under nonreducing conditions, (2) analysis under reducing conditions.

N-terminal sequencing of Cor a 8 was performed to confirm the identity and correct sequence of the protein. The first six amino acids SLTCPQ of the N-terminal part of Cor a 8 were successfully identified (Fig. 3.19.).

Cor a 8 (Q9ATH2):

```
         10         20         30         40         50         60
MGSLKLVCAV LLCMMVAAPV ARASLTCPQI KGNLTPCVLY LKNGGVLPPS CCKGVRAVND

         70         80         90        100        110
ASRTTSDRQS ACNCLKDTAK GIAGLNPNLA AGLPGKCGVN IPYKISPSTN CNNVK
```

Figure 3.19.: Theoretical amino acid sequence of Cor a 8 (Uniprot accession no.: Q9ATH2, aa 1-115). The signal sequence, which is post-translationally cleaved off is highlighted in grey. Cysteine residues of the protein that are linked via disulfide bonds are highlighted in yellow. Amino acid residues underlined in red were confirmed by N-terminal sequencing.

MALDI-TOF MS analysis of native Cor a 8 revealed a protein peak with a molecular weight of 9475.8 Da and some smaller peaks (Fig. 3.20., A). The identified mass of the major peak was close to the theoretical molecular mass of 9476 Da on Uniprot (accession no.: Q9ATH2, average mass, aa 24-115). Then the protein was incubated with the reducing agent DTT and alkylated with IAA. Thereafter the sample gave a major peak with 9887.9 Da, accompanied by a peak of 4736.7 Da corresponding to the doubly-charged protein Cor a 8 (Fig. 3.20., B). The mass difference of additional peaks that formed due to incomplete alkylation of the protein was about 57.05 and corresponds to carbamidomethyl residues.

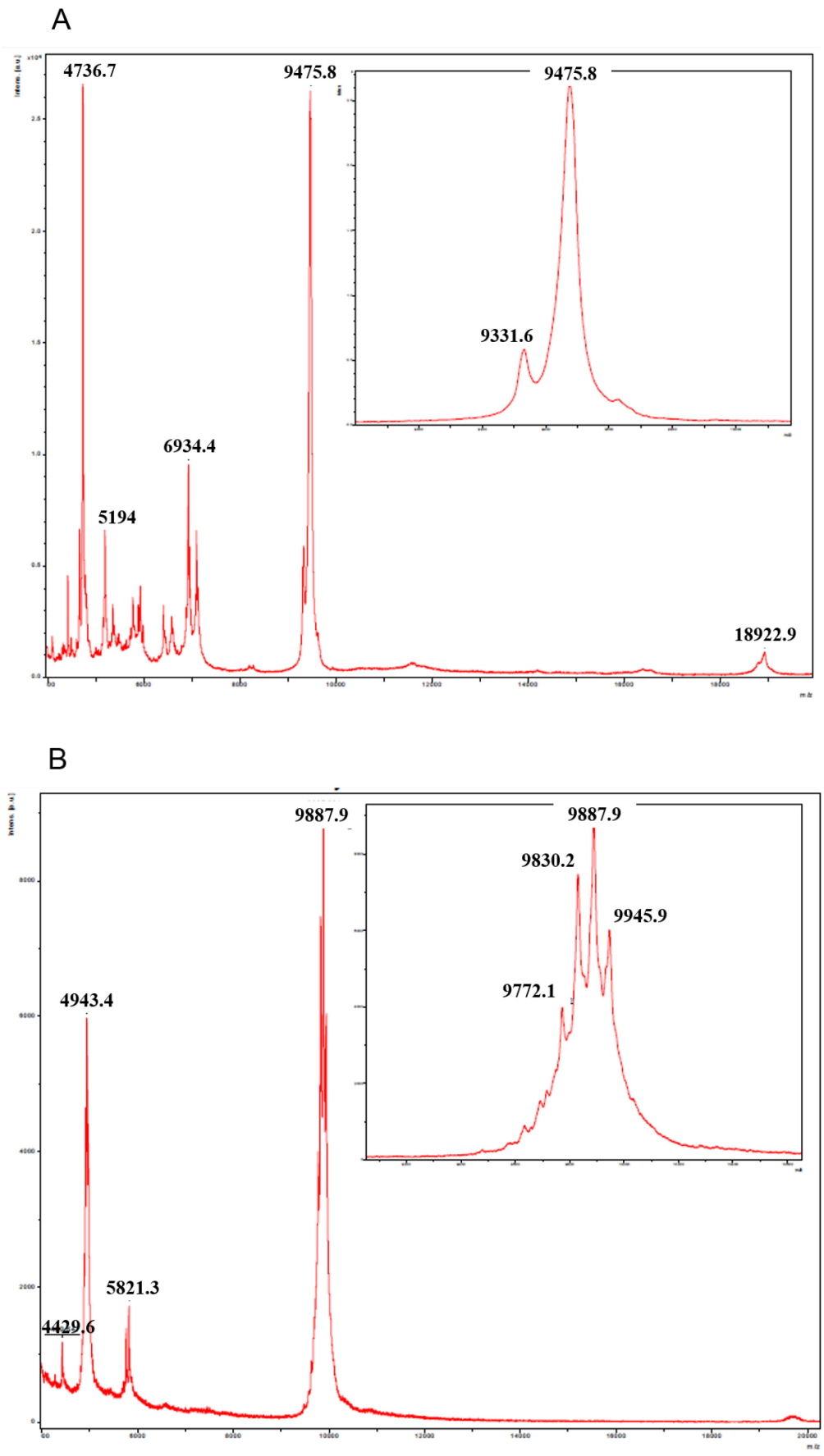


Figure 3.20.: Sequence analysis of purified Cor a 8. (A) native, (B) reduced and carbamidomethylated.

The secondary structure of purified Cor a 8 was analysed by CD spectroscopy (Fig. 3.21., A and B). The CD spectrum was consistent with the established α-helical structure of LTPs, showing a positive maximum around 195 nm and intense negative minima at 208 nm and 222 nm. [124] This result suggested that the purified allergen was in a native folded state and that it contained α-helices as predominant secondary structure. The molar ellipticity of Cor a 8 (Fig. 3.21, B) gave similar spectrum typical for largely α-helical proteins.

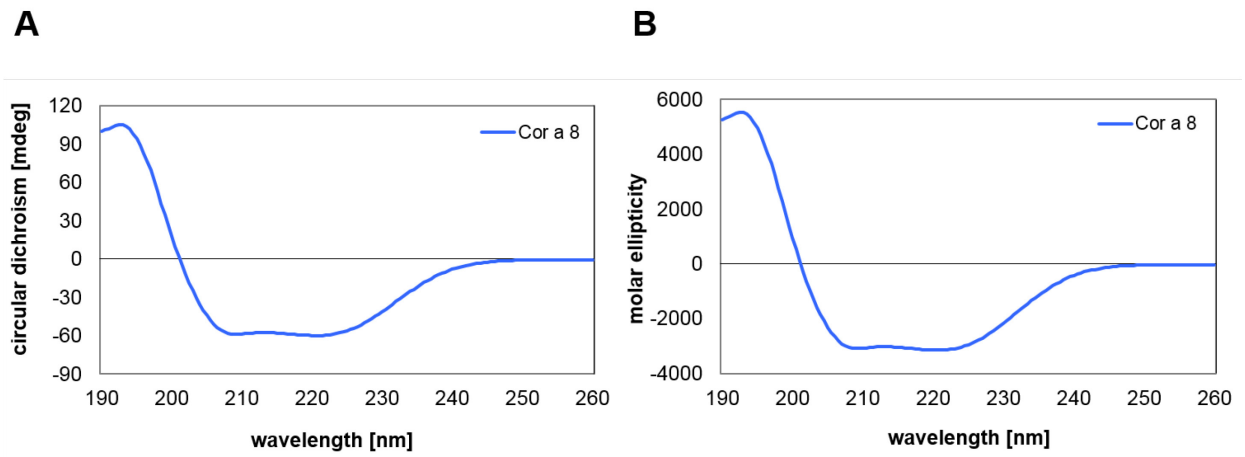


Figure 3.21.: CD spectra of purified nsLTP, Cor a 8. (A) CD spectrum (190-260nm) at RT, (B) molar ellipticity.

3.5. Immunological characterisation of hazelnut prolamins, Cor a 14 and Cor a 8

The allergenic activity of purified allergens was assessed in *in vitro* immunological assays by using selected sera of patients sensitized to Cor a 14 or Cor a 8, respectively (see chapter 2.1.2, Tab. 2.2).

3.5.1. IgE-Enzyme-Linked Immunosorbent Assay

All four batches of Cor a 14 were recognized by specific serum immunoglobulin E (sIgE) of ten hazelnut allergic patients, showing similar intensities dependent on the serum (Fig. 3.22, A-D). Most of the sera reacted with both, native as well as reduced/alkylated allergens, but differences in recognition of conformational and linear epitopes were observed.

ELISA performed with Cor a 14.1 by using serum 1 and 12 showed similar IgE-binding capacities for the native as well as for the denatured protein. In contrast, serum 13 displayed higher IgE-reactivity with

denatured Cor a 14.1. ELISAs performed with Cor a 14.2, Cor a 14.4 and Cor a 14.5 by using serum 1, revealed higher IgE-binding with conformational epitopes of the native protein.

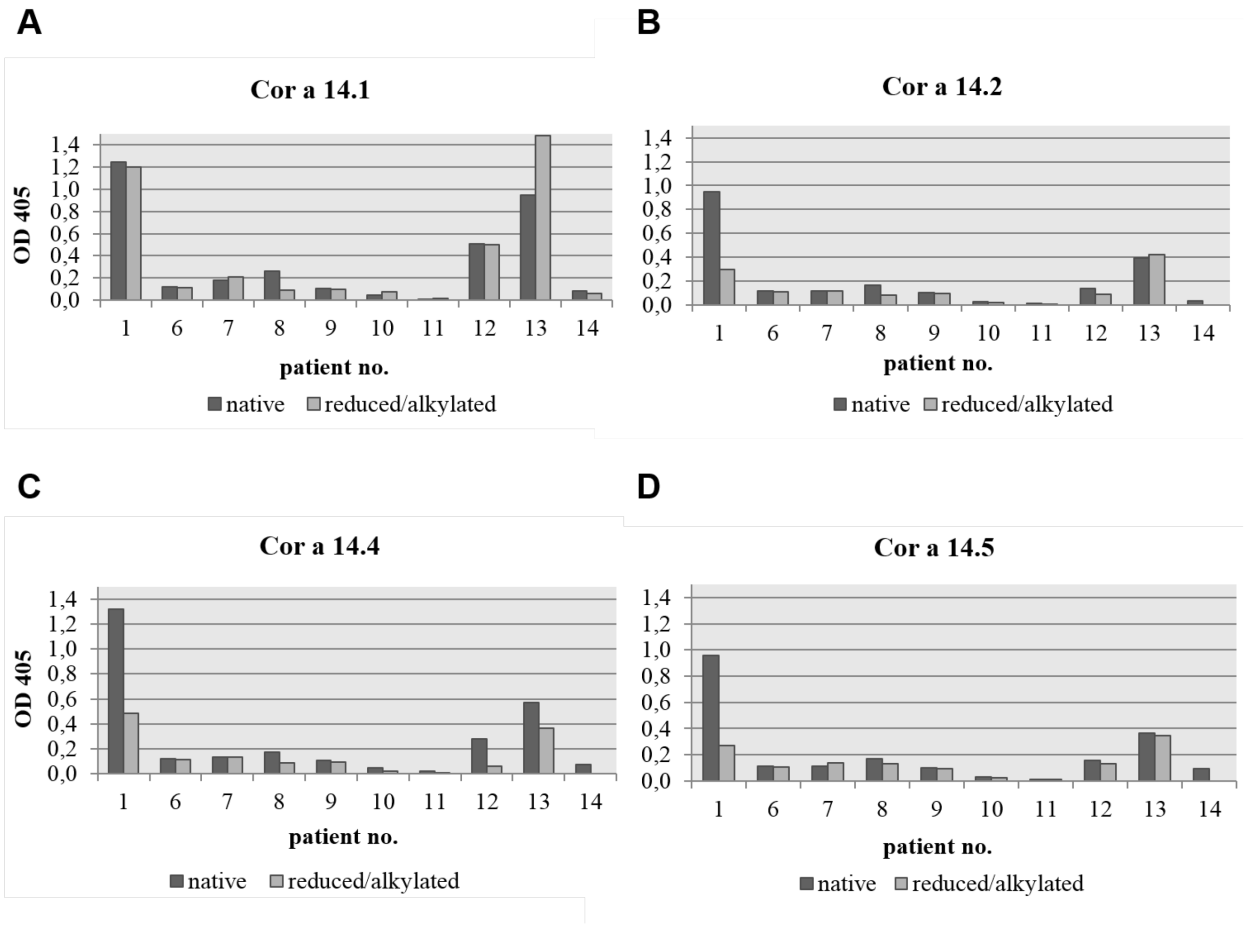


Figure 3.22.: IgE-ELISA analysis of (A) Cor a 14.1, (B) Cor a 14.2, (C) Cor a 14.4, (D) Cor a 14.5.

The IgE-reactivity of native and reduced/alkylated Cor a 8 was tested by using six sera from hazelnut allergic patients (Fig. 3.23). Serum IgE of all six tested sera recognized Cor a 8, but with similar intensities dependent on the serum. Native Cor a 8 displayed higher IgE-reactivity with serum 3, 4 and 5. In contrast, with serum 1, 2 and 12 IgE-binding was equal for the native as well as the denatured protein. In summary, the results from IgE-ELISA experiments suggested that Cor a 14 and Cor a 8 have been successfully purified in their biologically active form. The IgE-reactivity of both proteins was retained after reduction, demonstrating that also linear epitopes were able to bind specific serum IgE of hazelnut allergic patients.

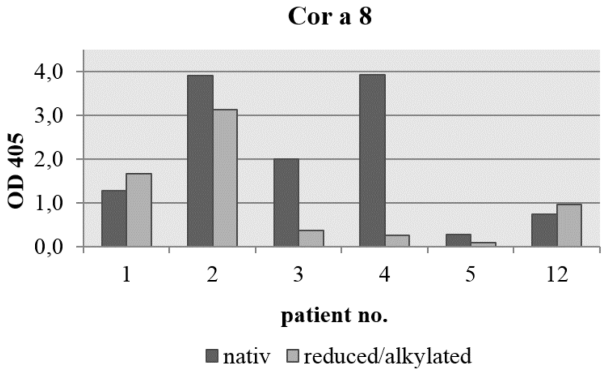


Figure 3.23.: IgE-ELISA analysis of Cor a 8.

3.5.2. Western blotting

Western blotting was performed with purified Cor a 14.1, Cor a 14.5 and Cor a 8 by using two different sera from hazelnut allergic patients and one control serum from a non-allergic donor (NHS), respectively. IgE-immunoblotting of native Cor a 14.1 showed no IgE-binding reactivity with both tested sera, whereas the reduced protein reacted with serum 1 (Fig. 3.24, A). Native Cor a 14.5 was recognized by specific IgE from serum 1, but after reduction no IgE-binding was observed (Fig. 3.24, B). These findings are in accordance with the results obtained by IgE-ELISA. Native protein Cor a 8 was also recognized by IgE from serum 1, but the reduced and alkylated allergen displayed no IgE-reactivity at all (Fig. 3.24, C). Western blotting using Cor a 8 gave a high background with serum 4 thus it was not possible to interpret the signal. The molecular weight of specifically recognized protein bands corresponds to the masses of Cor a 14 and Cor a 8 determined in SDS-PAGE analysis, respectively (Fig. 3.14 and Fig. 3.18.).

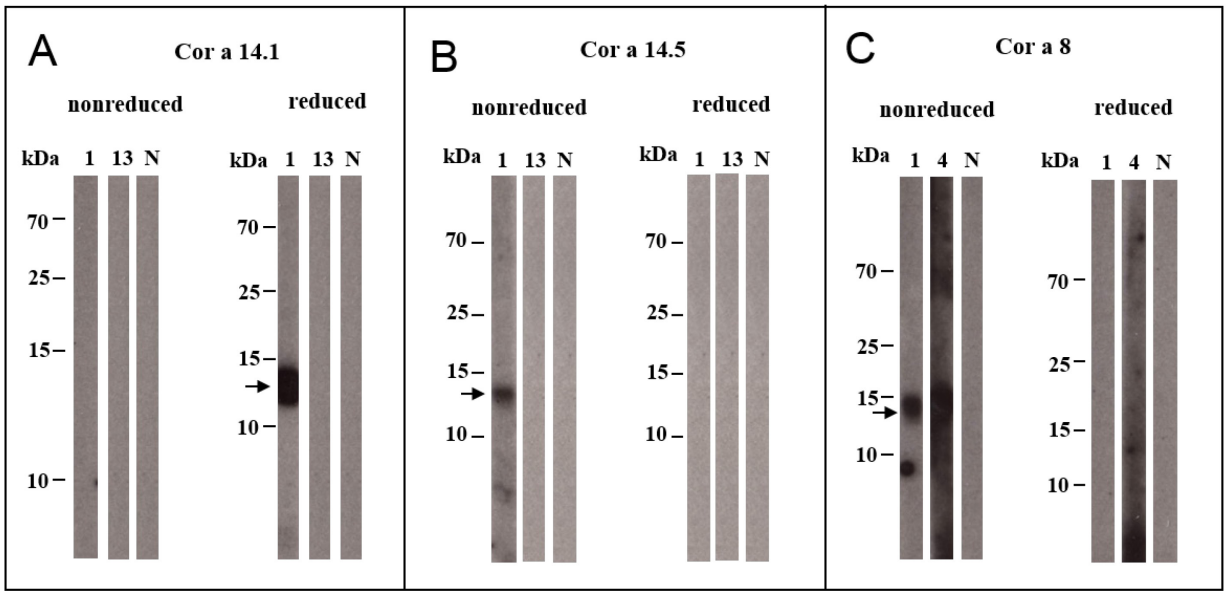


Figure 3.24.: IgE-immunoblot of native and reduced allergens from hazelnut (A) Cor a 14.1 (serum 1, serum 13, NHS), (B) Cor a 14.5 (serum 1, serum 13, NHS), (C) Cor a 8 (serum 1, serum 4, NHS). Recognized protein bands are indicated by arrows.

3.5.3. Rat basophil histamine release assay

After we had determined the IgE-binding of purified hazelnut allergens by *in vitro* immunological assays, their allergenic reactivity was further studied by a cellular assays based on rat basophilic leukemia cells. RS-ATL8 cells were passively sensitized with diluted sera (1:80, 1:20, 1:40, 1:10) of hazelnut allergic patients that contained different levels of allergen-specific IgE and total IgE (see chapter 2.1.2, Tab. 2.2). Sera that displayed highest IgE-reactivity with respective allergens in ELISAs were used for mediator release experiments. For RBL assays with Cor a 14.1 and Cor a 14.5, serum 1 and 13 were selected (Fig. 3.22), whereas for assays with Cor a 8, serum 1, 2 and 4 were chosen (Fig. 3.23). Degranulation was induced by challenging sensitized cells with hazelnut allergens in a dose-dependent manner (0.1-10,000 ng/ml or 1-100,000 ng/ml). In addition, RBL assays using allergens mixed with hazelnut derived oil, with a protein to oil ratio of 1:2, were performed to detect possible differences in allergenic reactivity.
Cor a 14.1 induced dose-dependent mediator release of humanised RS-ATL8 cells with maximum β-hexosaminidase release between 40-80% (Fig. 3.25, A-B, blue graph). Results indicated that degranulation activities of Cor a 14.1 and Cor a 14.1 mixed with hazelnut oil were similar (Fig. 3.25, A and B, red graph). RBL cells stimulated with Cor a 14.5 showed maximum β-hexosaminidase release of

about 30-70% (Fig. 3.25, C-D, blue graph). In case of serum 13, Cor a 14.5 displayed a slightly higher capacity to induce mediator release (approximately 15-20%) when hazelnut oil was added, compared to Cor a 14.5 alone (Fig. 3.25, C-D, red graph).

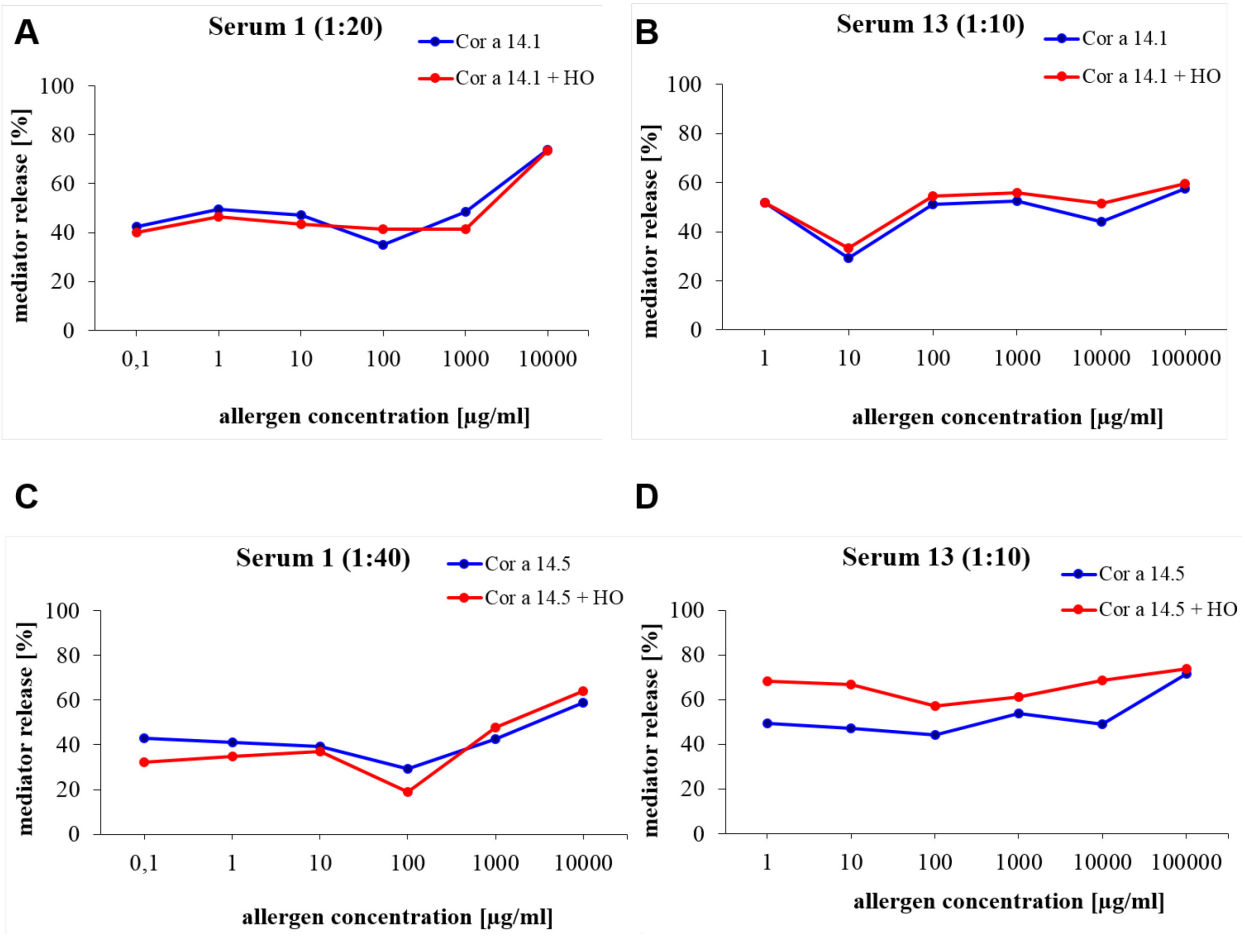


Figure 3.25.: RBL cell assays (RS-ATL8) performed with purified Cor a 14 and Cor a 14 mixed with hazelnut oil (HO) at a ratio of 1:2. Cells were sensitized with sera from hazelnut allergic patients and challenged with different concentrations of allergens (0.1-10,000; 1-100,000 ng/ml). (A and B) Cor a 14.1, serum 1 (1:20), serum 13 (1:10); (C and D) Cor a 14.5, serum 1 (1:40), serum 13 (1:10). Results are expressed as percent of specific β-hexosaminidase release from sensitized cells activated with allergens, relative to β-hexosaminidase activity of lysed cells (total release) and untreated cells (spontaneous release), calculated by $(OD_{experimental\ release} - OD_{spontaneous\ release}) / (OD_{total\ release} - OD_{spontaneous\ release}) \times 100)$.

Purified Cor a 8 was able to induce RBL cell degranulation in a dose-dependent manner (Fig. 3.25, A-C, blue graph). When Cor a 8 was mixed with hazelnut oil an increase in β-hexosaminidase release of approximately 10-20% could be measured for all three sera (Fig. 3.26, A and B, red graph). Result

obtained by mediator release assays demonstrated that hazelnut oil used as food matrix affected the allergenicity of Cor a 8, whereas no significant effect could be found for Cor a 14.

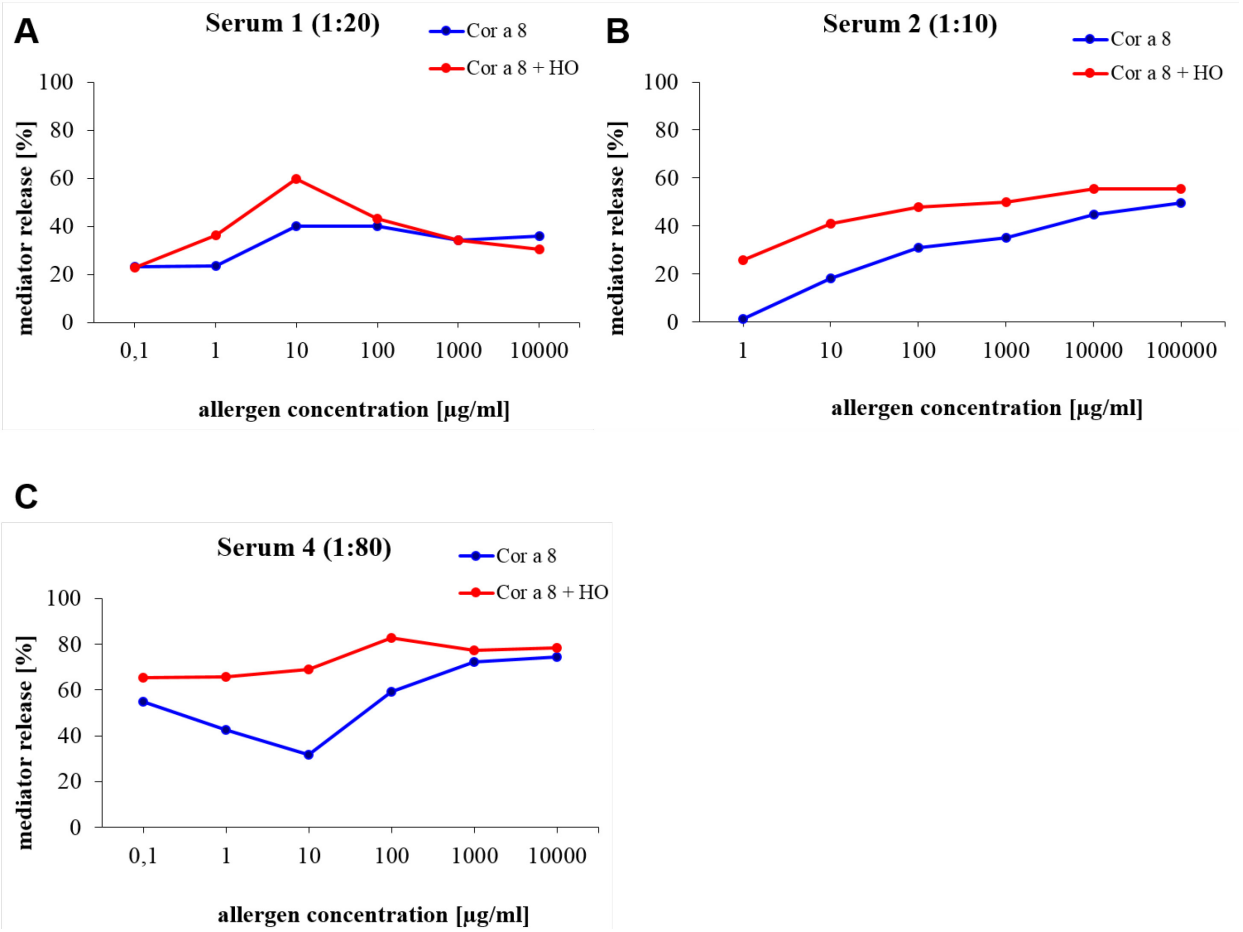


Figure 3.26.: RBL cell assays (RS-ATL8) performed with purified Cor a 8 and Cor a 8 mixed with hazelnut oil (HO) at a ratio of 1:2. Cells were sensitized with sera from hazelnut allergic patients and challenged with different concentrations of allergens (0.1-10,000; 1-100,000 ng/ml). (A-C) Cor a 8, serum 1 (1:20), serum 2 (1:10), serum 4 (1:80). Results are expressed as percent of specific β-hexosaminidase release from sensitized cells activated with allergens, relative to β-hexosaminidase activity of lysed cells (total release) and untreated cells (spontaneous release), calculated by $(OD_{experimental\ release} - OD_{spontaneous\ release}) / (OD_{total\ release} - OD_{spontaneous\ release}) \times 100)$.

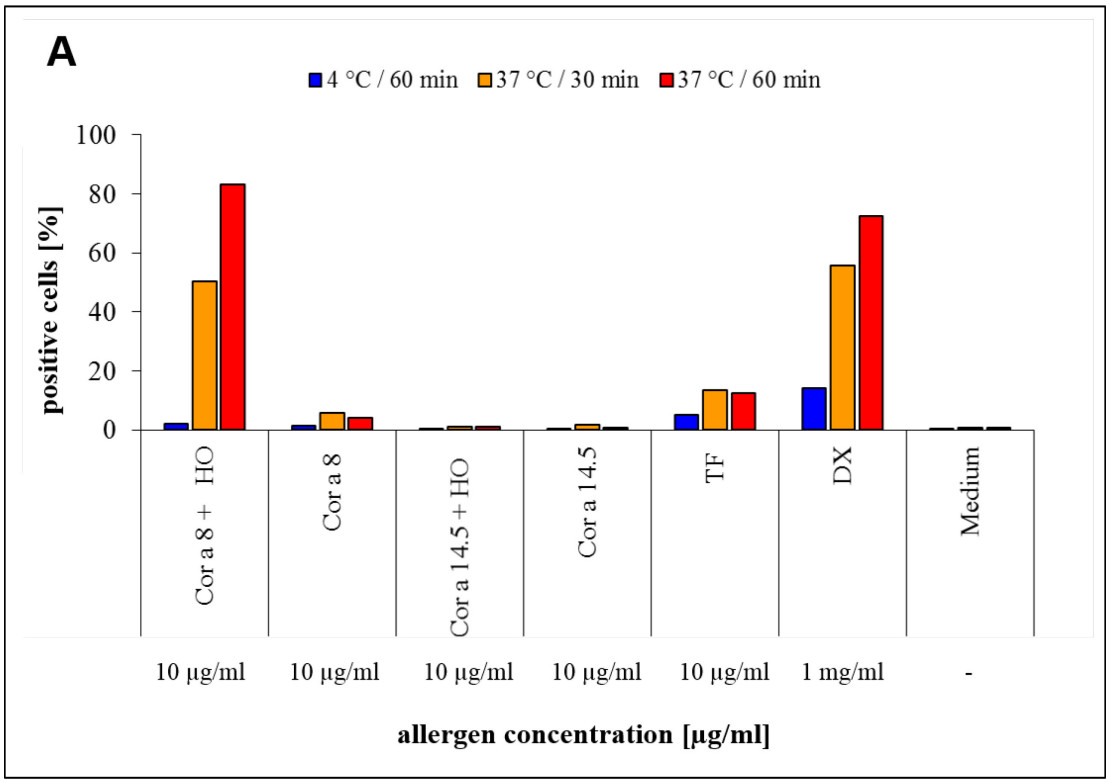
3.5.4. Protein uptake and surface marker expression experiments

Protein uptake of allergenic hazelnut proteins by immature monocyte-derived dendritic cells (MODCs) was investigated by using the cells from two non-allergic patients (NHS1, NHS2). The endotoxin concentration was about 47 EU/mg for purified Cor a 8 and 14 EU/mg for Cor a 14.5 determined by the EndoZyme® recombinant Factor C assay. In order to study the effect of different parameters, immature

MODCs were stimulated with various concentrations (1µg/ml, 10 µg/ml, 100 µg/ml) of fluorescent labelled proteins Cor a 14.5 and Cor a 8. Furthermore, uptake experiments using allergens mixed with hazelnut oil (HO) at a ratio of 1:2 were carried out. Internalisation of labelled proteins after different incubation times (30 min, 60 min) at 37 °C was measured by flow cytometry. To examine the binding of proteins to cell surface receptors, cells were incubated at 4 °C for 60 min. Positive controls for receptor mediated protein uptake (transferrin, TF) and non-receptor mediated protein uptake (dextran, DX) were included (Fig. 3.27, A and B).

It was shown that uptake of Cor a 8 was dose dependent, as the number of positive cells increased from concentrations of 1 µg/ml to 10 µg/ml (Fig. 3.27, B). By mixing Cor a 8 with hazelnut oil 1:2, the percentage of positive cells was higher than without oil, indicating that hazelnut lipids enhanced protein uptake (Fig. 3.27, A). Furthermore, uptake was also time dependent as internalisation of Cor a 8 in combination with hazelnut oil increased from 30 minutes to 60 minutes, with time of incubation.

Regarding Cor a 14.5, weak signals of positive cells were observed at a protein concentration of 10 µg/ml (Fig. 3.27, A). When Cor a 14.5 was mixed with hazelnut oil at a ratio of 1:2, the lowest allergen concentration at which a clear signal could be detected was 100 µg/ml (Fig. 3.27, B).



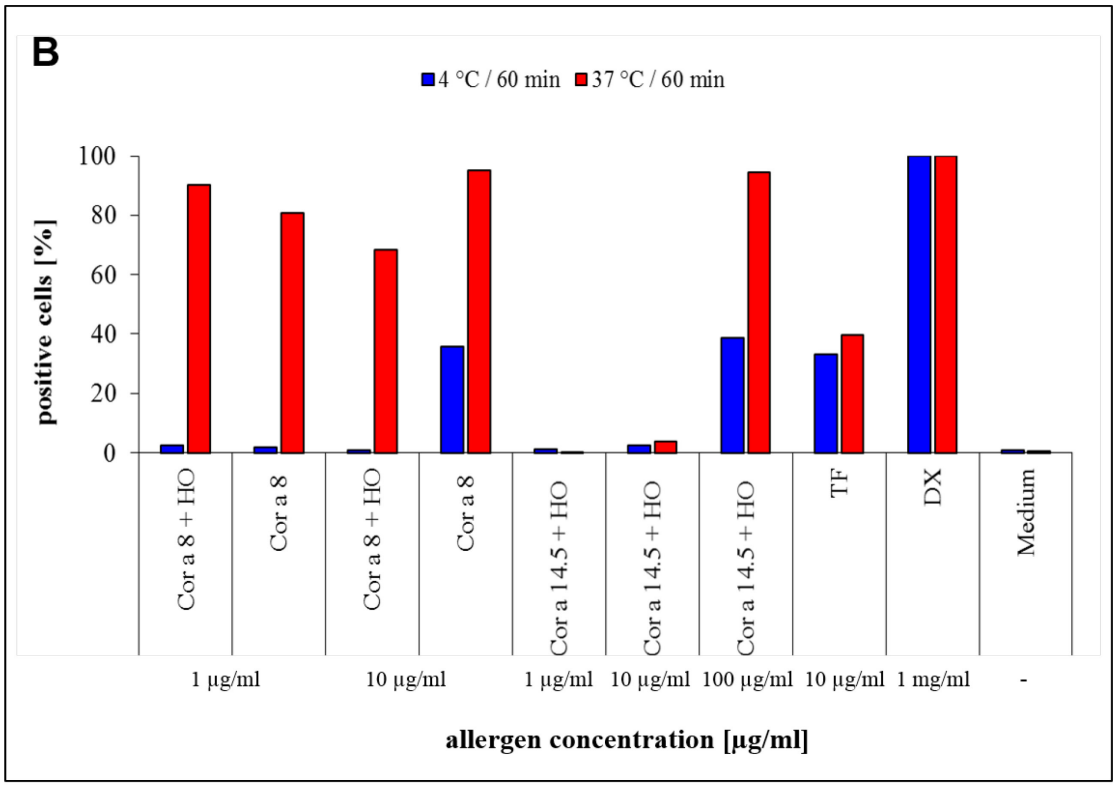


Figure 3.27.: Uptake of Cor a 8 and Cor a 14.5 by MODCs of two non-allergic donors (A) NHS1, (B) NHS2. Transferrin (TF) served as control for receptor mediated protein uptake and dextran (DX) served as control for non-receptor mediated uptake. Cells incubated with medium were used as negative control. Measurements were performed by flow cytometry and protein uptake was expressed as mean percentage of positive cells.

Furthermore, surface marker expression was investigated by using cells isolated from the blood of the same two non-allergic patients (NHS1, NHS2) already used for uptake experiments. Immature monocyte-derived dendritic cells (MODCs) were co-cultured with naïve $CD4^+$ T-cells and stimulated with purified allergens for two days. Therefore co-cultures were incubated with 10 µg/ml of hazelnut allergens (Cor a 14.5, Cor a 8) mixed with nut hazelnut oil 1:2 (10 mg/ml), allergens in combination with maturation factors (MF) as well as allergens mixed with lipids and maturation factors. Staining was carried out with antibodies against cell surface molecules before the surface marker expression was measured by flow cytometry (Fig. 3.28). As expected, MFs induced expression of DC-specific marker CD83 and both costimulatory molecules, CD80 and CD86. Compared to markers CD86, HLA-DR and CD11c, which were significantly upregulated, CD80 and CD83 showed distinct lower expression levels. Similar surface marker expression could be observed for cells, which were incubated with MFs, Cor a 14.5/Cor a 8 as well as with MFs, Cor a 14.5/Cor a 8 and HO. As apparent from Fig. 3.28, hazelnut oil (HO) was able to

activate maturation of dendritic cells. Markers CD86 (65%), HLA-DR (80%) and CD11c (100%) were upregulated, whereas for CD80 (20%) and CD83 (<5%) relatively low expression could be detected. The categories of cells treated with HO, LPS and MFs, showed equal upregulation of markers CD86, HLA-DR and CD11c. In contrast, expression of CD80 and CD83 was significantly lower with HO. When cells were cultured in the presence of Cor a 8 and hazelnut oil increased expression of CD86, HLA-DR and CD11c was apparent. Compared to Cor a 14.5 mixed with HO, major differences were visible for markers CD80 and CD83. Both markers were much stronger upregulated with Cor a 8 than with Cor a 14.

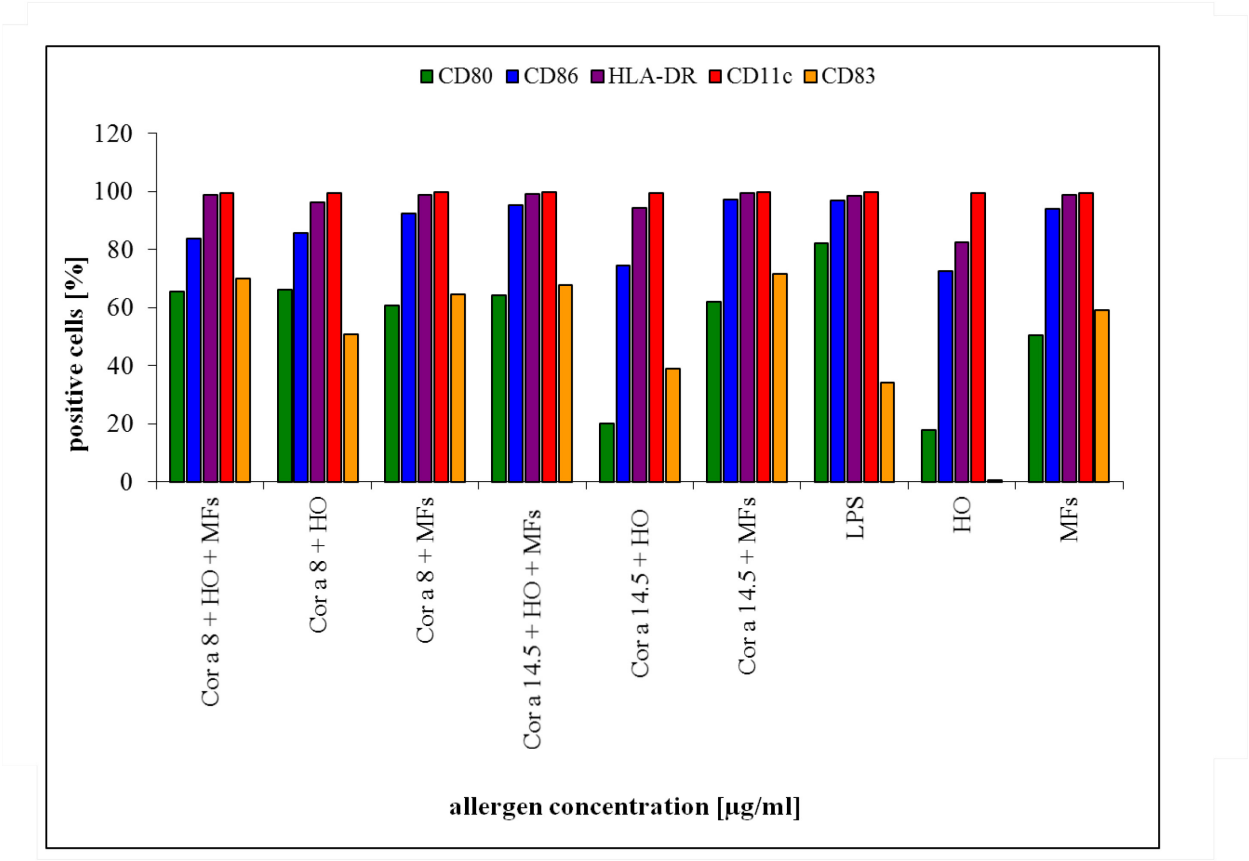


Figure 3.28.: Surface marker expression of MODCs from two non-allergic donors (n=2) stimulated with 10 µg/ml of Cor a 14.5 and Cor a 8 mixed with hazelnut oil (HO) and/or maturation factors (MFs). Results are indicated as mean percent of cells positive for the respective cell surface marker.

4. Discussion

In the present study we purified and characterized relevant hazelnuts allergens, the 2S albumin, Cor a 14 and the non-specific lipid transfer protein, Cor a 8. Subsequently, the allergenic activity of isolated protein batches was investigated in the presence and absence of hazelnut derived oil as food matrix.
We succeeded in purification of Cor a 14 and Cor a 8. The identity and correct sequence of both proteins could be confirmed by MALDI-TOF mass spectrometry and N-terminal sequencing, whereas circular dichroism spectroscopy verified that they were correctly folded. The IgE-binding of purified proteins was confirmed by IgE-ELISA and Western blotting, using sera of hazelnut allergic patients. Additionally, histamine release assays with rat basophilic leukemia cells (RBL-assays) approved the biological activity of both allergens. Furthermore, RBL-assays showed increased histamine release for Cor a 8, but not for Cor a 14, in the presence of hazelnut oil. Preliminary experiments with monocyte-derived dendritic cells (MODCs) indicated that the presence of hazelnut oil may also have an enhancing effect on protein uptake.

In the first set of experiments, 100 g of hazelnuts were defatted by n-hexane treatment before the total protein content was extracted. Both hazelnuts allergens, Cor a 14 and Cor a 8, belong to the superfamily of prolamins, which constitutes a group of plant food allergens soluble in alcohol.[20] Hence the prolamin fraction was isolated from the total protein extract by precipitating globulins upon addition of methanol. The concentration of the resulting prolamin extract was about 9.4 mg/ml in a volume of 50 ml, according to 7% of the total protein extract. Hazelnut oil was isolated from the n-hexane supernatant. Both prolamins, Cor a 14 and Cor a 8 were purified from the total prolamin extract by different ion exchange columns as well as gel filtration chromatography. Therefore, diverging pI values of Cor a 14 (pI 5.6) and Cor a 8 (pI 9.3), lying either in the acidic or basic range, were exploited for target protein separation. We isolated five different protein batches of Cor a 14, with concentrations in a range of 1.9% - 9.6% and one batch containing Cor a 8 with a concentration of 2.1 %, of the total prolamin fraction. The protein contents of Cor a 14 were comparable to those from other 2S albumins, such as Ara h 2 in peanut.[125]
The final, purified batches of Cor a 14 were characterized by physicochemical methods, including SDS-PAGE, 2D-SDS-PAGE, MALDI-TOF MS analysis and CD spectroscopy.

Cor a 14 has a theoretical molecular weight of 15 kDa and a pI of 5.6. The masses of purified proteins Cor a 14.1, Cor a 14.2, Cor a 14.3, Cor a 14.4 and Cor a 14.5 on Coomassie stained SDS-PAGE under nonreducing conditions were in accordance with the theoretical molecular weight of Cor a 14. After reduction, single protein bands split up into a double band of approximately 9 kDa and a smaller band of 6 kDa, which likely represented the two subunits of Cor a 14. These findings were in agreement with a previous study on the hazelnut 2S albumin.[37]

The exact molecular weight of two selected samples, Cor a 14.2 and Cor a 14.5, was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Native Cor a 14.2 had a molecular mass of 12 kDa, which corresponds to the theoretical molecular weight of Cor a 14 without signal sequence. Results of mass spectrometry gave evidence that C-terminal amino acids were lacking. The so called "C-terminal microheterogeneity" is a post-translational modification that has been observed for various other plant species, including Ber e 1, from Brazil nut[126], Ses i 1 from sesame[127] or 2S ASP-Ib from Ricinus communis seeds.[128] It is assumed that the formation of different isoforms may be attributed to shifted positions of the cleavage site during protein maturation, the presence of various protein precursors or due to carboxypeptidases.[129]

MALDI-TOF MS analysis after reduction and alkylation of Cor a 14.2 revealed the presence of two polypeptides with 8,000 Da and 4,000 Da, according to the correct sizes of both Cor a 14 subunits.[37] The cleavage of a large precursor polypeptide into two smaller covalently linked peptides, has been described for a number of other 2S albumins, such as Ber e 1 from Brazil nut[33] and Jug r 1 from walnut.[130]

2D-SDS-PAGE analysis of the Cor a 14 batches demonstrated that purified isoforms differed in their molecular masses and isoelectric points, which could be a result of different post-translational processing. For example, in the Brazil nut 2S albumin, deamination and cyclization of the glutamine residue located on the N-terminal part to pyroglutamic acid occurs, leading to loss of positive charge and lowering of the pI value. In a study of Moreno et al. it has been shown that amino acids at the C-terminal end of both subunits are cleaved off by carboxypeptidases after post-translational processing: serine (Ser) as well as glycine (Gly) are removed from the C-terminus of the large subunit and serine (Ser) from the small subunit.[126]

MALDI-TOF MS analysis of native Cor a 14.5 identified the protein as Cor a 14, with a molecular weight similar to that determined for Cor a 14.2, but additional peaks around 6,000 Da. The isoforms of native as well as reduced proteins Cor a 14.1, Cor a 14.3 and Cor a 14.4 are planned to be analysed in further studies.

Secondary structures of Cor a 14.1, Cor a 14.2, Cor a 14.4 and Cor a 14.5 determined by circular dichroism (CD) spectroscopy indicated that all proteins were correctly folded. Obtained CD spectra were characteristic of largely α-helical proteins, with a low content of β-sheets.[123, 126]

Purified Cor a 8 was characterized by physicochemical methods, including, SDS-PAGE, N-terminal sequencing, CD spectroscopy and MALDI-TOF MS analysis.

The nsLTP, Cor a 8 is an important allergen for hazelnut allergic subjects from the Mediterranean area, that has been described for the first time by Pastorello et al. in 2002.[62, 70] Cor a 8 has a theoretical molecular mass of 9.5 kDa and a pI of 9.3. In contrast to Cor a 14, only one isoform of Cor a 8 could be obtained from the prolamin extract. On SDS-PAGE, the protein migrated at about 14 kDa under nonreducing conditions and run slightly faster upon reduction. A molecular weight deviating from the theoretical mass has also been observed in other studies.[131] Although, the mass on SDS-PAGE was inconsistent with the theoretical value of 9.5 kDa, the correct first six amino acids (SLTCPQ) of the N-terminal part of Cor a 8 could be identified by N-terminal sequencing. In addition, MALDI-TOF mass spectrometry confirmed the identity as well as exact mass of 9.5 kDa. Circular dichroism (CD) spectroscopy demonstrated that the protein contained α-helices as predominant secondary structure according to the typical structure of the nsLTP, as previously described.[124]

The IgE antibody reactivity of purified Cor a 14 and Cor a 8 was examined by IgE-ELISA and Western blotting. Allergen-specific sIgE from most hazelnut allergic patients displayed IgE-binding towards both native allergens. Linear epitopes were generated by means of reduction and alkylation of native proteins. Interestingly, the destroyed protein structure of Cor a 14 and Cor a 8 was still recognized by some sera, which lead to the assumption that IgE-reactive conformational as well as linear epitopes could exist. Also reduced Cor a 14.1, which was visible as rather weak protein band in SDS-PAGE, exhibited relatively high IgE antibody binding. The existence of linear or sequential epitopes exhibiting weaker IgE-reactivity

compared to the conformational protein, has been found for other allergens, such as Pru p 3, the nsLTP from peach[132] and Jug r 1, the 2S albumin from English walnut.[133]

The allergenic reactivity of purified allergens was additionally confirmed in cellular assay with RS-ATL8 cells expressing α, β and γ-chains of the human high-affinity IgE receptor (FcεRI).[120] We found that all tested samples, Cor a 14.1, Cor a 14.5 and Cor a 8 were able to induce histamine release in a dose-dependent manner, by using sera containing Cor a 14 or Cor a 8 specific sIgE. Increased histamine release was observed, when Cor a 8 was associated with hazelnut oil in a stoichiometry of 1:2. In the presence of lipids the cross-linking capacity of the protein was 10-20% higher, indicating an influence of the food matrix. In contrast, degranulation activities measured for Cor a 14.1 and Cor a 14.5 were similar in the presence as well as in absence of hazelnut oil, respectively. Compared to Cor a 8, higher allergen concentrations were necessary to trigger histamine release from passively sensitized RBL cells.

Our experiments showed that the presence of hazelnut oil had different effects on the allergenic reactivity of Cor a 14 and Cor a 8. Firstly, it remains to be elucidated if proteins derive their allergenicity from an association with lipids, or simply due to the presence of these oily food matrix components. Various studies have depicted that 2S albumins from mustard seeds and sunflower seeds can associate with lipids[109, 110] and also nsLTPs are characterized lipid binding proteins.[40, 107] In addition, Bet v 1 like proteins were shown to associate with lipid membranes.[134] Furthermore, in an *in vitro* simulated digestion assay it has been confirmed that gastric phosphatidylcholine (PC) has a protective effect of on grape nsLTPs.[98] A previous study demonstrated that the presence of PC, influences the secondary structure of hazelnut allergen rCor a1 and other Bet v 1 homologous allergens.[135] Thus we speculated that maybe hazelnut oil could alter the protein structure of Cor a 8 in a certain way, leading to increased allergenicity of the protein. In order to reveal differences in structures of native allergens and protein-lipid complexes, three-dimensional protein structures should be determined by crystallography. A further possible explanation for the differing impact of lipids on Cor a 14 and Cor a 8 could be linked with varying contribution of lipids during the sensitization and/or effector phase. Hence it is important to explore the influence of lipids during sensitization and effector phase in allergic reactions towards individual proteins.

Initial protein uptake experiments were performed with immature monocyte-derived dendritic cells (MODCs) from non-allergic donors. Uptake of fluorescently labelled Cor a 14.5 and Cor a 8, analysed by flow cytometry showed that uptake of Cor a 8 was dose dependent and time dependent. For Cor a 8 an enhanced uptake was detected, when the allergenic protein was mixed with hazelnut oil in a stoichiometry of 1:2, suggesting an impact of the food matrix. Therefore a minimum concentration of 10 µg/ml Cor a 8 was necessary. Regarding Cor a 14.5 a tenfold higher protein concentrations was required to measure a clear signal. In contradiction to Cor a 8, no significant change in protein uptake by MODCs could be observed for Cor a 14. This study presents the first attempt to measure and compare the protein uptake of allergenic proteins from hazelnuts, in association with nut derived oil as model food matrix. Our findings demonstrated that this *in vitro* method could provide a useful tool to measure the sensitization potential of allergens in combination with lipids. Studies should be continued with a larger amount of sera from hazelnut allergic patients and improved experimental setup.

Moreover, MODCs of non-allergic donors were co-cultured with naïve $CD4^+$ T-cells and stimulated either with purified proteins (Cor a 14.5, Cor a 8) or proteins mixed with hazelnut oil. In these experiments no significant differences of surface marker expression in T-cells could be detected. Moreover, our data suggested that hazelnut oil activated cell maturation, resulting in upregulation of CD86 (65%), HLA-DR (80%) and CD11c (100%).

In summary, all data confirmed purity, identity, correct structural conformation and allergenic reactivity of purified hazelnut allergens Cor a 14 and Cor a 8. Furthermore, our results indicated increased allergenicity of Cor a 8 in the presence of hazelnut derived oil. Thus, it is important to consider the possible influence of lipids, which are present in food matrices of nuts and other foods, when the allergenicity of individual proteins is determined.

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5. Summary

Introduction: So far, little is known about food matrix-protein interactions and how they modulate immune responses. It has been hypothesized that food compounds, such as lipopolysaccharides, carbohydrates and lipids, may contribute to the allergenicity of certain proteins. Based on these considerations, we studied the impact of hazelnut derived lipids on the allergenic activity of two purified hazelnut allergens.

Methods: In the first set of experiments two important hazelnut (*Corylus avellana)* allergens belonging to the prolamin superfamily, the 2S albumin, Cor a 14 and nsLTP, Cor a 8, were purified by a multi-step chromatographic procedure. Subsequently, final protein batches, containing different isoforms of Cor a 14 and one isoforms of Cor a 8 were characterized by physicochemical and immunological methods, including SDS-PAGE, 2D-SDS-PAGE, CD spectroscopy, MALDI-TOF mass spectrometry, N-terminal sequencing, IgE-ELISA, Western blotting, histamine release assays based on rat basophilic leukemia cells (RBL assays) and protein uptake experiments using monocyte-derived dendritic cells (MODCs).

Results: The percentage of prolamin fraction was 7% of total hazelnut extract and contained about 5% of Cor a 14 and 2% of Cor a 8. We isolated five different batches of Cor a 14, which varied in their molecular weight and isoelectric point. MALDI-TOF MS and 2D-SDS-PAGE analysis of one of these isoforms, Cor a 14.2, showed that the mature protein had a molecular weight of 12 kDa and consisted of two subunits with molecular weights of 8 kDa and 4 kDa, linked by disulfide bonds. CD spectroscopy verified that Cor a 14.1, Cor a 14.2, Cor a 14.4 and Cor a 14.5 were correctly folded, containing α-helices as predominant secondary structure, which is typical for 2S albumins. For Cor a 8 we identified one isoform with a molecular weight of 9.4 kDa, which was also confirmed by N-terminal sequencing. All tested samples of Cor a 14 and Cor a 8 displayed IgE-binding capacity in IgE-ELISA by using sera of hazelnut allergic patients. Both allergens possessed conformational as well as linear epitopes as have been estimated by IgE-ELISA and Western blotting using native and denatured proteins.
Histamine release assays performed with Cor a 14.1, Cor a 14.5 and Cor a 8, demonstrated that all purified proteins induced dose-dependent degranulation of passively sensitized RBL cells *in vitro.* In

contrast to Cor a 14, in presence of hazelnut lipids Cor a 8 showed a 10-20% increased level of histamine release. Uptake experiments with immature monocyte-derived dendritic cells (MODCs) showed enhanced uptake of Cor a 8, when the protein was associated with hazelnut oil.

Conclusion: We established purification protocols for two important hazelnut allergens. Our preliminary experiments pointed out that hazelnut lipids play a role in the allergenic activity of Cor a 8, but not of Cor a 14. It remains to be investigated, how these allergens interact with hazelnut lipids and how lipids influence the sensitization and effector phase of IgE-mediated hazelnut allergy.

6. Zusammenfassung

Einleitung: Bisher weiß man wenig über die Interaktionen zwischen Proteinen und anderen Lebensmittelbestandteilen wie diese die Immunantwort beeinflussen können. Es wird vermutet, dass bestimmte Lebensmittelbestandteile, wie beispielsweise Lipopolysaccharide, Kohlenhydrate und Lipide zur allergenen Wirkung von Proteinen beitragen können. Basierend auf dieser Hypothese wurde der Einfluss von Lipiden der Haselnuss auf die Allergenizität von gereinigten Haselnussallergenen untersucht.

Methoden: In einer ersten Reihe von Experimenten wurden zwei Hauptallergene der Haselnuss (*Corylus avellana*), welche zur Familie der Prolamine gezählt werden, dem 2S Albumin, Cor a 14, und dem nsLTP, Cor a 8, mittels eines mehrstufigen chromatographischen Verfahrens gereinigt. Anschließend wurden die erhaltenen Fraktionen, von Cor a 14 und Cor a 8 durch physikalisch-chemische und immunologische Methoden, einschließlich SDS-PAGE, 2D-SDS-PAGE, Zirkulardichroismus Spektroskopie, MALDI-TOF Massenspektrometrie, N-terminale Sequenzierung, IgE-ELISA, Western blotting untersucht. Weiters wurden zelluläre Histaminfreisetzungsssays und Proteinaufnahme Experimente mit dendritischen Zellen (MODCs) durchgeführt.

Ergebnisse: Der Prozentanteil der Prolamin Fraktion aus dem gesamten Haselnussproteinextrakt betrug 7%, wovon 5% auf Cor a 14 und 2% auf Cor a 8 entfielen. Es konnten fünf unterschiedliche Protein-Isoformen von Cor a 14 mit voneinander abweichenden molekularen Massen und isoelektrischen Punkten isoliert werden. Die detaillierte Analyse einer der Isoformen, Cor a 14.2, mittels MALDI-TOF Massenspektrometrie und 2D-SDS-PAGE zeigte, dass das reife Protein ein Molekulargewicht von 12 kDa aufwies und aus zwei durch Disulfidbrücken verbundene Untereinheiten mit Molekulargewichten von 8 kDa and 4 kDa zusammengesetzt war. Anhand von Zirkulardichroismus-Spektroskopie konnte die korrekte Faltung der Proteine Cor a 14.1, Cor a 14.2, Cor a 14.4 und Cor a 14.5 mit einer Sekundärstruktur, die hauptsächlich aus für 2S Albumine typischen α-Helices bestand bestätigt werden. Von Cor a 8 konnte eine Isoform mit einem Molekulargewicht von 9.4 kDa isoliert werden, deren Identität durch N-terminale Sequenzierung verifiziert wurde. Die Bindung von IgE-Antikörpern konnte

für alle getesteten Proben von Cor a 14 und Cor a 8 durch Inkubation mit Seren von haselnussallergischen Patienten mittels ELISA nachgewiesen werden. Basierend auf Ergebnissen von IgE-ELISA und Western blotting kann angenommen werden, dass beide Allergene sowohl lineare als auch konformationelle Epitope beinhalten.
Histaminfreisetzungs-Assays mit passiv sensibilisierten Basophilen der Ratte zeigten *in vitro*, dass die gereinigten Proteine Cor a 14.1, Cor a 14.5 und Cor a 8 eine dosisabhängige Degranulation herbeiführten. Mit Haselnussöl, im Gegensatz zu Cor a 14, konnte für Cor a 8 eine um 10-20% erhöhte Histaminfreisetzung gemessen werden. Die durchgeführten Experimente mit MODCs erwiesen eine verstärkte Aufnahme von Cor a 8, wenn Haselnussöl hinzugefügt wurde.

Schlussfolgerung: Es wurden Reinigungsprotokolle für zwei wichtige Haselnussallergene etabliert. In vorläufigen Experimenten konnte nachgewiesen werden, dass Haselnusslipide einen Einfluss auf die Allergenizität von Cor a 8, aber nicht auf Cor a 14 zeigten. Es bleibt abzuklären, wie diese Allergene mit Haselnusslipiden interagieren und wie Lipide die Sensibilisierungsphase und Effektorphase der IgE-vermittelten Haselnussallergie beeinflussen.

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8. Appendix

8.1. List of illustrations

8.2. List of tables

8.3. Curriculum Vitae

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Education

10/2011-current	**Studies in Genetics and Molecular Pathology at the University of Vienna** Master Thesis at the Department of Pathophysiology and Allergy Research, Molecular Allergology at the Medical University of Vienna, Thesis on "Impact of the food matrix on allergenic properties of purified hazelnut allergens" in the group of Prof. Karin Hoffmann-Sommergruber
01/2013-02/2013	Internship at the Medical Division for Hematology and Oncology, Hanusch Hospital Vienna
03/2007-09/2011	**Studies in Microbiology and Genetics at the University of Vienna** Bachelor Thesis at Max F. Perutz Laboratories (MFPL), Biochemistry & Biophysics, Immunology & Pathogens at the University of Vienna, Thesis on "Molecular cloning and characterization of picornaviral proteins" in the group of Prof. Tim Skern
09/2005-12/2006	Nursing School, General Hospital Vienna
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