universität wien

DIPLOMARBEIT

“Characterization of proteins with potential allergenic activities from sunflower seeds“

Verfasserin

Claudia Jelinek

angestrebter akademischer Grad

Magistra der Pharmazie (Mag. pharm.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 449
Studienrichtung lt. Studienblatt:	Pharmazie
Betreuerin / Betreuer:	Assoc. Prof. Dr. Karin Hoffmann-Sommergruber

Danksagung

Im Rahmen dieser Diplomarbeit, möchte ich mich bei allen Menschen bedanken, die zum Erfolg dieser Arbeit beigetragen haben.

Ein großes „Danke“ an meine Eltern und meine liebe Familie für ihre finanzielle Unterstützung und dafür, dass sie immer an mich geglaubt haben.

Vielen lieben Dank auch an meinen Lebensgefährten, Gunther Pietsch, der mir immer ein offenes Ohr, unvorstellbar viel Liebe und emotionale Unterstützung gab.

Des Weiterem möchte ich mich bei all meinen lieben Freunden aus Europa und den USA bedanken, die mir immer wieder den notwendigen Ausgleich zum Studium gaben.

Ich freue mich sehr, dass diese interdisziplinäre Diplomarbeit entstehen konnte. In diesem Sinne möchte ich mich insbesondere bei Frau **PROF. DR. KARIN HOFFMANN-SOMMERGRUBER,** FRAU **PRIV. DOZ. MERIMA BUBLIN** vom Department für Pathophysiologie und Allergieforschung der Medizin. Universität Wien, Herrn **PROF. DR. MAG. PHARM. ERNST URBAN**, Herrn **PROF.DR. MAG. PHARM. HELMUT SPREITZER** vom Department Pharmazeutische Chemie, Universität Wien, für die gelungene Zusammenarbeit danken.

Table of Contents

Table of Contents

1 Introduction

1.1 Food Allergy

1.1.1 Classification and terminology of food allergy

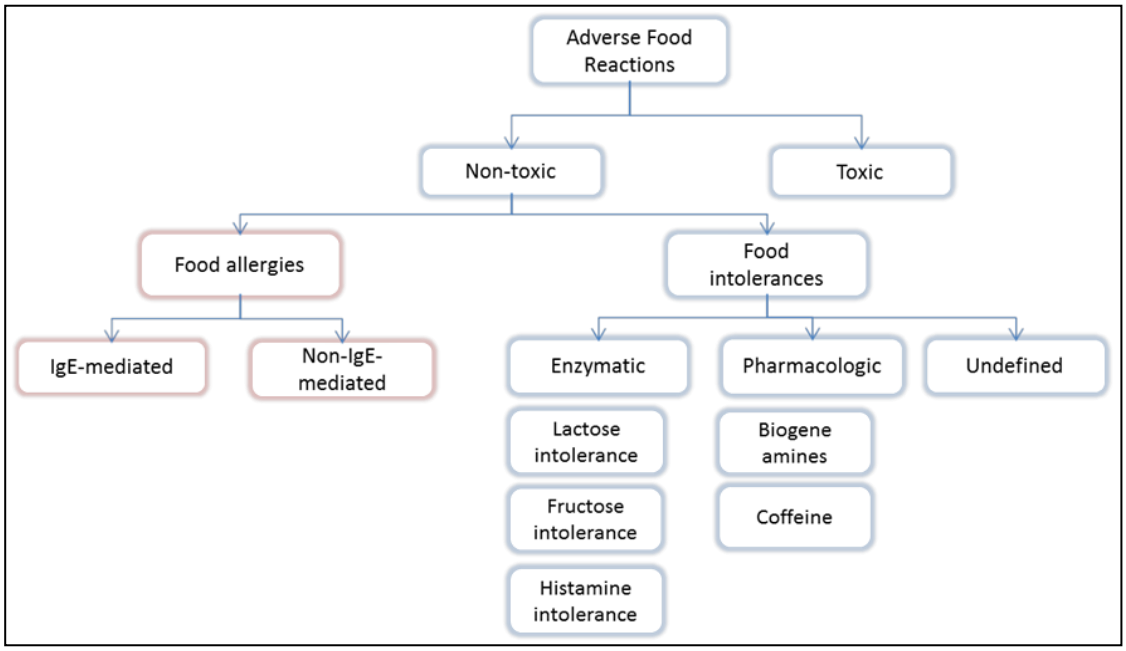


Figure 1.1: Classification of adverse food reactions

At first one can distinguish between toxic and non-toxic adverse food reactions (Figure 1.1). Toxic reactions affect every individual that gets exposed with an effectual amount. Food intoxications can occur from toxic metals such as arsenic, antimony or zinc-compounds. Besides toxic metals, also plants have toxins that can lead to life-threatening situations. For example solanin that can be found in verdant Solanaceae such as tomatoes and potatoes. Another well-known plant toxin is atropine that belongs to Atropa bella-donna. But also microorganism, fungi and fish have toxin we have to be cautious of.
In contrast non-toxic reactions depend on the individuals' susceptibility to certain foods. They can be subdivided into food intolerances and food allergies. Food intolerances aren't immune-mediated but can be matter of enzymatic, pharmacological or undefined issues. Important enzymatic food intolerances are lactose-, fructose- and histamine intolerances. During the last few years public

awareness of these intolerances has increased and certain food products have been developed to increase the quality of life for people with food intolerances. Pharmacological food intolerances can be triggered when food contains a high amount of biogenic amines, such as phenylethylamine, tryptamine and serotonin. But also caffeine can cause pharmacological reactions in some individuals.
Undefined or unspecific mast cell triggering can occur through consumption of lectins, some pharmaceuticals like acetylsalicylates or preservatives like benzoates and sorbic acid.
On the other hand immune-mediated adverse food reactions are called food allergies. They can be IgE-mediated or non-IgE-mediated [1].

1.1.2 Prevalence and possible causes of IgE-mediated food allergy

Food allergy can be provoked either through direct sensitization and subsequent elicitation to food, or cross-reactive IgE responses to inhalant allergens [2].
Genetic, environmental, developmental factors and allergen characteristics appear to influence the onset of food allergy. Family history of atopic diseases [3] and genetic polymorphisms [4] may be examples for genetic factors. The responsible alleles that indicate higher risk for allergies weren't found yet [5]. Studies showed that improved social standards, which correlate with higher hygiene standards, may increase the risk for developing food allergies, including food allergy [6]. For instance, it could be confirmed that children living on a farm with exposure to animals are less likely to develop allergies, than their counterparts living in the same geographic regions. This concept is called "hygiene hypothesis" [7, 8].
Commensal as well as pathogenic microorganisms are probably inducing the development of the gastrointestinal mucosal immune system by stimulating local B- and T-cells. Studies that kept animals in a germ-free environment could show, that their GI mucosal immune system was underdeveloped [9, 10].
Disturbances in the gut flora which can lead to disruption and irregularities in the permeability of the gut's epithelium is another precarious factor for the

development of food allergies, because the antigen transport across the gut barrier will increase [11]. This can be the result of reflux esophagitis [12], gastritis or gastric ulcer [13], and infectious diarrhea [14]. A similar but not pathophysiological condition can be found in infants. Their mucosal immune system is not fully developed yet: proteolytic activity and immature barrier function lead to a decreased luminal breakdown and an increased antigen uptake [15, 16].
The main food allergen sources - milk, wheat, egg, soy, peanut, tree nuts, fish and crustaceae, share common characteristics. All of them can cause allergic food reactions although they come from different origins. These characteristics include: a relatively small molecular weight mostly beyond 70 kDa [17], possible glycosylation residues [18] such as allergens from egg, shrimp, milk as well as inhalable ones are known to be heavily glycosylated. Also water solubility [19] and resistance against digestion and industrial processing [20] is a common feature among the main food allergens.

Epidemiological studies found out that around 2-4% of the population suffer from IgE-mediated food allergy. The prevalence in children is, with 5 - 8%, higher [21, 22]. Interestingly, if people are asked if they have food allergies, 3 - 38% they answer with yes. So there is a large gap between the percentage of people who think they have a food allergy and the percentage of people who are actually diagnosed [21, 22].

1.1.3 Symptoms in food allergy

The most common symptom in food allergy is the oral allergy syndrome (OAS). OAS affects lips, oral mucosa and pharynx [23, 24]. “Symptoms develop within minutes and typically include local itching of lips, tongue, throat, and/or ears and nose and/or swelling (angioedema) of the same areas”. It is often present especially in heat-labile/pepsin-labile plant proteins in patients with pollen-related food allergy. This would be a typical case of cross-reactivity between homologous plant proteins in pollen and vegetable food. Around 75%

of the birch-pollen allergic patients also experience oral allergy to raw fruits such as apple, nuts such as hazelnuts and walnuts and raw vegetable such as carrot and celery [25]. Most allergens that are involved in such cross-reactivity are heat and/or pepsin labile. So most of the patients with OAS can eat offending food cooked without having any symptoms.

IgE-mediated gastrointestinal symptoms may include nausea, diarrhoea, flatulence, vomiting, intestinal hyper-motility and abdominal pain to colonic spasm [26]. Food allergens that induce such symptoms in an individual have to be pepsin stable so their immunological epitopes are still intact.

Symptoms of allergic food reactions can be often seen on the skin. They can manifest in pruritus, urticaria, angioedema and rashes. Atopic dermatitis, a chronically relapsing inflammatory skin disease, is also a possible outcome and common in children with food allergy.

Another organ that can be concerned is the respiratory tract. Rhinoconjunctivitis and bronchospasm may occur in food-allergic patients following inhalation of food dusts or vapours, for example when cooking lobster.

Anaphylaxis is the most severe allergic reaction because it is systemic. It is caused by a massive release of mediators from mast cells and/or basophils throughout the body and can include the symptoms mentioned above, but also hypotension, fast heart rate, collapse and dysrhythmia. Anaphylaxis is always a medical emergency and may be fatal or near-fatal. Current advice to allergic patients is the avoidance of the offending food inclusive the food that might contain the allergen. Epinephrine is the first-line treatment when it comes to anaphylaxis. Patients with anaphylaxis not only have to be trained to wear their adrenalin pen (Epipen®) with them at all times but also to use it correctly. The Epipen® helps quickly but unfortunately Epinephrine gets metabolized really fast. This is due to its similarity to our body’s own adrenaline and therefore it may be necessary to apply another Epipen® [27, 28].

1.1.4 Diagnosis

The right diagnosis helps to prevent unnecessary avoidance of certain foods, which is important for the patient's nutrition. Diagnosis starts with taking a full medical history from the patient and concentrates on past allergic reactions. The next steps usually include skin prick testing and eventually blood tests. Skin prick testing (SPT) is most commonly used to detect food specific IgE. It is cheap, safe, and easy to perform and the test results are available within 15-30 min. To reduce interpatient variability, negative (saline) and positive (histamine 10 mg/ml) controls are included in each test. SPTs for milk, egg, fish or peanuts in children, with food allergies to these edibles, have an excellent sensitivity and negative predictive accuracy (generally >90%), but poor specificity and positive predictive accuracy (50-85%) [1]. The absence of a clinical allergy in the presence of specific IgE may be caused by very low levels of specific IgE, the absence of cofactors, low affinity of specific IgE or a high threshold [29]. Important factors for the reliability of the test is the stability of the allergen extracts, which is often difficult to ensure, especially in fruit and vegetable extracts, because enzymatic processes can still take place. Unfortunately there are no standard reagents for SPT testing [30]. If this criterium isn't fulfilled skin prick tests (SPTs) lead to more false-negative results. In these cases the prick-prick technique should be first choice, in which native foods are directly used [31]. By this method, the lancet will be plunged into the food several times immediately before pricking the patient's skin. The main drawbacks of the prick-prick method are the impossibility of standardization and its dependence on the availability of the fresh food in question.

Blood test are also used a lot to detect allergen-specific IgE-antibodies. Often an allergosorbent-type of assay is used, in which a serum is incubated with an immobilized allergen which is able to specifically bind antibodies. The bound antibodies will then be detected and quantified by an IgE-specific antibody detection reagent.

In another form of assay the serum antibodies will be captured by an immobilized anti-IgE antibody fraction and then covered by a labeled-allergen

reagent that makes the specific binding detectable. In contrast, another assay type, works without immobilization. Serum antibodies are allowed to bind a specific allergen freely in a fluid phase [29].
Afterwards these allergen-antibody-complexes get captured by a labeled anti-IgE reagent. For the accuracy of these assays a few criteria need to be observed, such as antibody isotype-specificity, level of background signals, minimal intra- and interassay variation, absence of nonspecific antibodies and adequate detection limit. And again the stability of the allergen must be maintained [29].
The presence of the structurally similar allergens in different foods and pollens can lead to specific antibody binding to allergens against which the patient doesn't react. It furthermore cannot distinguish between free IgE and IgE bound to mast cells. Only the bound IgE matters for the reactivity. Therefore the positive predictive value of IgE-based tests is generally lower than the negative predictive one.
Hoping for more accuracy, many researchers and medical doctors nowadays also try blood tests for the measurement of mast cell activity. One method would be to measure the amount of histamine release [29].
Oral food challenges are the golden standard in the diagnosis of food allergies, especially for patients who already had positive results to skin prick test and/or blood test. It can help to specify the allergy and therefore prevents an extensive elimination diet. They may be performed open, single - or double - blinded. The double-blind placebo-controlled food challenge (DBPCFC) is suitable mainly for older children and adults. Double-blind means that neither the patient nor the medical doctor/team knows whether or not the patient gets the actual allergen or the placebo. Hence, it is essential to hide the food with the allergen in another food matrix and to make sure that it isn't tasteable. Possible food matrices could be pancakes, pudding or cookies. Loss of allergen's stability through processing and preparation of the food is still a well-known problem in food challenges and must be minimized as good as possible.
Usually it involves giving patient increasing doses of suspected food with destabilization-time in between the doses. The test must be performed in a

facility with the necessary equipment and staff in case of a life-threatening occurrence [29].
Food challenges aren't standard tests yet because they are really time consuming, expensive in clinical resources and also more risky for the patient. They can produce false negative test result, because the challenge procedure usually cannot reproduce the circumstances under which the offending food is usually consumed.
A typical example is the food-dependent, exercise-induced anaphylaxis (FDEIA), in which allergic reactions only appear when the consumption of the allergen is followed by exercise. Other influences are cofactors, like medicine or food matrices, which can alter the absorption rate and release of the allergen [29, 32]. If the information is available, DBPCFCs should always use the most allergenic form of the food that can be given safely to the patient.
However, diagnosis is an important factor for allergic patient so they know the specific allergen that they are allergic to. This makes it easier for the medical doctor to recommend a diet. When the allergen is heat labile, it would be possible for the patient to cook or fry the food before consumption [29].

1.1.5 Therapy

Today the most important treatment for food allergies is abstention from the offending food allergens. A symptomatic therapy with pharmaceuticals is available. Antihistamines will be appropriate in mild forms as on-demand medication. Cromoglicic acid can also be used in mild forms to stabilize mast cells so histamine can't be released so easily. Glucocorticoids must be used together with adrenaline in anaphylaxis treatment but can also be used in severe allergic patients [33].

1.2 Plant Food Allergens

1.2.1 2S albumins as food allergens

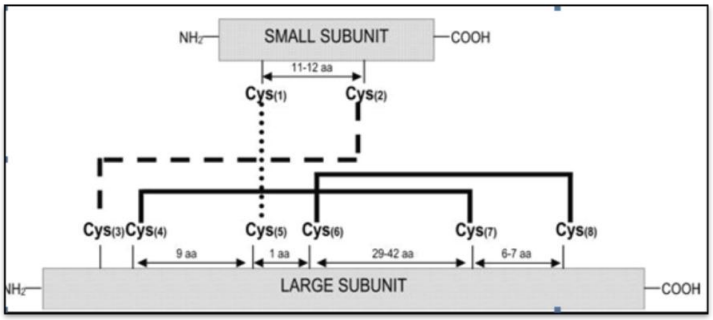


Figure 1.2:Schematic representation of the disulphide bond patterns formed between the eight conserved cysteine residues in the 2S albumin family (figure from the paper of Moreno et al, see reference 40).

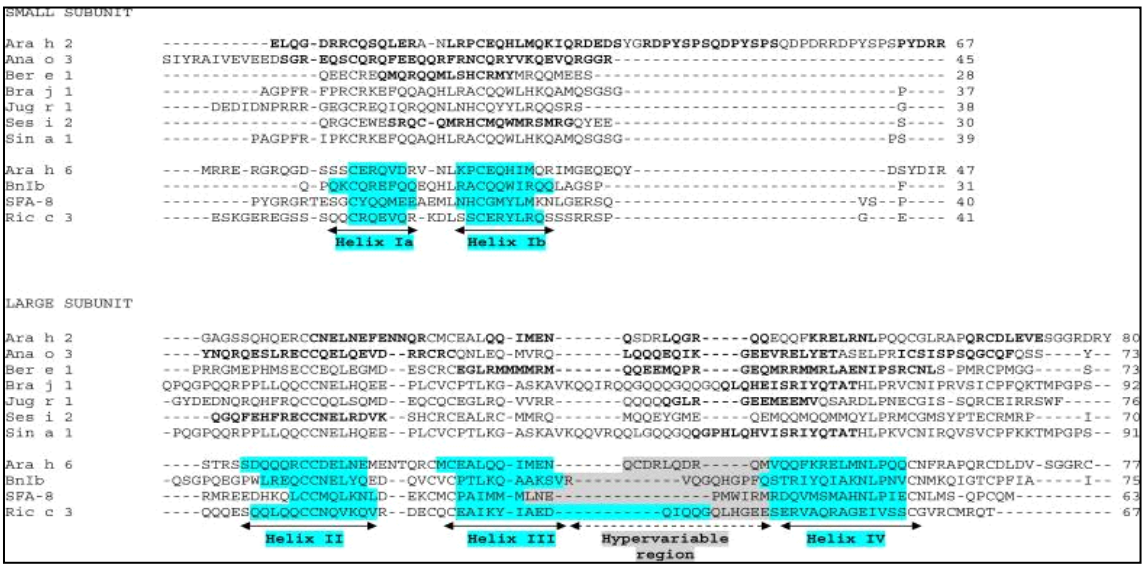


Figure 1.3: Alignment of the small and large subunits of allergenic 2S albumins whose linear IgE-binding epitopes (in bold) have been determined to date (Ara h 2, peanut; Ana o 3, cashew nut, Ber e 1, Brazil nut; Bra j 1, oriental mustard; Jug r 1, English walnut; Ses i 2, sesame seed; Sin a 1, yellow mustard). The alpha-helices Ia, Ib, II, III and IV (blue-shaded) and the hypervariable regions (grey-shaded) (figure from the paper of Moreno et al, see reference 40).

2S albumins belong to the prolamin-superfamily which also include the non-specific lipid transfer proteins , the alpha-amylase/trypsin inhibitors and the prolamin storage proteins [34]. They are a mayor group of seed storage proteins and can be found in both mono- and di-cotyledonous plants. All of the 2S albumins are cysteine-rich, water-soluble proteins with masses around Mr~ 12-15 kDa [35]. As storage proteins, the plant utilizes them as a nutrients source during germination and seedling growth. In recent years, some members

of the 2S albumin family were described as major food allergens. Their ability to bind IgE from allergic patients' sera could also be demonstrated [36, 37].
2S albumins thought to sensitize through the gastrointestinal tract (GIT) which means that they survive the harsh conditions within the GIT. 2S albumins from sunflower [38, 39] and other plants are stable under acidic conditions and maintain their three-dimensional structure (Figure 1.2). Intra-chain sulfide bonds and the characteristic conserved skeleton held to be responsible for that because they may be capable of holding the core protein together (Figure 1.3) [40]. A paper showed that they could also serve as defensive weapon against fungal attacks [41]. Studies showed that the association of 2S albumins with cell membranes or other food ingredients such as lipids or polysaccharides can alter their susceptibility for proteolysis [42]. Van Wijk et al. [43] could support this suggestion by demonstration that purified allergens alone don't induce per se an IgE response in animal models. They indicate that the food matrix has to be taken in account. Another study [44] could show that 2S albumins from Brazil nut and sunflower adsorb to model stomach emulsions which makes them resistant to the pepsin digestion. The binding to bile salts could be the reason which leads to impairment of the duodenal digestion. Within the plant,
2S albumins are synthesized as a larger precursor polypeptide of
Mr~18-21 kDa, which then is co-translationally transported in the endoplasmatic reticulum's lumen. There the formation of four intra-chain disulphide bonds is performed. Afterwards the folded protein gets transported into a vacuole, in which it is subsequently processed to a polypeptide (Mr~12-14 kDa) and eventually to the large and small subunits of Mr~8-10 and 3-4 kDa [45]. But the small and large subunits remain associated by two intermolecular disulphide bonds. An exception of this synthesis would be the sunflower's 2S albumine SFA 8. Its post-translational processing is limited to the removal from the signal-peptide and the pro-region [46].
Fourier transform infrared (FT-IR) spectroscopic and circular dichroism (CD) have shown that 2S albumins from many different plant species, e.g. sunflower [38, 47, 48] and sesame [49] are rich in alpha-helix contents (35-50%). The hypervariable region, a relatively short segment that connects

the alpha-helices III and IV, has been assumed to be the most important allergenic region in 2S albumins [40]. Cross-reactivity in 2S albumins is uncommon although they have a high structural homology. The reason for that could be that they do not resemble each other in the hypervariable region, which is thought of being important for the IgE-binding [50, 51].

1.2.2 Non-specific Lipid-transfer proteins (nsLTP) as food allergens

Lipid transfer proteins are small molecules with a Mr~9 kDa, which share an similar three-dimensional structure, which includes four alpha-helices kept together by four disulfide bridges and is furthermore linked by flexible loops [52]. This feature provides a hydrophobic cavity that can house different lipids, such as phospholipids, and transfers them across membranes. This makes LTPs important in formation of the cellular membrane, but studies could also show that they play a role in the antibacterial and antifungal defense [53]. LTPs can usually be found in the outer cell layers, and show induced production when confronted with endogenous stress hormones, which emerge from the plant in response to osmotic stress, low temperature or after wounding [54]. Cause of their stability to heat and low pH, and their resistance to proteolysis they can be considered “ideal” food allergens, capable of sensitizing the GALT [55]. Cross-reactivity among LTPs from different plants could be documented and found in many LTP-allergic patients. Sensitization to LTPs is especially common in Mediterranean areas but less frequent in Northern Europe [54]. Recently, a case study in Japan presented a patient with anaphylaxis due to LTP in sunflower seeds [56].

1.3 Helianthus annuus (sunflower)

Helianthus annuus belongs to the botanic family of Compositae. It is one of the most relevant oil crops globally and is grown on more than 21 million hectares worldwide [57]. Sunflower seeds are widely used for the production of

margarine, cooking and salad oils, as an ingredient in cereal and bread and as emulsions-stabilizer, cause of its ability to form stable emulsion in oil/water mixtures [58]. In Mediterranean countries sunflower with hull are roasted and salted and used as snack.

To date, no specific allergen has been characterized to be the major one in the sunflower seeds. 2S albumins and LTPs, which are main food allergens in other seeds and nuts, such as Brazil nuts [59] and walnuts [60], could also be found in sunflower seeds and therefore get focused in research for their allergenicity. Studies found out that different sunflower allergic patients recognize different IgE-binding proteins in sunflower seed extract. One has to mention that there aren't a lot of sunflower allergic patients, which makes it difficult to determine a major allergen. However, a few anaphylactic reactions have been reported after consuming sunflower seeds [61], sunflower oil [62] and honey containing sunflower pollen [63]. Rhinitis and asthma could be seen in workers exposed to sunflower pollen [64, 65], people living in sunflower-growing areas [65] or are exposed to sunflower seed dust [66], such as baker but also people with pet birds [67].

Mature sunflower seeds contain about 20 – 40% protein, of which the 2S albumins build the major component [48]. RP-HPLC allows us to separate about 13 different 2S albumins from sunflower seeds [46, 68]. Two 2S albumins, SFA 7 and SFA 8, are closely related to each other, having similar masses (about 12,1 kDa), similar amino acid compositions [46] and identical N-terminus amino acid sequences [48].

Microcalorimetry measurements indicated that SFA 8 is thermo stable above 100°C. LTP from sunflower seeds is also stable to heat but showed structural changes after cooling to 20°C [69].

LTP has the shortest retention time when using RP-HPLC, meaning that it has a lower surface hydrophobicity than the other 2S albumins in sunflower seeds [69]. Due to its exclusion from the interface [58], LTP could be shown to be less surface active and therefore has a limited emulsifying capacity.

Conversely, SFA 8, has the longest retention time and was capable of forming stable emulsions with small droplet size [69]. Previous studies found out that SFA 8 not only has a high proportion of hydrophobic residues, including 16 methionines out of 103 residues, but also possess a single tryptophan residue, which is exposed and inserted into the oil phase, following adsorption to the oil/water interface [48]. The indole group of Trp76 is surrounded by 4 of these methionines, forming a crown [70]. The surface activities from 2S albumin fractions, A, B and C were in between of LTP and SFA 8 [69].
Another test was carried out to assess the stability of SFA 8 in simulated gastric fluid (SGF). Native SFA 8 was significantly more stable that BSA, persisting up to 30 min after the SGF's addition.

1.4 Aim

The aim of this diploma thesis was establishing an easy and efficient purification protocol for low molecular proteins from sunflower seeds which are known to be hypoallergenic. We then characterized the purified proteins by various methods like mass analysis.
With immunological characteristics, such as IgE binding and interaction of the proteins with human immune systems, we will compare the uptaking of these purified sunflower proteins to highly allergenic food proteins, such as Ara h 2.

2 Materials and Methods

2.1 Materials

Chemicals were obtained from the suppliers listed in the following table.

2.1.1 Chemicals

Material	Supplier
PlusOne DryStrip cover fluid SP Sepharose™ Fast Flow	Amersham Bioscience AB, Uppsala, Sweden
AKP Mouse Anti-Human IgE	BD Pharmingen, San Diego, Ca
BCIP (5-Bromo-4-chloro-3-indolylphosphat) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate) DTT (ditiothreitol) Glycine NBT (Nitro blue tetrazolium) Tris(hydroxymethyl)aminomethane, ultra pure	Biomol GmbH, Hamburg, Germany
Acetonitrile Coomassie Brilliant Blue SDS ultra pure Sodium acetate TEMED Urea	Carl Roth GmbH and CO.KG, Karlsruhe, Germany
Swine anti-rabbit-Immunoglobulins	Dako A/S, Denmark
IPG buffer 3-10 Phenyl Sepharose™ Fast Flow Q Sepharose Fast Flow	GE Healthcare, Uppsala, Sweden
Ammoniumperoxidosulfate (APS)	Gibco BRL, MD, USA

Cellstar®Tubes 50 ml	Greiner bio-one
Filter papers	Macherey-Nagel, GmbH&Co KG, Germany
Acetone Acrylamide Ammonium sulfate Bis-acrylamide Bromophenolblue CertiPUR® ph7 and ph9 Dimethylformamide HCl, 37% Iodoactamide Isopropanol Methanol $MgCl_2\,6H_2O$ $Na_2HPO_4\,2H_2O$ NaCl $NaH_2PO_4\,H_2O$ $NaHCO_3$ NaN_3 NaOH n-Hexane Thiourea	Merck, KGaA, Darmstadt, Germany
Centrifugal Filter Units Amicon® Ultra	Millipore, Ireland
BSA (bovine serum albumin)	PAA, Pasching, Austria
Parafilm	Pechiney Plastic Packaging, Menasha, WI, USA
Trifluoracetic acid	Pierce, Rockford, Illinois, USA
SIGMAFAST™ p-Nitrophenyl phosphate Tablets Tween 20	Sigma-Aldrich Co., St. Luis, MO, USA
Spectra/Por® Dialysis Membran,	Spectrum Laboratories Inc., Rancho

MWCO: 3,500	Dominguez, CA
Agarose	StarLab GmbH, Ahrensburg, Germany
Page Ruler Prestained Protein Ladder	Thermo Scientific
Centrifuge tubes 15 ml	TPP, Switzerland
Acetic acid Ethanol absolute	VWR, Fontenay-sous-Bois, France

Table 2.1.

2.1.2 Patient's sera

Out of the serum bank from the Dept. of Pathophysiology we used the serum from a 44 year old male patient who showed clinical symptoms of allergy after being exposed to pumpkin, sunflower and linseeds. ImmunoCap analysis determined, > 100 kU/l of sunflower specific IgE. (The cutoff value is < 0.1 kU/l). As a negative control, we used three different sera from non-allergic subjects. Storage temperature for all serum samples was -20 C.

2.1.3 Allergens and Extracts

Sunflower 2S albumins: SFA 7 and SFA 8

SFA 7/8 were kindly provided by EuroPrevall (EuroPrevall project (FOOD-CT 2005 - 514000)). Purified SFA 7 and SFA 8 were kindly provided by Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK. In addition we used a purified SFA 8 which was kindly provided by Prof. Marcos Alcocer, School of Bioscience, University of Nottingham, UK.

Sunflower nsLTP

Sunflower nsLTP was kindly provided by EuroPrevall (EuroPrevall project (FOOD-CT 2005 - 514000)).

2.2 Methods

2.2.1 Protein extraction from sunflower seeds

Protein extract from sunflower seeds was prepared by defatting sunflower flour, following extraction in aqueous buffers and precipitation with ammonium sulfate.

Sunflower seeds (540 g) were frozen immediately with N_2 and stored at -80°C for 2 days. Then the seeds were grinded to a homogenous powder and defatted with n-Hexane. Per 100 g sunflower seed flour, 300 ml n-Hexane was used. This suspension was stirred for 30 min under the hood and was then centrifuged (Ultracentrifuge SORVALL) at 12,000 rpm for 10 min at room temperature. Afterwards n-Hexane was disposed and, the procedure repeated twice.
After extraction, the wet sunflower seed flour was spread onto a horde for drying. The dried flour was then extracted with 4,000 ml 50 mM sodium phosphate buffer overnight at 4°C under constant stirring.

Sodium phosphate buffer

> 50 mM sodium phosphate buffer, pH 7.0 containing:
> di-sodiumhydrogenphosphate-dodecahydrate ($Na_2HPO_4\ 12H_2O$)
> adjusting the pH with sodium-dihydrogenphosphate-dihydrate ($NaH_2PO_4\ 2H_2O$) diluted with water

On the next day, the extract was centrifuged at 12,000 rpm for 60 min at room temperature. The supernatant was then filtrated.

Ammonium sulfate precipitation

Ammonium sulfate is an often used precipitant for salting out proteins. When adding a high salt concentration the protein surfaces neutralize, which leads to their aggregation. They tend to build large complexes and precipitate easily. Since proteins differ in their solubilities at high ionic strength, the ammonium sulfate precipitation is a useful tool for the purification of desired proteins. The concentrated ammonium sulfate solution is also protecting the proteins against bacterial growth and denaturation.
Ammonium sulfate precipitation was carried out by adding the salt to the filtered extract solution, using 90% saturation.

$(NH_4)SO_4$ precipitation (90%)

90% solid $(NH_4)SO_4$ was added stepwise to the supernatant (1045.97 g $(NH_4)SO_4$ to 1600 ml supernatant) under constant stirring. After adding all the salt, the mixture was stirred for another 30 min.
The precipitated fraction was separated from the supernatant by centrifugation at 12,000 rpm for 20 min at room temperature and then dissolved in 20 mM Tris/HCl, pH 7.8. The tubes with dissolved pellets were stored at -20°C.

Tris/HCl buffer

20 mM Tris/HCl buffer, pH 7.8 containing:
20 m Tris (Tris(hydroxymethyl)aminomethane)
adjust pH to 7.8 with HCl (Hydrochloric acid)
add ddH_2O to 1,000 ml

The pellets were defrosted, resuspended and centrifuged again at 18,000 rpm for 60 min at 4°C. Developing pellets were disposed and the supernatant was sterile-filtrated before desalted by dialysis.

2.2.2 Protein purification

Methanol and Acetone precipitation

Pellets were defrosted, resuspended and centrifuged at 12,000 rpm for 60 min at 4°C. Then the supernatant was dialysed in 20 L 20 mM Tris/HCl-buffer, pH 7.8 + 0,5 M NaCl overnight.
On the following day, the dialysed sample and methanol were both cooled to 0°C, prior to the addition of cold methanol to precipitate sunflower seed globulins. This mixture was then left stirring at 4°C for about 30 min. Afterwards it was centrifuged (Ultracentrifuge SORVALL) at 12,000 rpm for 30 min at 2°C. The albumin-rich supernatant was furthermore precipitated with 3 volumes ice cold acetone (-20°C) and incubated for 16 h at -20°C. Incubation was followed by centrifugation at 12,000 rpm for 30 min at 2°C, and extensive dialysis against water for 48 h.
After dialysis the sample was again centrifuged as mentioned above, before lyophilisation.

Desalting by Dialysis

Dialysis is a well known method for desalting or changing buffers.
Dialysis-membranes come in different cut-off sizes. They allow molecules beneath the MWCO,(molecular weight cuff-off), to diffuse, while they hold back molecules above the MWCO.
Dialysis membranes are often conserved in NaN_3 and therefore it has to be rinsed carefully before usage. The protein sample can then be directly filled into the dialyse membrane-tube. It is recommended to only fill up to two - thirds of the tube because sample volume can increase during dialysis process. Now it can be placed into a beaker glass with the desired buffer. The diffusion rate is determined by diffusion gradient, surface size of the membrane and temperature.
For an efficient desalting, constant stirring but also buffer change is essential [71].

The supernatant was dialyzed (Molecular porous Dialysis Membrane, MWCO: 3,500) against 10 L of 20 mM Tris-HCl buffer, pH 8.0 at 4°C overnight. Another centrifugation (Ultracentrifuge SORVALL) followed at 18,000 rpm for 30 min at 4°C. Afterwards pH was controlled and again and the sample again sterile-filtrated.

Ion exchange chromatography

Ion-exchange chromatography can separate molecules by their charge. Separation is based on reversible interactions between the charged molecules and an oppositely charged chromatography medium. Anion and cation exchange columns are available, which differ in the charge of the covalently linked molecule on the matrix.
First, conditions are selected to let the molecules of interest bind to the medium during loading the sample into the column. Then conditions are changed, mostly through increasing ionic strength (salt concentration), so the bound molecules can be eluted [72].

Procedure:

Q Sepharose™ Fast Flow Chromatography column matrix (GE Healthcare, Uppsala, Sweden) was used to pack a 20 cm long column with a diameter of 1 cm.
This strong anion exchanger works with a quaternary amine group covalently bound to a highly cross-linked agarose base matrix. The column was closed on the bottom and its leak-tightness was tested before the medium was mixed with water and filled in the prepared column. The column matrix was washed with water and equilibrated with buffer A and B using a peristaltic pump.

Buffer A: 20 mM Tris/HCl, pH 8.0
Buffer B: 20 mM Tris/HCl, pH 8.0 + 1 M NaCl

The buffers were both filtered and degassed before applying to the column.
The prewashed column was connected to Äkta, a fast-performance liquid chromatography (FPLC) apparatus (GE Healthcare Amersham Bioscience AB, Uppsala, Sweden).
The dialyzed extract from sunflower seeds was loaded onto the column at a flow rate of 1 ml/min. While loading flow-through and wash were collected separately.
After elution of unbound proteins with start buffer, proteins were eluted by a linear gradient from 0 to 50% buffer B within 100 min at a flow rate of 1 ml/min and samples were collected in 1 ml fractions. Finally, the column was washed with 100% B eluent for 14 min with a flow rate of 1 ml/min. A single path monitor UV-1 (GE Healthcare) recorded the absorbance of the eluting fractions at 280 nm.
The column was re-equilibrated with the peristaltic pump with ddH_2O and stored in 20% ethanol.

HighTrap Capto Q is a strong anion exchanger column. Dynamic binding capacity is >100 mg BSA/ml medium or >150 mg ovalbumin/ml. Its maximum back pressure is up to 0,3 MPa (3 bar). It is a strong quaternary ammonium anion exchanger linked to a chemically modified high-flow agarose matrix.
This chromatography was performed at a FLPC wash station (GE Healthcare).

Buffer A: 20 mM Tris/HCl, pH 7.5
Buffer B: 20 mM Tris/HCl, pH 7.5 + 1 M NaCl

After connecting to the FLPC, the column was loaded with elution buffer and washed with start buffer. The sample was applied and flow-through and wash were collected.
Proteins were then eluted by a linear gradient from 0 to 50% buffer B within 20 min at a flow rate of 1 ml/min. 1 ml fractions were collected.
The column was re-equilibrated with ddH_2O and stored in 20% ethanol.

Hydrophobic Interaction Chromatography (HIC)

Protein surfaces have hydrophilic as well as hydrophobic areas. Therefore hydrophobic ligands on HIC-medium can capture these hydrophobic areas of proteins. In pure water, the hydrophobic effects would be too weak for any protein-HIC medium interactions. To enhance the adsorption to the medium, salt, such as ammonium sulfate, will be added to the sample and to buffer A. Buffer B can then be used to decrease the salt concentration and therefore loosen the binding.

Procedure:
The column was packed with Phenyl-SepharoseTM 6 Fast flow material (procedure as described above) and equilibrated with buffer A by peristaltic pump.

Buffer A: 20 mM Tris/HCl, ph 8.0 + 1 M ammonium sulfate
Buffer B: 20 mM Tris/HCl, ph 8.0
The buffers were filtered and degassed before applying to the column.

The prewashed column was connected to Äkta, FPLC apparatus (GE Healthcare, Amersham Bioscience AB, Uppsala, Sweden).
1 M ammonium sulfate was then added to the dialyzed and selected, pooled fractions from the Sepharose Q column. The pool was loaded onto the column at a flow rate of 1 ml/min. After loading the sample flow-through and wash were collected separately in 14 ml tubes. At the same time a single path monitor UV-1 (GE Healthcare) recorded the absorbance of the eluting fractions at 280 nm.
After elution of unbound proteins with start buffer, proteins were eluted by a linear gradient from 0 to 50% buffer B within 100 min at a flow rate of 1 ml/min. 1 ml fractions were collected.
A 100% B elution was collected in the end for another 14 min with a flow rate of 1 ml/min.

The column was connected to the peristaltic pump and re-equilibrated with the peristaltic pump with ddH_2O and stored in 20% ethanol.

Gel filtration

Procedure:

In gel filtration molecules are separated according to their size. The column material is porous, which means, that little molecules fit into these pores and therefore get retarded, while bigger ones do not fit and pass through the column much quicker. So, unlike IEX, there is no binding to the column matrix involved and moreover only one buffer is needed. This makes gel filtration the mildest chromatography technique [73].

FLPC was performed on Äkta (GE Healthcare, Amersham Bioscience AB, Uppsala, Sweden) with a HiPrep 16/60 Sephacryl S-300 HR column. Possible sample volume is up to 5 ml. Its maximum back pressure is up to 0.15 MPa (1.5 bar).

Buffer: 20 mM Tris/HCl, pH 7.5 + 0.3 M NaCl

After connecting to FLPC, the column was washed with start buffer and loaded with elution buffer. The sample was applied and flow-through and wash was collected. Proteins were then eluted by the buffer within 20 min at a flow rate of 1 ml/min. 1 ml fractions were collected.
The column was re-equilibrated with ddH_2O and stored in 20% ethanol.

Reversed-phase High-performance-liquid chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) separates molecules through differences in hydrophobicity. Hydrophobic molecules bind onto the hydrophobic ligands of the stationary phase, while the hydrophilic molecules pass through the column together with the mobile phase. The bound molecules are eventually eluted with increasing the organic buffer over time. It is an important tool for purification of proteins because it has an excellent resolution which makes it possible to distinguish even proteins with very similar structures.

The column used is a Jupiter 5u C5 300 A from phenomenex and its maximum back pressure is up to 13 MPa (130 bar).

Buffer A: ddH_2O with 0.07% TFA (Trifluoroacetic acid)
Buffer B: Acetonitril with 0.05% TFA

Firstly a test run with pure SFA 8/7 and sunflower-LTP was performed.
The column was pre-equilibrated with buffer A. An aliquot of the prepared lyophilisate was dissolved in water and centrifuged at 3,200 rpm for 10 min at room temperature. The eluent was monitored at 215 nm and 280 nm. Sunflower proteins, including LTP and 2S albumins, were separated on our RP-HPLC apparatus (Shimadzu, Corporation Analytical Instruments Division, Kyoto, Japan). This procedure was performed according to the protocol from Burnett et al [48].

Lyophilisation

Lyophilisation is the dehydration of frozen materials with vacuum. The drying process will be performed through sublimation, which means that the frozen material doesn't have to liquefy before becoming a gas. The lyophilisate is good soluble in water and the material, such as proteins, won't be altered through the lyophilisation process. Furthermore, lyophilisation preserves proteins because enzymatic, bacterial or chemical reactions are prohibited.

Procedure:

HPLC-fractions, which contained proteins diluted in acetonitril with trifluoroacetic acid, were shock-frozen in 2 ml Eppendorf-tubes N_2 for about 30 min. Every lid of every tube was pinched with a needle a few times to ensure the lyophilisation process later on. The lyophilisator (Christ® Alpha 1-4) and the vacuum pump were also turned on, for the system to warm up. After 30 min the samples were placed on the platform and the MV-pressure bottom was pushed. Lyophilisation was stopped on the next day, when the samples were dry.

2.2.3 Molecular Characterization of Sunflower proteins

Proteins were analysed under reducing, non-reducing and native conditions with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent, which denaturizes the proteins and gives them a negative charge. The samples are loaded onto the stacking gel, which is a large-pore gel. It is able to concentrate the proteins before they enter the separating gel. The separating gel then separates the proteins according to their molecular mass. To get a good separation the pore sizes of the separating gel should be adjusted to the proteins' molecular mass [74].

Analysis of Proteins by SDS - Polyacrylamide Gel Electrophoresis

Lower buffer:

1.5 M Tris pure
0.4% SDS
adjust pH 8.8 with HCl

Upper buffer:

0.5 M Tris pure
0.4% SDS
adjust pH 6.8 with HCl

Reagent C:

29.2% Acrylamide
0.8% Bis-Acrylamide
Filtrate through folded filter

10% APS (Ammonium persulfate)

1 g for 10 ml dH_2O

For 4 gels with 0.75 mm spacer

	Separating gel 8%	Separating gel 12%	Separating gel 15%	Stacking gel 4,5%
Regent C	5.4 ml	8.0 ml	10.0 ml	1.2 ml
Lower Buffer	5.0 ml	5.0 ml	5.0 ml	-
Upper Buffer	-	-	-	2.0 ml
Aqua dest.	9.6 ml	7.0 ml	5.0 ml	4.8 ml
TEMED	10.0 µl	10.0 µl	10.0 µl	4.0 µl
10% APS	100.0 µl	100.0 µl	100.0 µl	80.0 µl

Table 2.2: Guidance for the preparation of stacking and separating gel in different concentrations.

Two glass plates were hold together by a glass plate sandwich. The separating gel was prepared (Table 2.2) and poured between the glass plates up to the marker line. The gel mixture was then overlaid with isopropanol to exclude oxygen from the surface and to generate a straight line between the stacking and separating gel. After the polymerization of the separating gel is completed, the isopropanol was drained and removed with paper towels. The stacking gel (Table 2.2) was then overlaid with the stacking gel and a comb with 10 or 15 slots was inserted. The best resolution was received when the gels polymerized overnight.

Sample Preparation

For SDS-PAGE, the protein samples were mixed with 4x sample buffer, denatured by heating at 95°C for 5 min and centrifuged for 10 min.

4x sample buffer:

200 mM Tris pure
300 mM DTT (dithiothreitol)
ddH_2O
adjust to pH 6.8 with HCl
4% SDS
40% glycerol
0.02% bromophenol blue

SDS is used for solubility and denaturation of proteins for accurate molecular mass determination. DTT breaks down disulfide bonds to make sure that polypeptide denaturation and binding of SDS is sufficient. Glycerol is mainly used to increase the density of the sample volume. Bromophenol blue marks the track so the progression of the gel electrophoresis can be followed visually.

Sample Loading and Electrophoresis:

10x Electrophoresis buffer:

250 mM Tris pure
192 mM Glycine
1% SDS

For gel electrophoresis a dilution 1:10 for a 1x electrophoresis buffer is used. For 3 µl protein sample, 0.75 µl 4x sample buffer (reducing, non-reducing or native) was used. Then the mixture was heated and centrifuged. 3 µl were loaded into the slots of the gel. To start the gel electrophoresis the 1x electrophoresis buffer was poured into the gel apparatus. The

electrophoresis was carried out at 160 V for about 50 min. For the determination of the molecular weights a Page ruler was used (Figure 2.1).

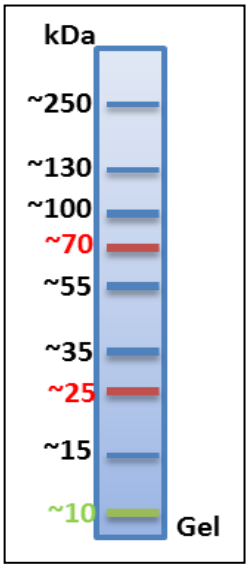


Figure 2.1:Page Ruler Puls Prestained Protein Ladder 10-250 kDa.

Coomassie Brilliant Blue R - 250, detects protein bands by nonspecific binding to aromatic amino acids. Through ionic interactions between the dye's sulfonic groups and the protein's amino acids as well as through Van der Waals forces the protein bands get visualized on the gel. The length of the staining process depends on the thickness of the gel and also the gel concentration. The dye stains the gel as well and therefore Coomassie-Destainer helps to remove the dye not bound to any protein bands. The staining and destaining process can be accelerated by letting the gel shake at higher temperatures [75, 76].

Procedure:

After gel electrophoresis the gel was put into a plastic tray and was stained for about 40 min while shaking on a rocking platform. Afterwards it was put into the destainer solution until the protein bands could be seen clearly.
The 2D-gel was stained for 1 day on the shaking platform. To accelerate the destaining process, the 2D gel was given into a large glass beaker and shaken in a heated water bath.

Coomassie stainer and destainer solutions contain methanol and were disposed in a special Coomassie refuse.

Coomassie Brilliant Blue Staining Solution:

0.125% Coomassie Blue R-250
50% Methanol
10% Acetic acid
dissolved in ddH_2O and filtered

Destaining Solution:

15% Acetic acid (100%)
50% Methanol

Protein concentration determination: Bicinchoninic Acid (BCA) protein assay

The BCA Protein Assay enables quantification of the amount of the protein by colorimetric reaction. Peptide bonds are able to reduce Cu^{2+} to Cu^{+}, which can then be complexed by two molecules of bicinchoninic acid. A purple-colored product is formed and can be detected at 562 nm. The concentration can then be determined by comparing the absorption of the samples to the absorption of reference-solutions. As a reference bovine serum albumin (BSA) with known concentrations is run in parallel [77, 78].

Procedure:

Different concentrations for the standards and samples were prepared. The standards were prepared (Table 2.3) by using the same buffers in which the samples are in, together with BSA-stock solution (c = 2 mg mL^{-1}). The dilutions were mixed well and then pipetted in duplicates into polystyrene microplates (Greiner Bio-One GmbH, Frickenhauser, Germany). 200 µl working buffer, consisting of 50:1 reagent A: reagent B, was added to each well. The plate was incubated for 30 min at 37°C before it was measured by 562 nm by the plate

reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). The Soft Max Pro 4.8 software (Molecular Devices, Sunnyvale, CA) was then calculating the standard curve and the sample concentration for each dilution.

Standard	Buffer	BSA (2 mg·mL^{-1})	Final conc.
A	70 µl	10 µl	250 µg mL^{-1}
B	72 µl	8 µl	200 µg mL^{-1}
C	148 µl	12 µl	150 µg mL^{-1}
D	152 µl	8 µl	100 µg mL^{-1}
E	156 µl	4 µl	50 µg mL^{-1}
F	160 µl	-	0 µg mL^{-1}

Table 2.3:Table for the preparation of the BCA standards.

2.2.4 Immunological Characterization of Sunflower proteins

Western Blotting

Western blotting transfers the gel onto a nitrocellulose- or PVDF-membrane. One possible method would be semidry-electroblotting. With mono- or polyclonal antibodies, which can be either from an allergic patient's serum or pure, specific antigens can be detected. Before this detection, the membrane has to be incubated with either a protein, such as BSA, or a detergent blocking agent, in order to block unspecific binding sites. After blocking, the membrane can be incubated with the specific antibody solution, which consists of the primary antibody which recognizes the target antigen and a secondary antibody that specifically binds the primary antibody. The secondary antibody is usually labeled with an enzyme, which catalyzes a colorimetric reaction, after adding its substrate.

Procedure:

After SDS-PAGE, the gel was transferred onto a nitrocellulose membrane. Therefore, the gel, four Whatman papers and a nitrocellulose membrane were equilibrated in 1x transfer buffer.

10x Transfer buffer:

250 mM Tris pure
1.92 M Glycine

1x Transfer buffer:

10% 10x transfer buffer
20% Methanol

Two Whatman papers build the bottom. Then the nitrocellulose membrane and the gel follow on top. And the top of the sandwich is built by the other two

Whatman papers (Figure 2.2). Between the layers the air bubbles are carefully removed by rolling over the sandwich with a plastic tube. It was then placed into the semidry blotter and the electroblotting was done by applying a voltage of mA per for about 35 min. The time depends upon the thickness of the gel. Afterwards the page ruler was traced with a ball pen and then blocked for 1 h in 8 ml TBST, 3% BSA. In the meanwhile, primary and secondary antibodies were diluted in 1% TBST in concentrations 1:1,000 and 1:10,000. After blocking the membrane was washed with TBST and directly incubated with antibody solution while shaking on the rocking platform overnight. The next day the membrane was washed three times for 20 min, again under shaking conditions. After washing, the membrane was transferred into a glass plate and covered with the alkaline-phosphate-solution, which has alkaline-posphate-buffer and 4-Nitro blue tetrazodium chloride (NBT) as well as 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) as its ingredients. The colorimetric reactions proceeded in the dark and stopped with water.

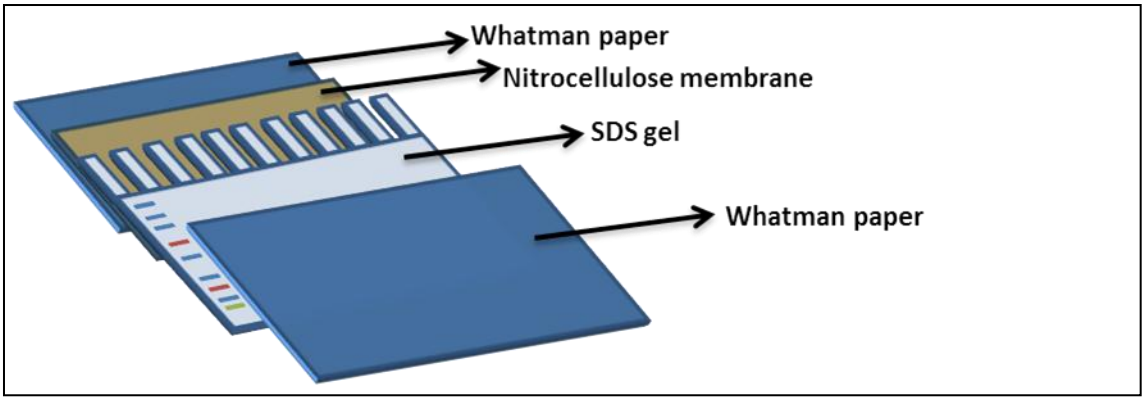


Figure 2.2: Western blotting

10x Tris buffered saline (TBS):

0.5 M Tris/HCl, pH 7,4

1.5 M NaCl

0.5% NaN_3

dissolved in ddH_2O

Tris-buffered saline with Tween 20 (TBST):

1x TBS + 0.5% Tween 20

AP-buffer (alkaline phosphate):

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM $MgCl_2$ 6 H_2O

dissolved in ddH_2O

4-Nitro blue tetrazodium chloride (NBT):

50 mg/mL^{-1} NBT

dissolved in 70% DMF/H_2O

5-Bromo-4-chloro-3-indolyl-phosphate (BCIP):

25 $mg\ mL^{-1}$ BCIP

dissolved in ddH_2O

2D-Gel electrophoresis

First dimension:

With the 2D gel electrophoresis the proteins are separated by two dimensions. First, the proteins are distinguished by their isoelectric point (pI) by electrofocusing. Every protein applied to the gel gets a charge, depending on the local pH, and starts moving through the pH-gradient of the electric field until it reaches the pH region that matches its pI. At this point it has no net charge and therefore stops the migration [71].

Procedure:

Three volumes of ice cold pure acetone (-20°C) were mixed with one volume protein sample and immediately precipitate at -20°C for 30 min. By low protein concentrations, water is also added to the protein volume. After precipitation,

the mixture was centrifuged at 4°C with 15,000 rpm. The supernatant was decanted and the pellet covered with 50 µl ice cold 90% acetone. Then the mixture was centrifuged at 4°C with 15,000 rpm for another 10 min. The supernatant was wasted again and the pellet air dried. The dry pellet was then suspended in 125 µl of isoelectric focusing-sample buffer (IEF-SB without any DTT) and the mixture was shaken for 10 min. The total volume was pipetted into the middle of a strip holder, without causing any air bubbles. Now the plus-pol of the strip (Immobiline^{TM} DryStrip pH 3-10, GE-Healthcare, Little Chalfont, UK) was carefully slid towards the plus pol of the strip holder, making sure that the sample was equally wetting the strip's gel side. 400 µl mineral oil was added onto the strip before the protective cover was put on top. The first dimension of the 2D gel electrophoresis was carried out by using the Ettan IPGphorII Isoelectric Focusing System, which provides rehydration and IEF. A strip holder consists of thermally conductive ceramic with platinum electrodes and a transparent cover.

IEF-SB:

7 M Urea
2 M Thiourea
2% CHAPS
0.5% IPG buffer 3-10
0.002% Bromophenol blue
adding water to 10 ml

When reducing conditions are favored, 65 mM Dithiothreitol (DTT) (65 µl from a 1 M stock solution) is added, right before using the IEF-SB.
Carrier ampholytes improves the solubility of proteins, without disturbing the IEF itself. *Bromophenol blue* acts again as a control dye. *Urea* disrupts hydrogen and hydrophobic bonds. *DTT*, as a reducing agent, denatures proteins by breaking their disulphide bridges. If non-reducing conditions are wanted, DTT can be left out.

Second dimension:

For the second dimension the strips have to be equilibrated. Under reductive conditions, Solution I and II are available and will be used one after the other, to equilibrate the strip for about 15 min. If non reductive conditions are favored, the strip should only be equilibrated in 1x electrophoresis buffer instead. Afterwards the equilibrated strip can be put onto the 2D-gel and the gel electrophoresis can be carried out.

Equilibration stock solution:

6 M Urea
2% SDS
30% Glycerol 87%
0.002% Bromophenol Blue
divided into 2x 200 ml for Solution I and II

Solution I:

65 mM DTT

Solution II:

25 mg/ml Iodoacetamide

Both solutions are stored in 10 ml aliquots at -20°C.

Urea (6 M) and *Glycerol (30%)* increase the viscosity of the buffer, which helps to reduce the elctroendosmosis effects. Electroendosmosis occur due to the fixed charges, which are present on the IEF strip in the electric field. This can handicap the protein transfer from the strip to the 2D-gel. Concentrated urea furthermore helps to solute hydrophobic proteins by bringing them into a single conformation and by avoiding any protein-protein interactions.

DTT helps to preserve the already reduced conditions, while *SDS* charges the protein negatively. This makes the proteins separable for the successional gel electrophoresis.
Iodoacetamide alkylates all thiol-groups present in proteins, which prevents the rebuilding of disulfide bridges.

Agarose sealing solution:

0.5% Agarose
0.002% Bromophenol blue
melt agarose, cool to 60°C and store in 1 ml aliquots.

Procedure:

After IEF, the strip taken out of the strip holder and carefully drained onto a paper towel. Next the strip was incubated with equilibration Sol-I for 15 min followed by equilibration with Sol-II for 15 min. The strip was then placed onto the 2D-gel together with a piece Whatman paper, which had been soaked with 4 µl Page Ruler (Mass Ruler Prestained Protein Ladder Fermentas) before. The strip was now covered with agarose-sealing solution to ensure an efficient SDS-PAGE. In the first few minutes 15 mA per gel was used to support the proteins' transfusion from the strip into the gel. Then the electricity was raised to 40 mA per gel. Thereafter the gel was either stained with Coomassie as described in section 2.2.3 or was blotted onto a NC-membrane as described in section 2.2.4.

IgE ELISA (Enzyme Linked Immunosorbent Assay)

With this method, proteins get immobilized on the inert surface of the ELISA microtiter-plate. Then these proteins can be recognized by specific antibodies. The antibody-protein complexes can then be detected through chromomeric detection (Figure 2.3).

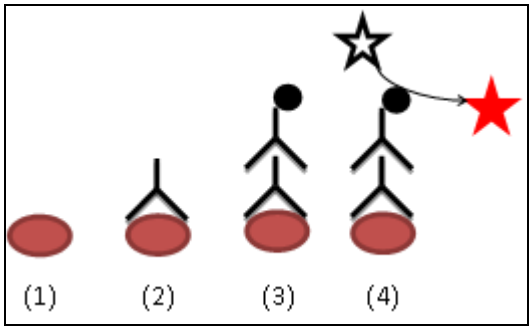


Figure 2.3: ELISA

Procedure:

100 µl protein-sample (1 µl/ml protein diluted in ELISA coating buffer, 50 mM Na-carbonate-buffer pH 9,4) was coated into microtiter-plates (Maxi Sorp Immuno Plate, Nalge Nunc International, Roskilde, Denmark) and incubated over night at 4°C. On the next day, the plate was washed with TBST and each well was blocked with 200 µl TBST + 3% BSA for 2 h at room temperature.
In the meantime, TBST+ 1% BSA and α-human-IgE-AKP (Alkaline phosphate conjugates mouse anti-human IgE monoclonal antibody, BD Bioscience Pharmingen, San Diego, US) were mixed in a relation 1,000:1. Sera were prepared with 10 volumes of TBST+1% BSA/ α-human-IgE-AKP and 1 volume of patients' sera, control-NHS or buffer as control.
After 2 h the plate was again washed with TBST and was then coated with 100 µl of diluted patients' sera, control-NHS or control-buffer. Then the plate was again stored at 4°C overnight.
Finally, the wells were washed 7 times with TBST and then the specific antigen-antibody-binding was visualized with a p-Nitrophenylphosphate Tablete set (Sigma fast Nitrophenylphosphate Tablete Sets; set to prepare 5 or 10 ml) by adding 100 µl per well.

The plate was incubated in the dark and the absorbance was measured at 405 nm all 20–30 min.
The actually absorbance-values are calculated by subtracting the absorbance of the controls' median plus two times standard deviation.

Sample concentration

Centrifugal filter units help to concentrate samples through centrifugation. Typical processing time is 15 to 60 min, depending on the molecular weight cutoff (MWCO). A physical dead stop device within the filter of the tubes prevents that the samples run dry and therefore potential sample loss. Maximum sample volume for one centrifugal filter unit is 15 ml.

Procedure:

For the concentration of protein samples, centrifugal filter units (Millipore™, Amicon® Ultra-15, Billerica, MA) with MWCO of 3,000 were used.
Centrifugation time was about 30 to 50 min, depending on the sample amount and the end volume that was wanted to be reached.

3 Results

3.1 Extraction and purification of low molecular weight proteins from sunflower seeds

Two multi-step purification protocols were established to purify low molecular weight allergens of sunflower seeds. After defatting, the sunflower seed flour was mixed overnight with aqueous sodium-phosphate buffer in order to extract the salt-soluble low molecular proteins. Approximately one-third of the total salt-soluble proteins are 2S albumins [70].The proteins were precipitated with 90% ammonium sulfate and centrifuged. This ammonium sulfate concentration was chosen because previous experiments show that the 90% ammonium sulfate precipitation is able to precipitate especially the desired low molecular weight proteins(Figure 3.1).

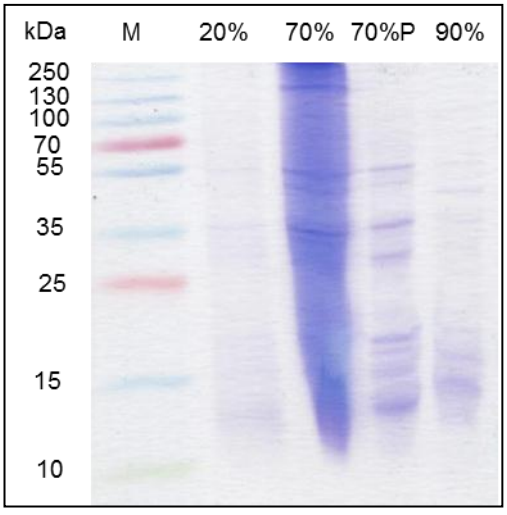


Figure 3.1:SDS-PAGE analysis of ammonium sulfate precipitations with 20%, 70% and 90% saturation (P, pellet; M, marker).

3.1.1 Purification method 1

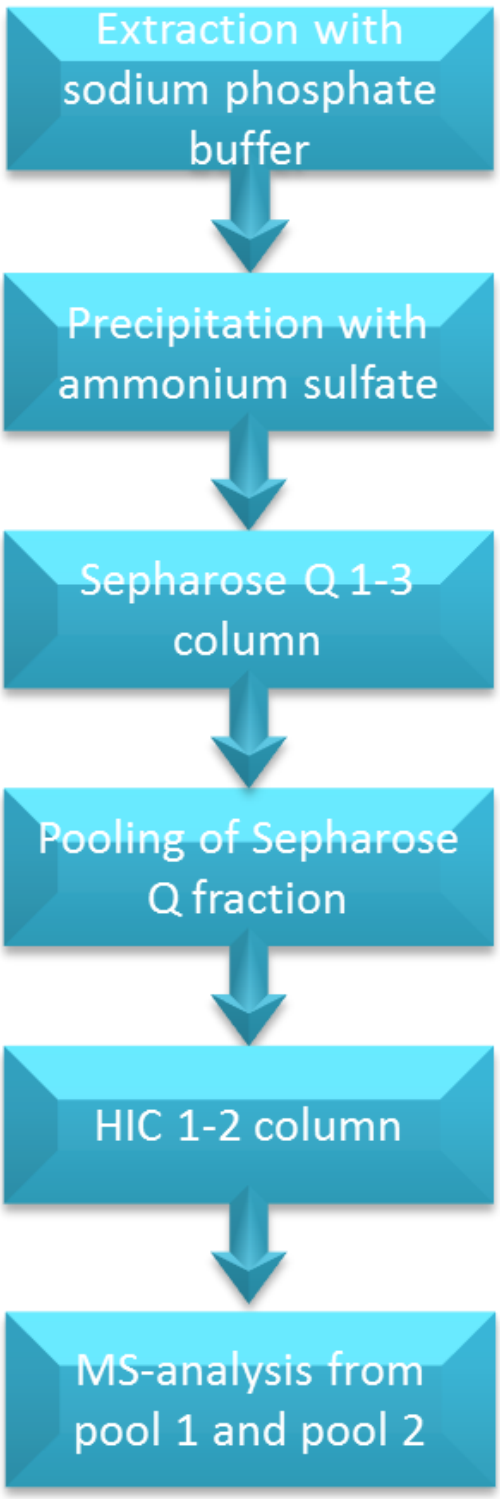


Figure 3.2:Flow chart for the purification of 2S albumins in sunflower seeds.

The pellet of 90% ammonium sulfate precipitate was dissolved in 20 mM Tris/HCl-buffer, pH 8.0. In order to remove high molecular weight proteins and ammonium sulfate salt, the extract was dialyzed against 10 L of 20 mM Tris-HCl buffer, pH 8.0, using a dialysis membrane with a cut off of 3,500 kDa. The buffer-pH of 8 charges 2S albumins and LTP. SFA 8 has a pI of 5.9 and will be positively charged, while LTP has a pI of 9 and therefore will be charged negatively. This creates perfect condition to separate the low weight proteins by a strong anionic exchanger.

Separating of proteins by Sepharose Q

In the next step, the dialyzed sample was then loaded onto a self-packed Sepharose Q column (GE-Healthcare). This was done three times to make sure, that the column won't be overloaded. One milliliter Sepharose Q fast flow can bind approximately 0.18-0.25 mmol Cl^-/ml, so the 8 ml column matrix could bind about 1.44-2.00 mmol/ml. During chromatography the column material was dyed dark. This was caused due to the extensive amount of chlorogenic acids and polyphenolics in the extract [48]. The removal of the pigments was a great advantage since they always interfered with analytic tests, such as SDS-PAGE (as shown in Figure 3.1. 70% ammonium sulfate saturation). The eluted Sepharose Q 1-3 fractions were collected and analyzed with 15% SDS-PAGE gels and Coomassie-blue staining. Finally, fractions 10-20 from Sepharose Q 1, wash 1 and 2 and fraction 5 from Sepharose Q 2, and wash 1-6 and fraction 7 from Sepharose Q 3 were pooled (Figure 3.3 to 3.10).

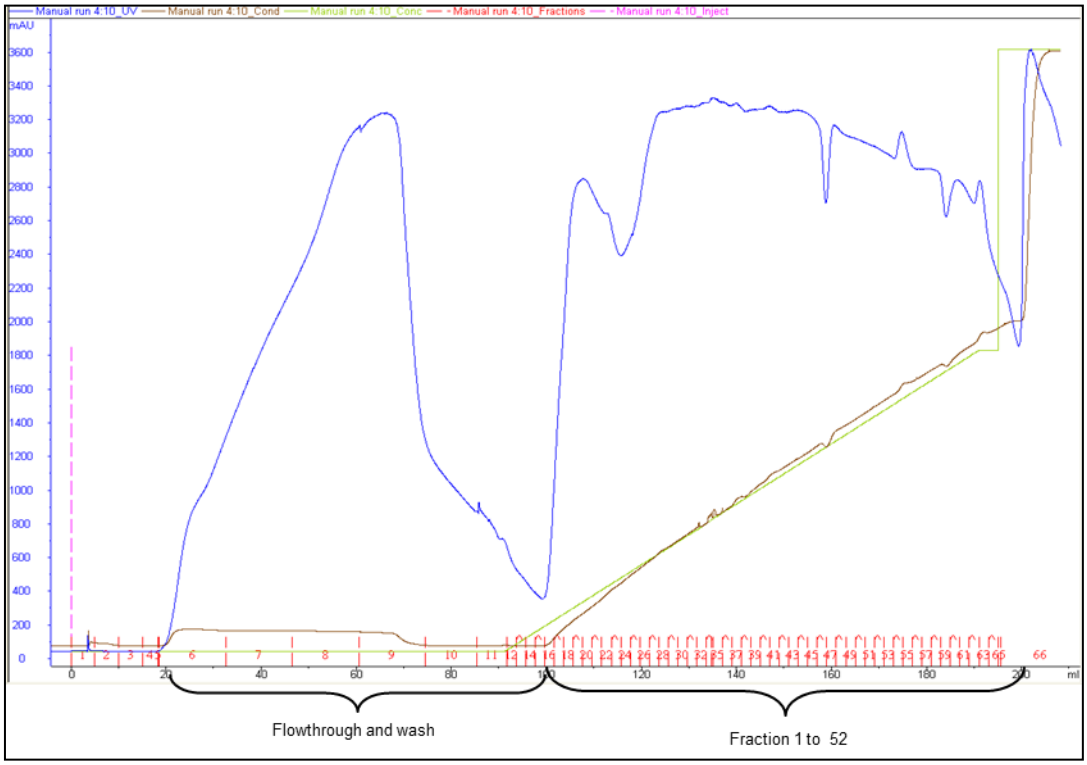


Figure 3.3:Chromatogram of Sepharose Q shows the separation and purification of 2S albumins and sunflower LTP during. Gradient 0-50% with buffer B.

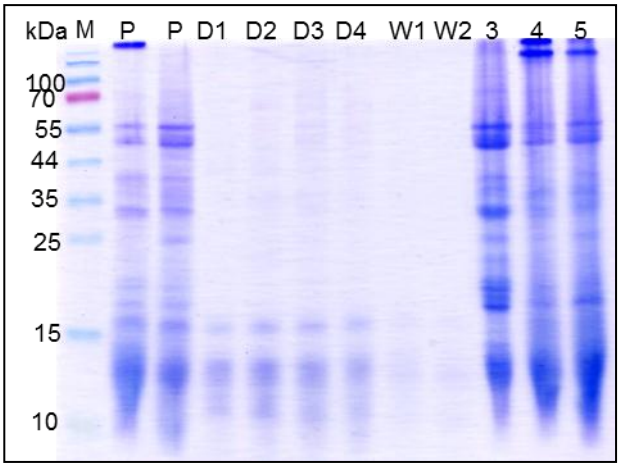


Figure 3.4:SDS-PAGE analysis of Sepharose Q 1 chromatography of sunflower seed proteins. Flowthrough 1 to 4 (D1 to D4), wash 1 and 2, and fractions 3 to 5, (P, stands for sample before separation).

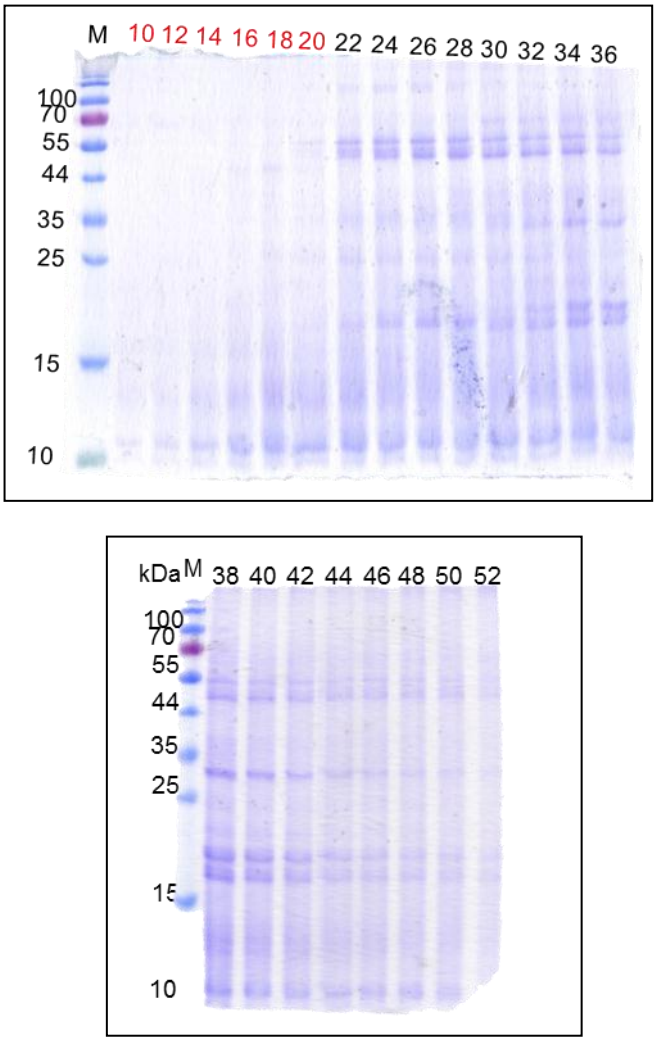


Figure 3.5: SDS-PAGE analysis of Sepharose Q 1 chromatography. It shows the even numbered fractions from 10 to 52, (red numbered fractions were pooled).

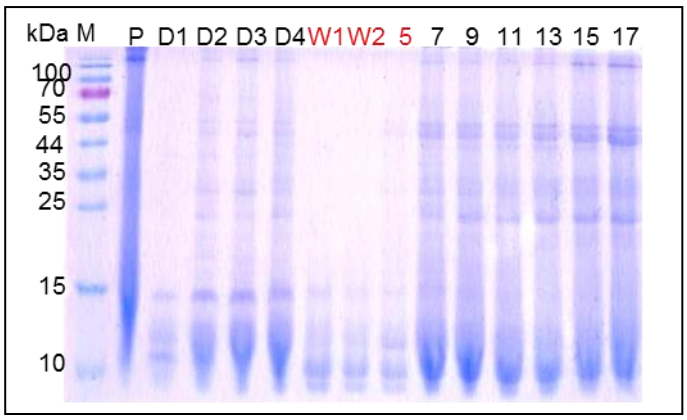


Figure 3.6: SDS-PAGE analysis of Sepharose Q 2 chromatography. It shows the flowthrough 1 to 4, wash 1 and 2, and odd numbered fractions from 5 to 17, (red numbered fractions were pooled).

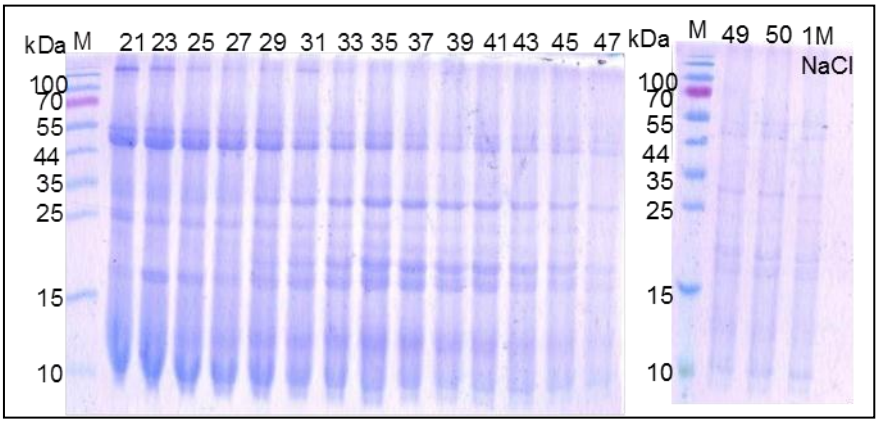


Figure 3.7: SDS-PAGE analysis of Sepharose Q 2 chromatography. It shows the odd numbered fractions from 21 to 50. 1M NaCl eluation was used for the last 14 ml.

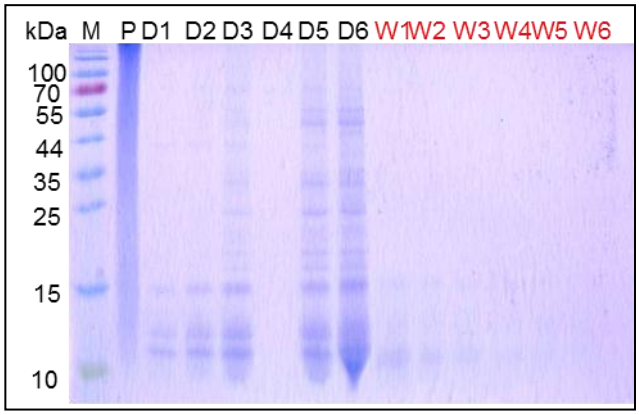


Figure 3.8: SDS-PAGE analysis of Sepharose Q 3 chromatography. It shows the flowthrough 1 to 6, and wash 1 to 6, (red numbered fractions were pooled).

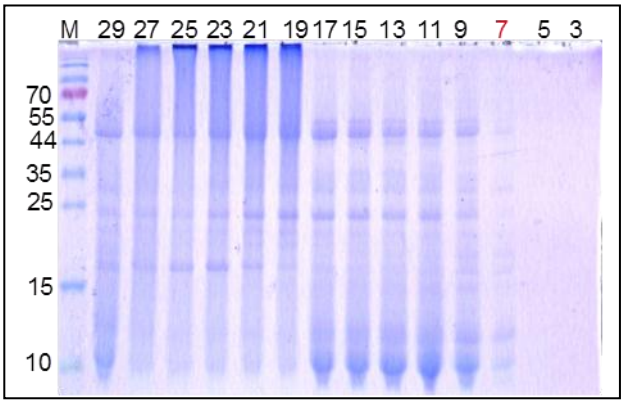


Figure 3.9: SDS-PAGE analysis of Sepharose Q 3 chromatography. It shows the odd numbered fractions from 3 to 29, (red numbered fractions were pooled).

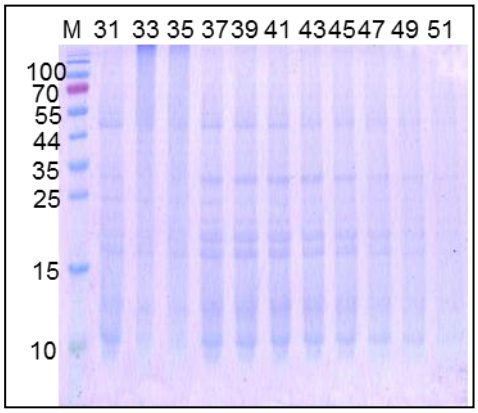


Figure 3.10: SDS-PAGE analysis of Sepharose Q3 chromatography. It shows the odd numbered fractions from 31 to 51.

Hydrophobic interaction chromatography

The next step was to separate the pooled proteins by their hydrophobicity (Figure 3.11). To do so, 1 M ammonium sulfate was added before loading onto a HIC-column. Previous studies showed that LTP of sunflower has the lowest surface hydrophobicity, followed by 2S albumin fractions and SFA 8, which has the highest surface hydrophobicity [48, 79]. The eluted fractions were again characterized by SDS-PAGE. The fractions with proteins of similar molecular mass were pooled into three pools: pool 1 included wash 2 - 4, pool 2 fractions 1 - 25 and pool 3 fractions 26 - 49. Pool 1 and 2 show protein bands mostly between 8 and 15 kDa. The pools were desalted by dialysis against 20 mM Tris/HCl, pH 8 overnight. Gel electrophoresis showed that in pool 1 and 2 are the desired low molecular proteins (Figure 3.12).

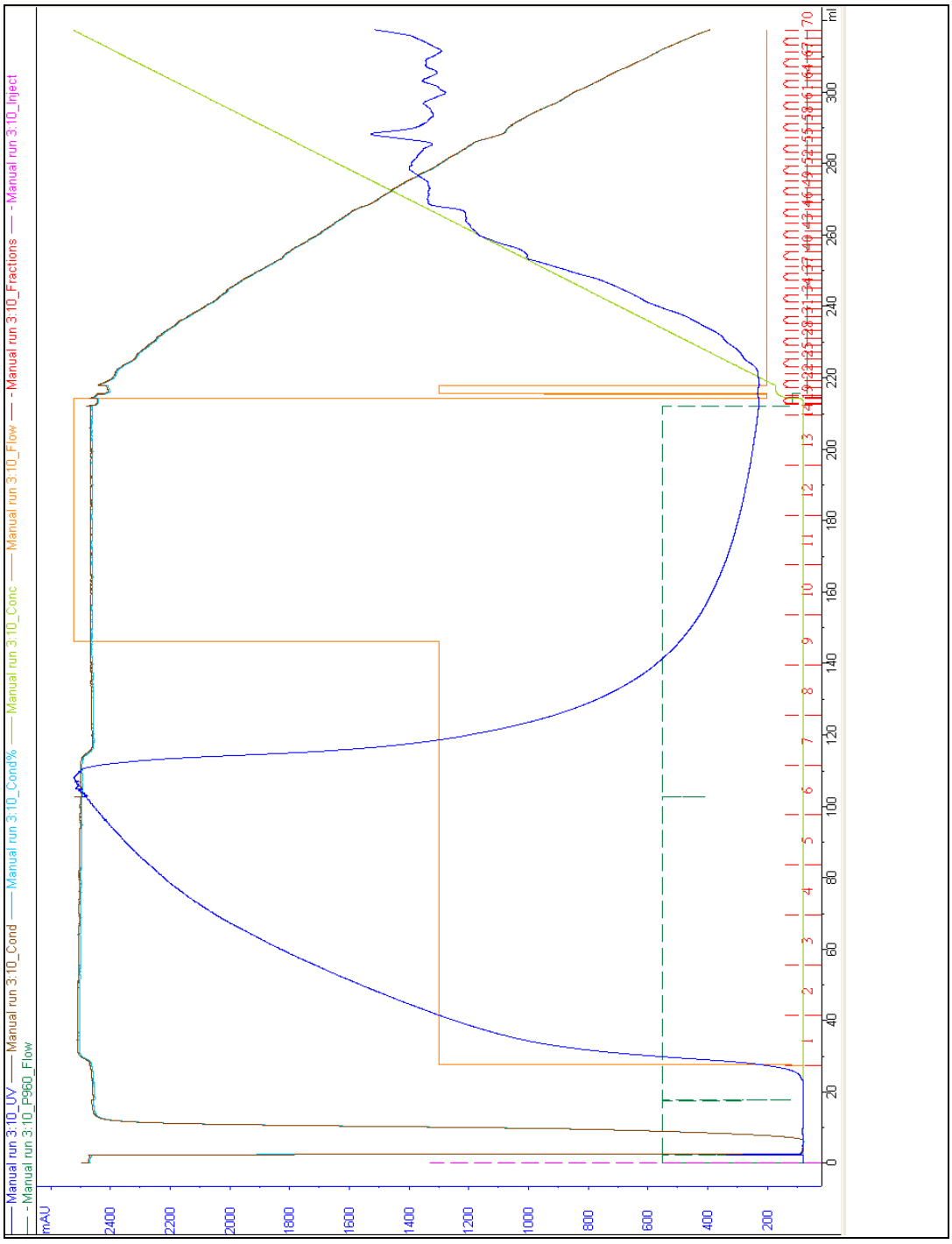


Figure 3.11: Chromatogram of the separation and purification of 2S albumins and LTP from sunflower seeds by hydrophobic interaction chromatography.

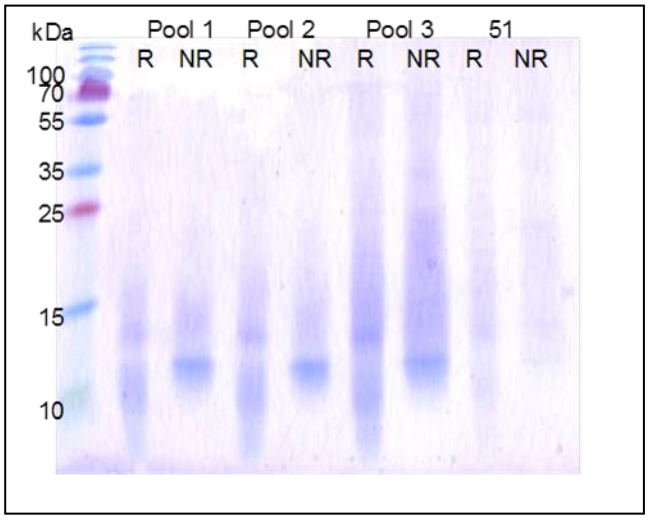


Figure 3.12: SDS-PAGE analysis of pooled fractions after HIC-chromatography. It shows the Pool 1, including wash 2 to wash 4; Pool 2 including fraction 1 to 25,and Pool 3, including fraction 26 to 49. Pool 1 and Pool 2 contain mostly low molecular weight proteins, while Pool 3 has proteins with high and low molecular weight.

Immunological characterization and quantification of pool 1 and pool 2

For further characterization, 2D-gel electrophoresis and immunoblots were performed.

With pool 2, 2D-gel electrophoresis was performed in order to confirm the purity and isoelectric point. Coomassie staining, shown in Figure 3.13, revealed 3 spots of same molecular weight but slightly different pI. Each dot represents an isoform of the purified protein. The 2D-gel was also blotted onto NC-membrane to test anti-SFA antibody binding, which significantly bound the three protein isoforms (Figure 3.14). Pool 1 and 2 were both immunoblotted with anti-SFA 8-antibody after SDS-PAGE (Figure 3.14). Anti-SFA 8-antibody strongly recognized in both pools a protein of about 12 kDa.

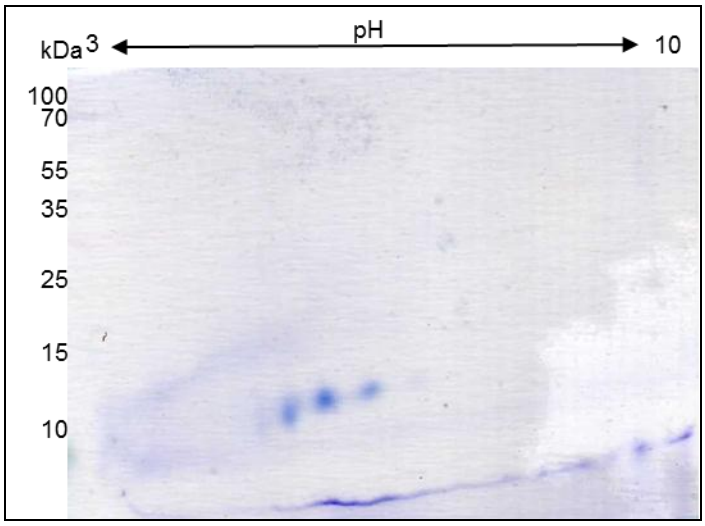


Figure 3.13: 2D gel electrophoresis and staining with CBB from Pool 2. Three spots are visible at about 12 kDa and pI of 5.1, 5.6 and 6.5 (using a pH-strip of pH 3-10.)

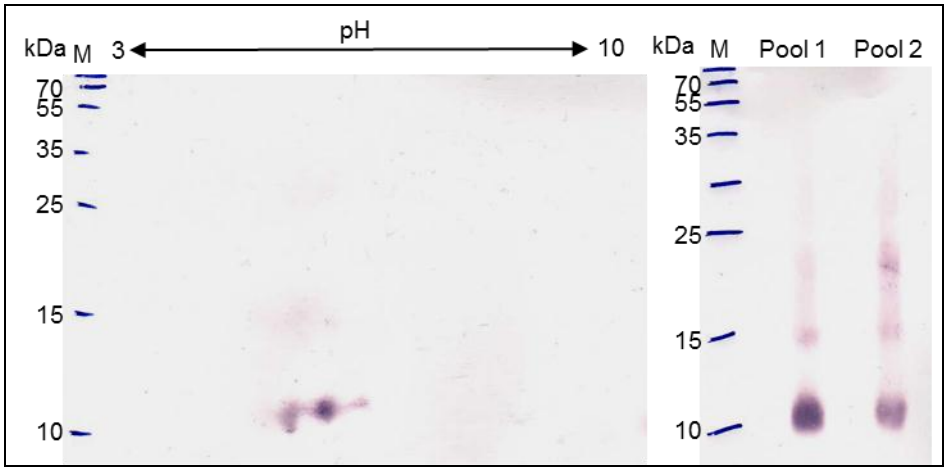


Figure 3.14: Immunoblot after 2D gel electrophoresis of Pool 2. Anti-SFA 8 recognized the three protein isomers that could also be seen in the CBB-staining. In the right picture the immunoblot of SDS PAGE from Pool 1 and Pool 2 is shown.

To quantify the amount of total protein in pool 1 and 2, a BCA was performed. Pool 1, with wash 2 - 4 showed a concentration of 243.87 µg/ml, and pool 2, with the fraction 1 - 25 a concentration of 360.57 µg/ml.

Mass spectrometry of pool 1 and 2

After quantification a SDS-PAGE gel electrophoresis was performed with pool 1, 2, pure SFA 8 and 7. Purified SFA 8 and 7 (reference material provided by P.Shewry). Figure 3.15 shows that pure SFA 8 (kindly provided by Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK) runs different on the gel than our pool 1 and pool 2. The band of the reduced form of pure SFA 8 has Mr~12.5 kDa, while the non-reduced form of the pure SFA 8 has Mr~11 kDa. So the reduced form shows a higher molecular mass than the non-reduced. With the pool 1 and 2 it is the other way round. There non-reduced form has a higher molecular mass than the reduced form although both are, with a Mr~10.9 and 10.7 kDa, smaller than the pure SFA 8. To get more information about their sequence, we sent the pools to Mag. Dr. Gabriele Gadermaier (University of Salzburg, Dept. of Molecular Biology), who kindly performed a mass analysis for us. The main component of pool 1 is P15461, also called HAG 5. Also P23110, which is known as SFA 8, could be found in a lower concentration (Figure 3.16). The peptides printed in bold, representing 34% for HAG 5 and 40% for SFA 8 of the whole sequence, could be found after trypsin digestion.

Pool 2 has only a low amount of 2S albumins. The main component was an unknown protein.

P15461 is a 2S seed storage protein and has a total length of 295 amino acids. Its molecular mass is 34,071 Da (Figure 3.16 A)

P23110 is a complete 2S seed storage protein and has a total length of 141 amino acids. Its molecular weight is 16,090 Da (Figure 3.16 B).

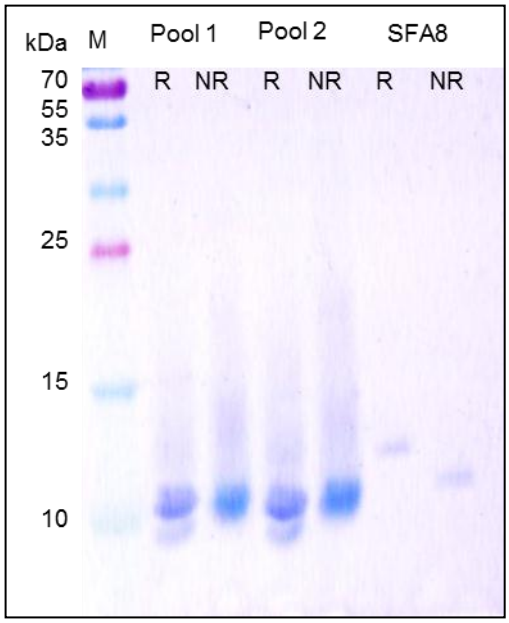


Figure 3.15: SDS-PAGE gel electrophoresis with Pool 1, Pool 2 and pure SFA 8. (SFA 8, kindly provided from Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK).

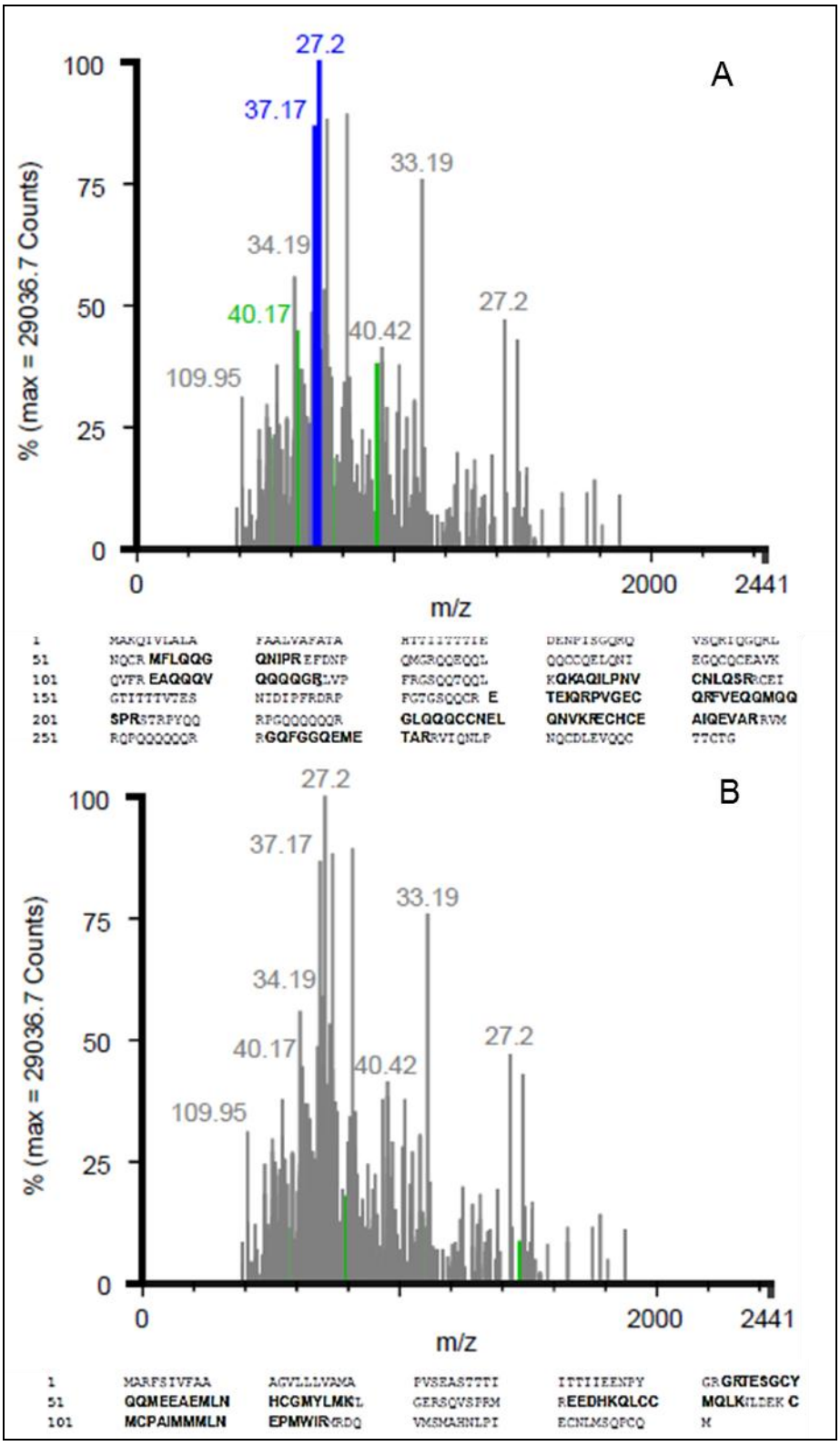


Figure 3.16: Mass analysis of Pool 1 proteins and peptide fingerprinting showing P15461, also known as HAG 5 (A) and P23110, also known as SFA 8 (B).

3.1.2 Purification method 2

This method was described earlier by Burnett et al [48].

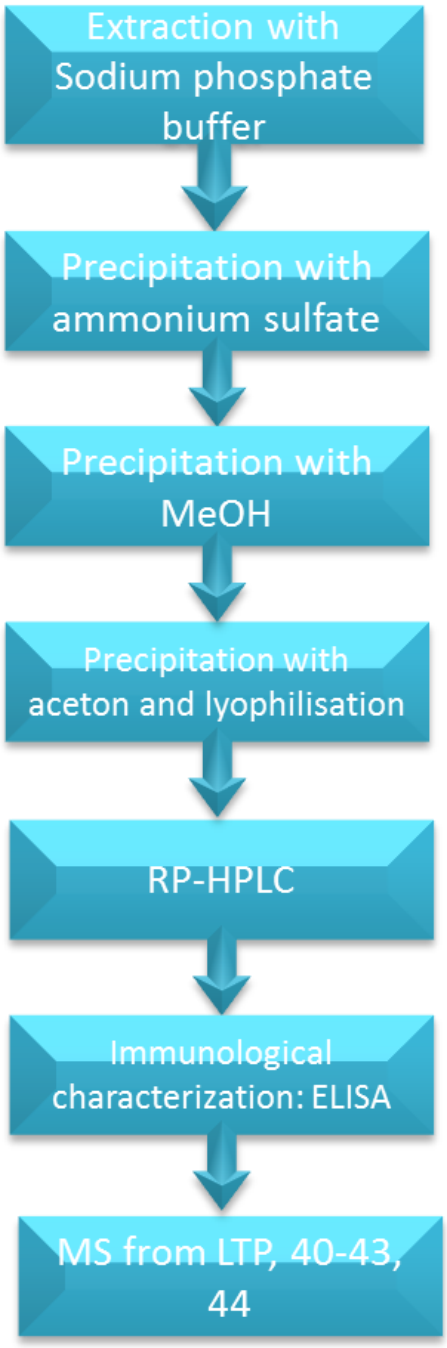


Figure 3.17:Flowchart of purification method 2 in order to purify 2S albumins and LTP from sunflower seeds.

After centrifugation, ammonium sulfate pellets (described in chapter 3.1.1) were diluted and dialyzed against 20 mM Tris/HCl + 1 M NaCl, pH 7.8 at 4°C overnight.

Precipitation with Methanol

For the methanol-precipitation the dialysate was cooled to 0°C prior to the addition of 0°C cold methanol. Methanol only precipitates sunflower globulins, while 2S albumins stay in solution. Separation was guaranteed through centrifugation.

Precipitation with ice cold acetone and lyophilisation

Methanol precipitation was followed acetone precipitation. 3 volumes of ice cold acetone precipitated 1 volume supernatant with 2S albumins at -20°C for 16 h. The 2S albumin fraction was then recovered by centrifugation. The pellets were then dissolved and dialyzed against water for another 48 h. During dialysis chlorogenic acid and polyphenolics precipitated and could entirely be removed through following centrifugation. The soluble protein fraction was then lyophilised (Figure 3.18).

Reversed-phase HPLC

At first pure SFA 8, and pure sunflower LTP were subjected to reversed-phase HPLC in order to recognize nsLTP and 2S albumins from our own sunflower fractions (Figure 3.19 and 3.20).
An aliquot of the lyophilisate was dissolved in 2 ml water (Figure 3.18). 500 µl and approximately 11.88 mg protein was injected into the column per run. During the first 5 min the column was eluted with 10% buffer A, and from minute 5 to 40 with 10-50% buffer B. This rising amount of acetonitrile eluted the proteins from least hydrophobic to most hydrophobic. Therefore sunflower LTP was eluted first, followed by 2S albumin fraction, SFA 7 and in the end SFA 8.

Peaks, similar to the purified LTP and SFA 7/8 run, could be found also in the prepared lyophilisate (Figure 3.21). Eluted fractions were then lyophilisated and additionally controlled with SDS-PAGE (Figure 3.22 to 3.26). Protein fractions with the same molecular weight could then be pooled. Due to the reproducibility of RP-HPLC, the exact same fractions could be collected with 13 more runs. The same proteins from the different runs were then pooled and analysed by SDS-PAGE.

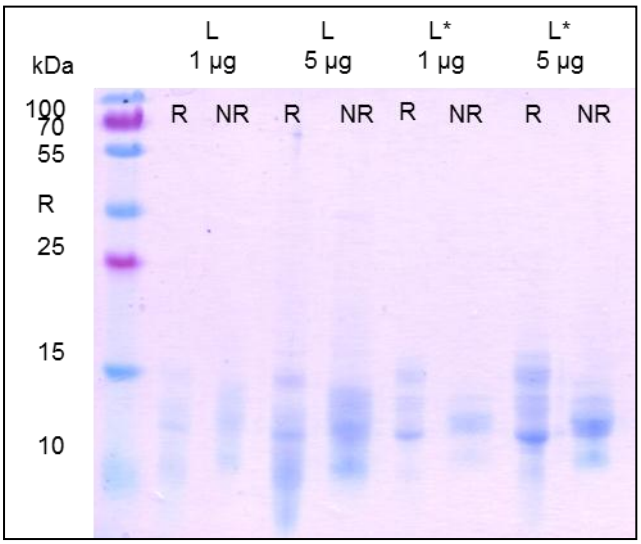


Figure 3.18:SDS PAGE and CBB-staining of L, lyophilisate (concentration 1 µg and 5 µg) before RP-HPLC and L, lyophilisate (concentration 1 µg and 5 µg) before RP-HPLC, kindly provided from Burnett et al.*

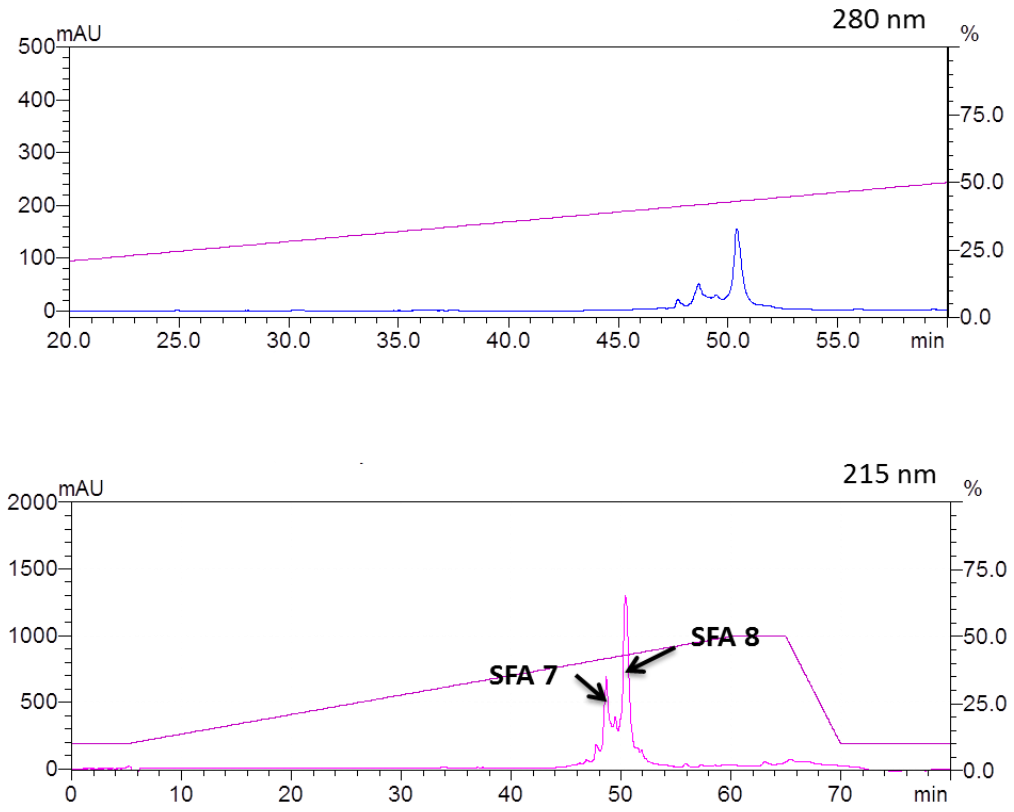


Figure 3.19:Chromatogram of RP-HPLC shows pure sunflower SFA 7/8. The retention time is approximately 50 min and the peak is shown by 280 and 215 nm.

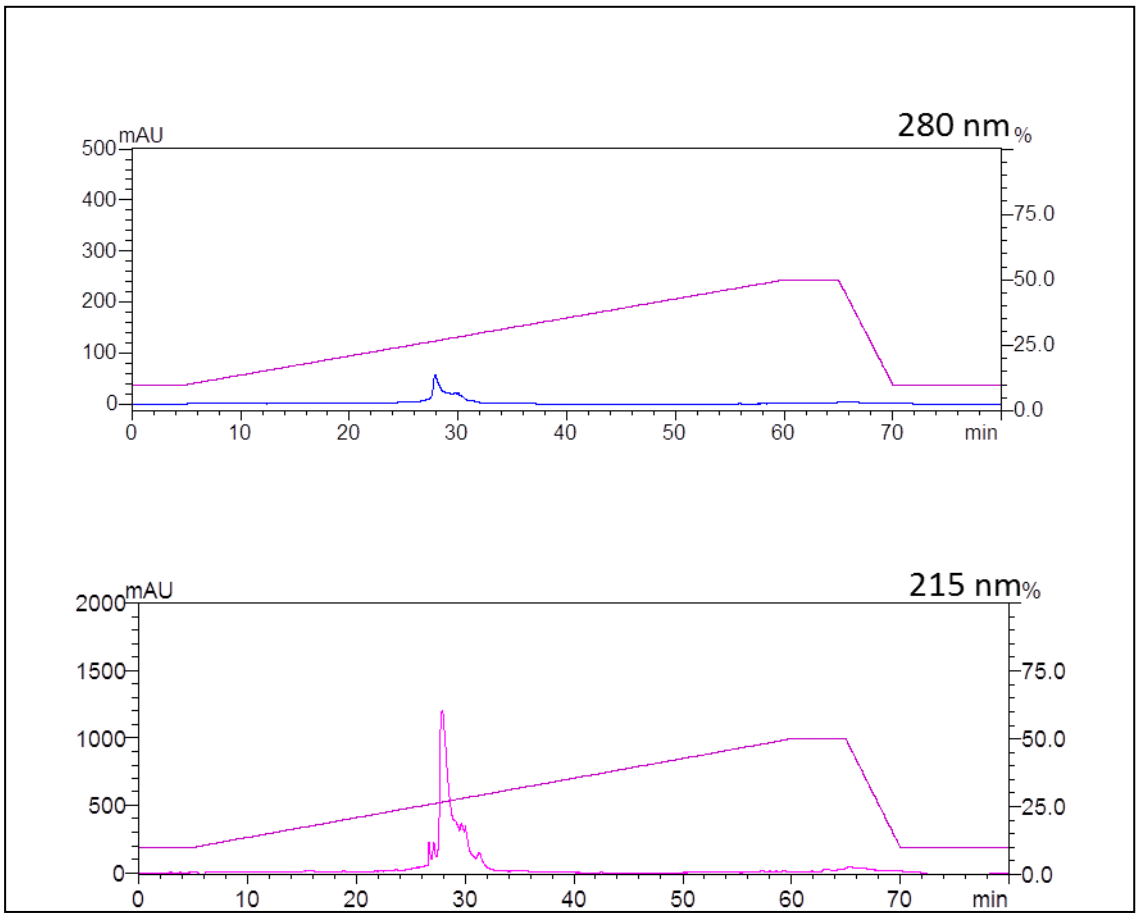


Figure 3.20: Chromatogram of RP HPLC shows pure sunflower LTP. The retention time is approximately 28 min and the peak is shown by 280 and 215 nm.

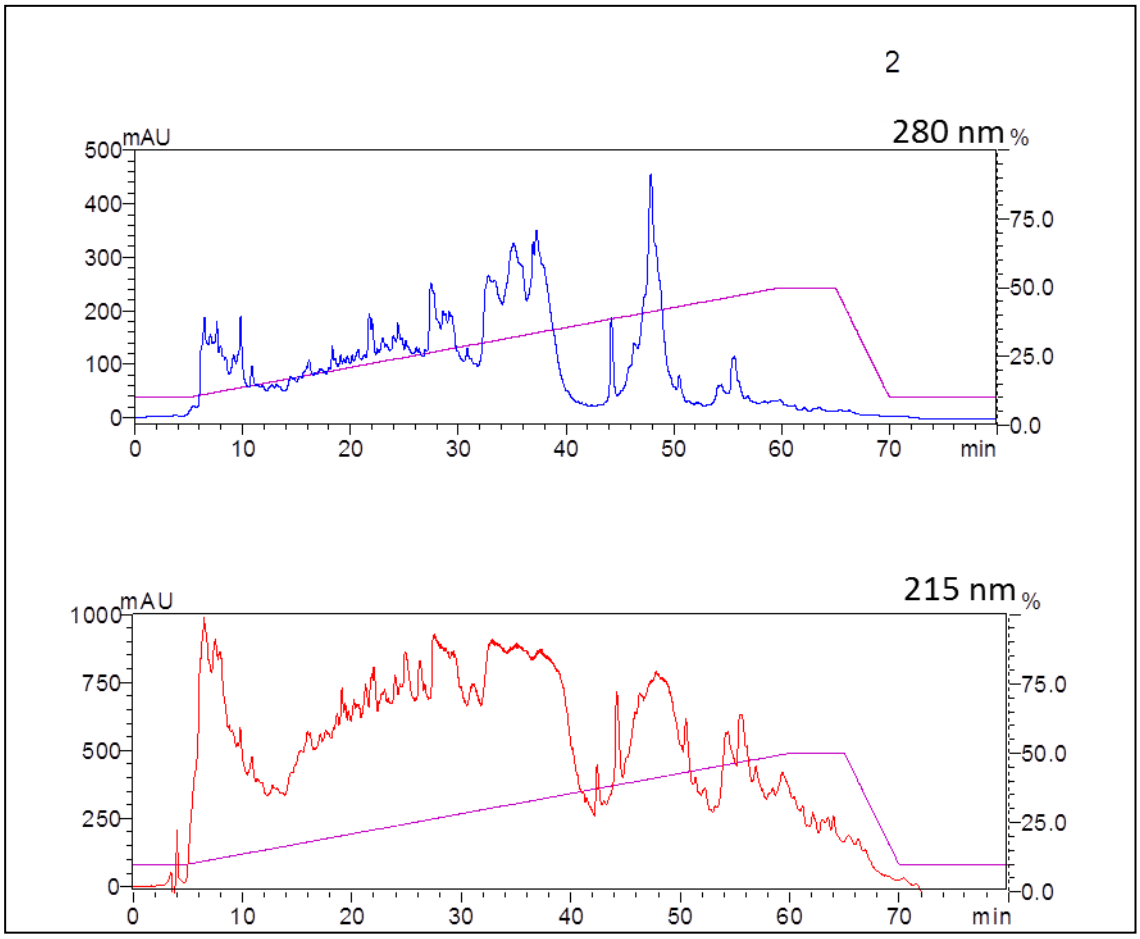


Figure 3.21: Chromatogram of RP-HPLC from lyophilisate shows various peaks between 10 and 60 min, shown by 280 and 215 nm.

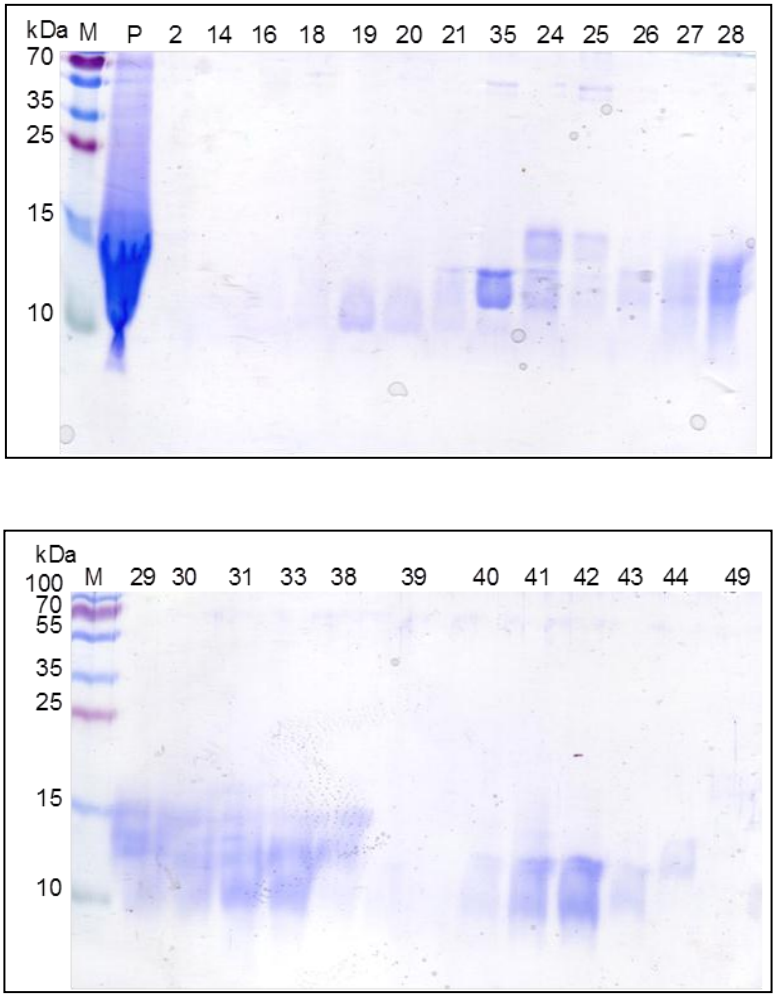


Figure 3.22: SDS-PAGE analysis of selected fractions from RP-HPLC..

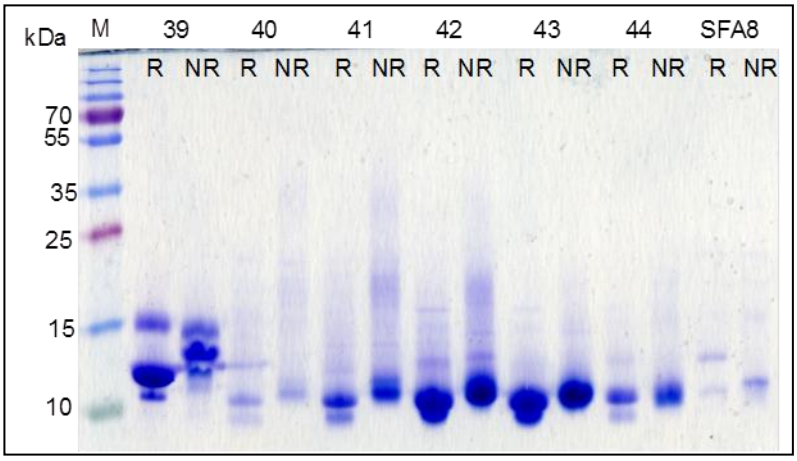


Figure 3.23: SDS-PAGE analysis of selected fractions from RP-HPLC run 13 and 14 and reference material. (SFA 8, kindly provided from Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK).

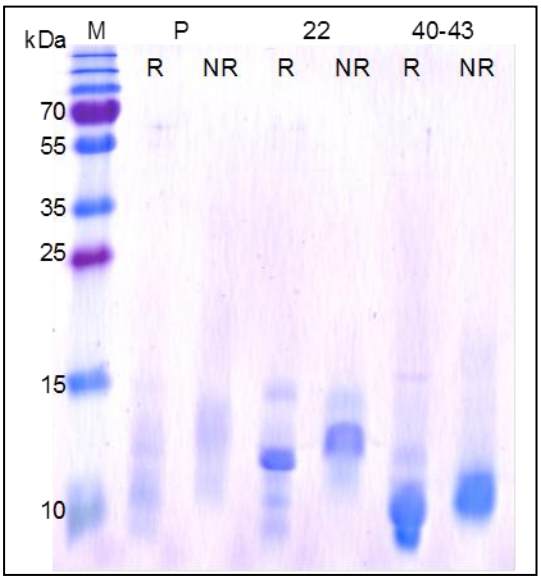


Figure 3.24: SDS-PAGE analysis of pooled fractions from RP-HPLC. (P, sample before RP-HPLC separation).

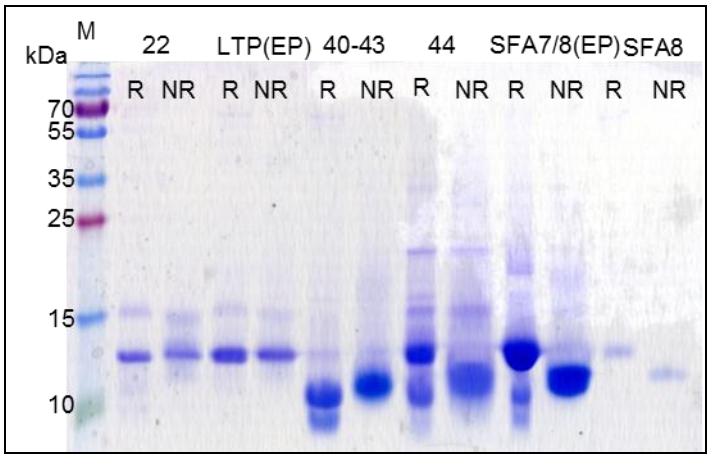


Figure 3.25: SDS-PAGE analysis of pooled RP-HPLC fractions, comparing them to purified LTP and SFA 8(22(EP), sunflower LTP and SFA 7/8 (EP), from EuroPrevall project (FOOD CT 2005-514000); SFA 8, kindly provided from Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK).

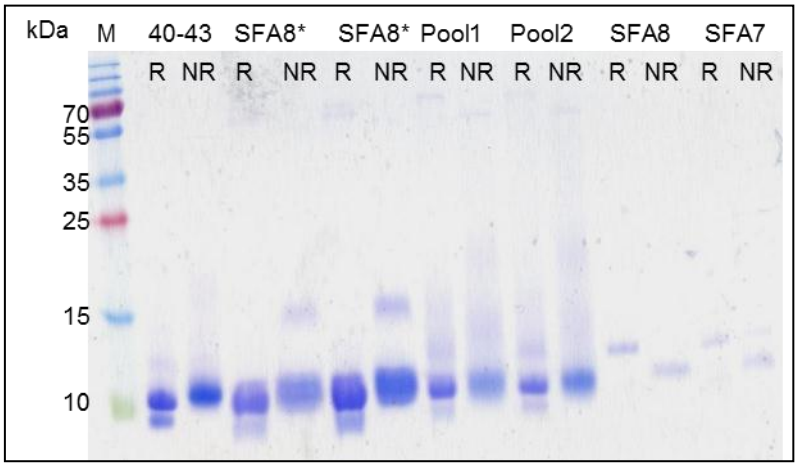


Figure 3.26: SDS-PAGE analysis of 2S albumins, in fraction 40-43 from RP-HPLC, SFA 8 and SFA 7. (SFA 8 in two different concentrations, kindly provided from Prof. Marcos Alcocer, School of Bioscience, University of Nottingham, UK; SFA 8 and SFA 7, kindly provided from Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts Al5 2JQ, UK).*

3.2 Molecular characterization of purified proteins from sunflower seeds

With SDS-PAGE analysis of RP-HPLC we compared pool 1 and pool 2 from protocol 1 to fraction 22, 40-43 and 44 from protocol 2, but also to the pure SFA 8 (from P.Shewry and M. Alcocer) and purified sunflower LTP and SFA7/8 (from EuroPrevall).

Fraction 44 looks really similar to the purified SFA 7/8 from EuroPrevall but also the purified SFA 8 and SFA 7 from Prof. Peter Shewry. That's because it is the only one of our isolated fractions, where the reduced form has a higher molecular weight than the non-reduced form.

Fraction 40-43, on the other hand, shows similarity to SFA 8* from Prof. Marcos Alcocer. Fraction 22, our potential sunflower LTP, looks much like the sunflower LTP provided by EuroPrevall.

Further characterization of our RP-HPLC fractions, were performed by Mag. Dr. Gabriele Gadermaier (University of Salzburg, Dept. of Molecular Biology), who through mass spectrometry analysis.

Fraction 22 could be identified as P82007, also known as sunflower LTP. The bold amino acids, representing 42% of the whole sequence, could be found after trypsin digesting (Figure 3.27). HAG 5 and SFA 8 could be found in fraction 40-43 (Figure 3.16 A and B). Purified SFA 8 could be found in fraction 44 (Figure 3.16 A).

P82007 is also called non-specific lipid-transfer protein AP10 and has 116 amino acids. Its molecular mass is 11,954 Da. BCA showed that the total amount of sunflower LTP, from fraction 22, is 4.8 mg.

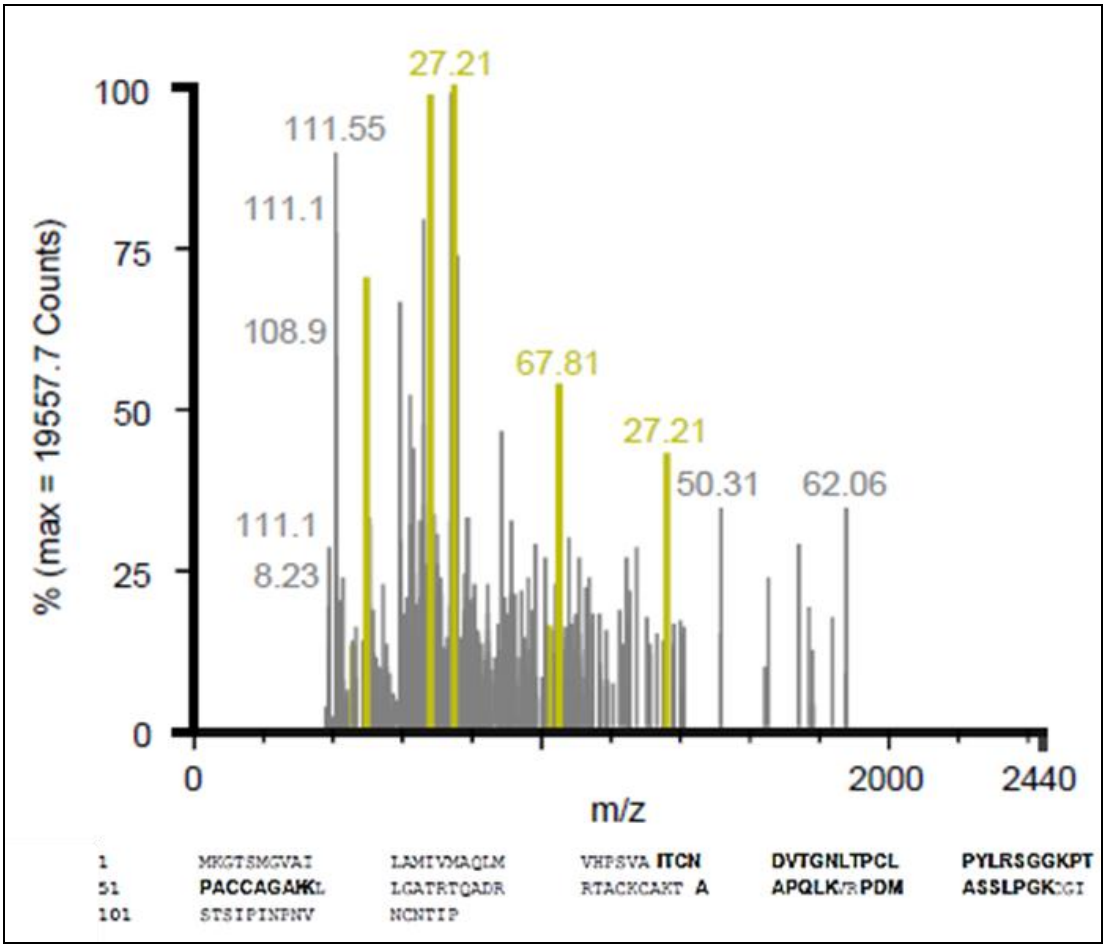
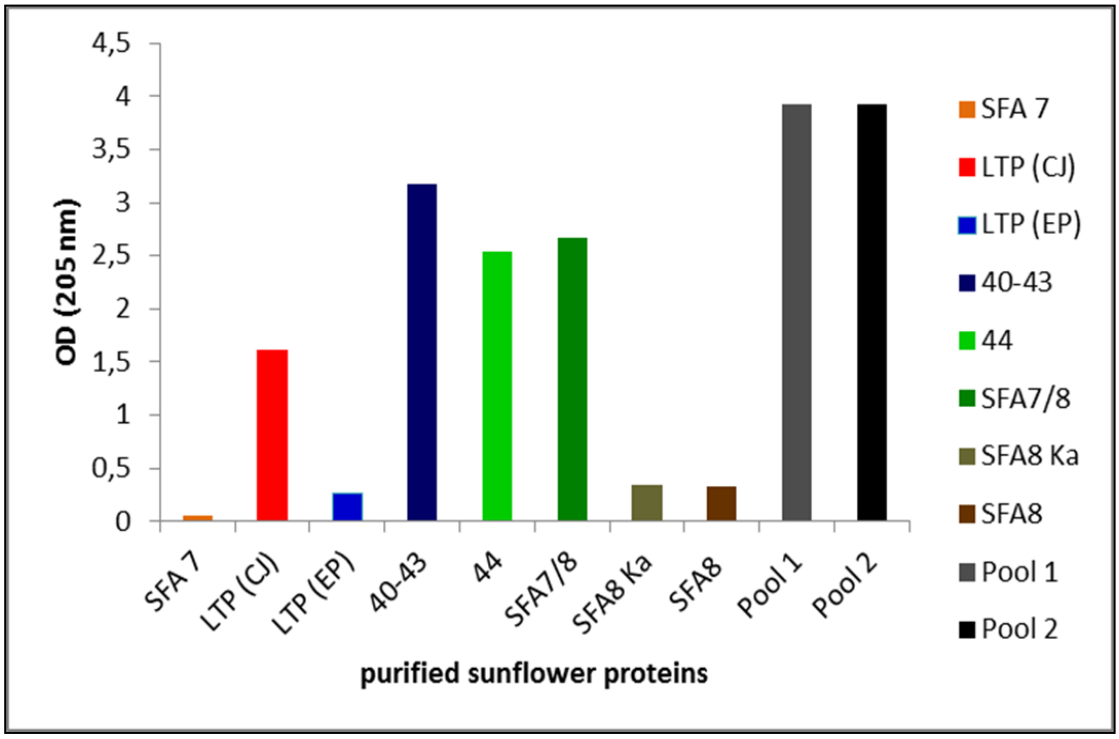


Figure 3.27: Mass spectrometry analysis of fraction 22 proteins and peptide fingerprinting showing P82007, also known as sunflower LTP. The bold amino acids, making 42% of the whole sequence, could be found after digesting with trypsin.

Immunological characterization of LTP, 2S albumins through IgE-ELISA

IgE-binding was tested for sunflower LTP, pool 40-43 from RP-HPLC, fraction 44 from RP-HPLC, SFA 7/8 from EuroPrevall, SFA 8 provided from Prof. Alcocer, SFA 8 and SFA 7 provided from Prof. Shewry, pool 1 and pool 2 from purification protocol 1 by ELISA (Figure 3.28).



SFA 7	0,055
LTP (CJ)	1,619
LTP (EP)	0,268
40-43	3,181
44	2,5445
SFA7/8	2,672
SFA8 Ka	0,341
SFA8	0,325
Pool 1	3,923
Pool 2	3,923

Figure 3.28:Different sunflower protein preparation were tested for IgE- binding. .

P15461:

Entry	Organism	Identity
Q9AUD1	*Sesamum indicum* (Oriental sesam)	33,0%
B6EBI1	*Sesamum indicum* (Oriental sesam)	33,0%
D0PWG2	*Corylus avellana* (European hazel)	39,0%
P0C8Y8	*Bertholletia excelsa* (Brazil nut)	34,0%

Table 3.1: Identity comparison from P15461 to other 2S albumins.

Figure 3.28 shows that IgE from the sunflower allergic patient recognized all different samples, while the control sera didn't. Fraction 44 and SFA 7/8 from EuroPrevall show similar IgE-binding capacity. Pool 1, pool 2 and fraction 40-43 had higher OD values than all the other samples. In table 3.1, HAG 5's identity was compared to other 2S albumins. It shows 30% identity to sesame allergen, Q9AUD1 and B6EBI1 and 39% to D0PWG2, from European hazel. The blood test of our sunflower allergic patient also registered 1,2 kU/l of sesame seed extract specific IgE. He furthermore showed a positive response to a nut mix, which included peanut, hazel nut, Brazil nut, almond and coconut, in vitro.

4 Discussion

To date, no specific allergen from sunflower seeds could be characterized to be highly allergenic [80]. Only few studies mentioned allergic reactions towards sunflower proteins, such as SFA 8 or sunflower LTP [56, 61-67].
SFA 8, a 2S albumin, but also sunflower LTP belong to the prolamin-superfamily, which is a major group of seed storage proteins and can be found in many plants.
2S albumins are often major food allergens in food, for example Brazil nut or sesame. They are cysteine-rich, water-soluble proteins with a molecular mass of about 12-15 kDa. SFA 8 could be found to be highly stable against acidic condition, but also against heat and guanidinium chloride [38]. These results were expected, since the intra-chain sulfide bonds and the characteristic conserved skeleton of 2S albumins give them a compact structure that holds the core protein together. Within the plants, 2S albumins are synthesized as precursor proteins, which then get folded and transported through the endoplasmatic reticulum and Golgi apparatus, until they finally are sorted into vesicles for their transport to the vacuole [81]. All 2S albumins are finally processed into the large and the small subunit with one exception, SFA 8.

Non-specific lipid transfer proteins (nsLTPs) are also known to be major allergens in different foods. They are small molecules of an approximately molecular mass of 9 kDa and share a similar three-dimensional structure that provides a hydrophobic cavity.
Also nsLTPs are stable against heat and low pH, which makes them “perfect” for the sensitization through the GIT. Kean et al, demonstrated the differential polarization of immune responses through Ber e 1 and SFA 8. SFA 8 induced IL-12-producing dendritic cells, which then could promote a Th1 response and reduce the Th2 response. In contrast, Ber e 1did not induces IL-12 or TNF-alpha production through DC. Previous studies could show that IL-12 might play a major role in preventing allergic responses [80].

Immunological responses in our body are highly complex and so there are still many questions unanswered. For a better understanding, more research has to be done, and therefore it is important to establish easy, qualitative and quantitative purification protocols in order to receive highly purified proteins for further testing. This was also our aim, and so we established two purification protocols for low molecular weight proteins in sunflower seeds. Further testing with the purified proteins will be done by Dr. Ashjaei, Medical University of Vienna.

Our first purification protocol was established to purify the low molecular mass proteins from sunflower seeds, such as 2S albumins and sunflower LTP, avoiding harsh treatment of the proteins. After extraction with sodium phosphate buffer, precipitation with ammonium sulfate, ion exchange chromatography and hydrophobic interaction chromatography, we finally received two different pools. Pool 1 includes wash 2 to 4, pool 2 fractions 1-25. A 2D gel electrophoresis was then performed with pool 2, which includes fraction 1 to 25. The two-dimensional gel showed three spots with pI 5.1, 5.6 and 6.5. All three spots have a molecular weight of about 12 kDa. The immunoblot of the 2D-gel showed that the polyclonal anti-SFA 8-antibody recognized exclusively the three spots (Figure 3.13 and 3.14). A one-dimensional immunoblot with anti-SFA 8-antibody was also performed with pool 1 and pool 2. Also in this blot a band with a molecular weight of 12 kDa could be seen (Figure 3.14).

Despite our expectations, pure SFA 8 (kindly provided by Prof. Peter Shewry, Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK) runs different on the gel compared to our pool 1 and pool 2 (Figure 3.15). The band of the reduced form of pure SFA 8 has Mr~12.5 kDa, while the non-reduced form of the pure SFA 8 has Mr~11 kDa. So the reduced form shows a higher molecular weight than the non-reduced. With the pool 1 and 2 is it the other way round. There non-reduced form has a higher molecular weight than the reduced form although both are, with a Mr~10.9 and 10.7 kDa, smaller than the pure SFA 8. our samples behaved different in SDS-PAGE than the

purified SFA 8, which was provided by Prof. Shewry, (Dept. of Plant Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK).
For identification, pool 1 and pool 2 were sent for mass spectrometry analysis to Mag.Dr. Gabriele Gadermaier (University of Salzburg, Dept. of Molecular Biology). The main component of pool 1 is P15461, also called HAG 5. Also P23110, which is known as SFA 8, could be found in a lower concentration. (Figure 3.16). Pool 2 has only a low amount of 2S albumins. The main component was an unknown protein.

For the immunological characterization pool 1 and 2 were used to perform an IgE-ELISA with a sunflower-allergic patient serum and control sera. Figure 3.30 shows that IgE from the sunflower allergic patient recognized all different samples, while the control sera couldn't. Fraction 44 and SFA 7/8 from EuroPrevall show similar IgE-binding. Pool 1 and pool 2 created a higher IgE-absorption than all the other samples. This might be due to the present of P15461, HAG 5. In table 3.1, HAG 5's identity was compared to other 2S albumins. It shows 30% identity to sesame allergen, Q9AUD1 and B6EBI1 but also to D0PWG2, from European hazel.
The blood test of our sunflower allergic patient also registered 1,2 kU/l of sesame seed extract specific IgE. He furthermore showed a positive response to a nut mix, which included peanut, hazel nut, Brazil nut, almond and coconut. So it is possible that HAG 5 cross-reacted with the patient's IgE, directed against sesame or other nuts. Another possible cause for the higher IgE-absorption of our extracted proteins is, a lower concentration of proteins in purified SFA 8 and SFA 7, which were kindly provided by Prof. Peter Shewry.

The second purification protocol we tried, was described previously in the paper from Burnett et al [48]. The extractions steps from our two purification protocols are different to the paper from Burnett et al: For the defatting process we used n-Hexane instead of Petroleum and the air-dried flour was then extracted with 50 mM Sodium-phosphate buffer, pH 7 overnight at 4°C under constant stirring,

while Burnett et al. extracted sunflower proteins with 20 mM Tris-HCl containing 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 h at RT. The dried flour was then extracted with 4,000 ml 50 mM Sodium phosphate buffer overnight at 4°C under constant stirring. Our extract was furthermore precipitated with 90% ammonium sulfate. The globulin precipitation through cold methanol, the 2S albumin precipitation through ice cold acetone, the extensively dialysis for 48 h and the following lyophilisation was performed as described in the paper of Burnett et al. RP-HPLC was carried out by a Phenomenex Jupiter C5 column, instead of a Phenomenex Jupiter C4 column, using a 10-50% acetonitrile gradient. A single run took 80 min, the last 20 min being the washing and regenerating of the column for the next run. The eluted fractions were monitored at 215 nm but also 280 nm. After RP-HPLC the fractions were immediately frozen with N_2 for about 30 min and lyophilsated. Burnett et al. furthermore performed a size exclusion chromatography [48]. Before we injected our own sample, we ran test-runs with purified sunflower LTP and SFA 7/8 from EuroPrevall to see the expected retention time. Retention time of sunflower LTP was about 28 min, being less hydrophobic than SFA 7/8, which was eluted not till 50 min. RP-HPLC was able to separate SFA 7 and 8, because SFA 7 is a little bit less hydrophobic. Our sunflower seeds (from Natur pur, Spar, origin:China) had less SFA 8 then the hybrid variety, Aphasol (Cargil seeds), Burnett et al used [48]. In the paper from Anisimova et al. [82], 103 sunflower accessions were tested with RP-HPLC. The various genotypes showed a different SFA 7, SFA 8 and total albumin fraction levels.

With SDS-PAGE we detected fractions of similar molecular mass, which could then be pooled. In fraction 22 we found sunflower LTP, and in fraction 40-43 we expect sunflower 2S albumins. These fractions were compared with purified LTP, from EuroPrevall, purified SFA 7/8 from EuroPrevall, Prof. Peter Shewry and Prof. Marcos Alcocer, on SDS-PAGE with CBB-staining (Figure 3.25 and 3.26). Our sunflower LTP was identically to sunflower LTP from EuroPrevall (Figure 3.25), and our 2S albumin fraction could also be found to be highly similar to the SFA 7/8 from Prof. Marcos Alcocer. But the SFA 7/8 from Prof.

Marcos Alcocer and our own 2S albumin fraction couldn't be compared to the purified SFA 8 from Prof. Peter Shewry.
Further characterization of our RP-HPLC fractions, were again performed through mass spectrometry analysis. Fraction 22 could be identified as P82007, also known as sunflower LTP (Figure 3.27). HAG 5 and SFA 8 could be found in fraction 40-43 (Figure 3.16 A and B). Purified SFA 8 could also be found in fraction 44 (Figure 3.16 A).

Comparing the two purification protocols, the second one, after Burnett et al. [48], is easier to perform because it only needs a single chromatography. Although it is a harsh method, with using methanol, acetone and also trifluoroacetic acid, it was found to be very suitable for sunflower seed proteins. The other purification protocol tried to provide protein-safe conditions, in order to receive native proteins. It is much more time-consuming, and there was less of the purified protein in the end. Since, sunflower proteins, 2S albumins as well as sunflower LTP, are highly-stable, the purification protocol of Burnett et al. [48] can be recommended. With immunological characteristics, such as IgE-binding and interaction of the proteins with human immune systems, we will compare the uptaking of these purified sunflower proteins to highly allergenic food proteins, such as Ara h 2.

5 Summary

Many major food allergens known today belong to the 2S albumins- and nsLTPs-family. Examples for 2S albumins are, Ber e 1 from Brazil nut, Ara h 2 and Ara h 6 from peanut, and Ses i 1 from sesame.
Examples for LTP allergens are, Prup p 3 from peach, Cor a 8 from hazelnut, or Act d 10 from kiwi. All of them can cause life-threatening allergic reactions.
Until today, there couldn't be found a major sunflower seed allergen. This might be due to the fact, that there aren't many people allergic to sunflower seeds, and the few people who are, recognize different allergens.
Most of the reported allergic reactions to sunflower seeds are mild, however, a few cases showed anaphylaxis as an allergic reaction. Reports have been suggesting that sunflower seed sensitization occurs via inhalation rather than ingestion. This affects especially baker, people with caged birds which are fed with sunflower seeds, but also people working or living near sunflower fields.
So, sunflower LTP and SFA 7/8 are quite interesting for food allergy research, cause despite their stability against heat and gastric acid, they are not identified as food allergens.
Researchers could find differences between SFA 8 and other 2S albumins, which could be the reasons for their different allergenicity. Despite of most other 2S albumins, SFA 8, as well as Ara h 2, is not processed into a small and large subunit.
The aim of this study was to establish an easy-performed, qualitative and quantitative purification protocol for low molecular weight proteins of sunflower seeds.
The first protocol was performed in order to keep proteins as native as possible, while the second one, described before in Burnett et al [48]., was rather harsh.
With the purification protocol 1 we isolated P15461(HAG 5) and P23110, (SFA 8), shown in Figure 3.16.
In the purification protocol 2, we isolated sunflower LTP, SFA 8 and HAG 5. Sunflower LTP and SFA 8 could be found highly pure (Figure 3.27) and

furthermore the amount of SFA 8 was significantly higher than in pool 1 and pool 2.

The purified proteins will be used to investigate dendritic cells' uptaking in comparison to major food allergens.
Purification of potential allergens is and will be important for the understanding of food allergies and the following allergic mechanism in our body.

6 Zusammenfassung

Viele Nahrungsmittelallergene, die wir heutzutage kennen, gehören zu den Familien der 2S Albumine und nicht spezifischen Lipid-Transfer-Proteinen (nsLTPs). Wichtige Nahrungsmittelallergene der 2S Albumine sind beispielsweise, Ber e 1 (Paranuss), Ara h 2 und Ara h 6 (Erdnuss) und Ses i 1 (Sesam).
Zu den nsLTPs gehören zum Beispiel, Prup p 3 (Pfirsich), Cor a 8 (Haselnuss) und Act d 10 (Kiwi). Ihnen allen ist gemein, dass sie lebensgefährliche allergische Reaktionen in sensibilisierten Nahrungsmittelallergikern auslösen können. Auch Sonnenblumenkerne besitzen 2S Albumine und nsLTPs und wurden deshalb bereits eingehend auf ihre Allergenität untersucht. Allerdings konnte bis dato, kein Hauptallergen in Sonnenblumenkernen gefunden werden. Dies könnte vor allem daran liegen, dass es nicht viele Sonnenblumenallergiker gibt und, dass diese zusätzlich unterschiedliche Allergene erkennen. Die meisten allergischen Reaktionen, die durch Sonnenblumenkerne ausgelöst werden sind zudem mild. Es gibt aber auch einzelne dokumentierte Fälle von Anaphylaxie. Einige Publikationen zeigen, dass die Sensibilisierung auf Sonnenblumenkerne eher durch Inhalation der Sonnenblumenpollen oder des Sonnenblumenkernmehl erfolgen könnte. Dies betrifft vor allem Bäcker, Vogelbesitzer (Vogelfutter enthält oft Sonnenblumenkerne) und auch Menschen, die auf Sonnenblumenfeldern arbeiten oder in der Nähe leben.
Im Gegensatz zu den meisten 2S Albuminen wird SFA 8 nicht in große und kleine Untereinheiten prozessiert.
Das Ziel dieser Diplomarbeit war ein Reinigungsprotokoll für niedermolekulare Sonnenblumenkernproteine zu erstellen, das leicht durchführbar, aber auch qualitativ und quantitativ hochwertig ist. Es wurden zwei Reinigungsprotokolle ausgearbeitet und miteinander verglichen. Während das eine Protokoll sich zur Aufgabe gemacht hat möglichst native Bedingungen für Proteine zu gewährleisten, sind beim zweiten Aufreinigungsprotokoll eher aggressive Methoden zu finden [48].

Mit dem Protokol 1 konnten v.a. P15461(HAG 5) und P23110, (SFA 8), (Figure 3.16) aufreinigen. Für das Protokoll 2 zeigte das Massenspektrum die erfolgreiche Aufreinigung von Sonnenblumen-LTP, SFA 8 und HAG 5. Sonnenblumen-LTP und SFA 8 konnte besonders rein gefunden werden. Mit Protokoll 2 erhielten wir siginifikant mehr SFA 8 (Figure 3.27).
Die weiteren wissenschaftlichen Arbeiten mit diesen aufgereinigten Proteinen umfassen die Untersuchung der Aufnahme in Dendritischen Zellen, im Vergleich zu stark wirksamen Proteinen.
Die Aufreinigung von potentiellen Allergenen wird auch noch in Zukunft wichtig sein, um Allergien besser zu verstehen.
.

List of illustrations

List of tables

7 References

1. *Boyce, J.A., et al., Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol, 2010.* ***126****(6 Suppl): p. S1-58.*
2. *Ismail, I.H. and M.L. Tang, Oral immunotherapy for the treatment of food allergy. Isr Med Assoc J, 2012.* ***14****(1): p. 63-9.*
3. *Murch, S.H., The immunologic basis for intestinal food allergy. Curr Opin Gastroenterol, 2000.* ***16****(6): p. 552-7.*
4. *Rothenberg, M.E., Eosinophilic gastrointestinal disorders (EGID). J Allergy Clin Immunol, 2004.* ***113****(1): p. 11-28; quiz 29.*
5. *Vickery, B.P., S. Chin, and A.W. Burks, Pathophysiology of food allergy. Pediatr Clin North Am, 2011.* ***58****(2): p. 363-76, ix-x.*
6. *Fishbein, A.B. and R.L. Fuleihan, The hygiene hypothesis revisited: does exposure to infectious agents protect us from allergy? Curr Opin Pediatr, 2012.* ***24****(1): p. 98-102.*
7. *Ege, M.J., et al., Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol, 2006.* ***117****(4): p. 817-23.*
8. *Alfven, T., et al., Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle--the PARSIFAL study. Allergy, 2006.* ***61****(4): p. 414-21.*
9. *Cebra, J.J., Influences of microbiota on intestinal immune system development. Am J Clin Nutr, 1999.* ***69****(5): p. 1046S-1051S.*
10. *MacPherson, G.G. and L.M. Liu, Dendritic cells and Langerhans cells in the uptake of mucosal antigens. Curr Top Microbiol Immunol, 1999.* ***236****: p. 33-53.*
11. *Metcalfe, D.D., H.A. Sampson, and R.A. Simon, Food Allergy: Adverse Reactions to Foods and Food Additives 4th edition ed. 2008: Blackwell Publishing. 623.*
12. *Nordenstedt, H., M. Nilsson, and R. Johnson, Helicobacter pylori infection and gastroesophageal reflux in a population-based study (The HUNT Study). Helicobacter 2007.* ***12****: p. 16-22.*
13. *Figura, N., A. Perrone, and C. Gennari, CagA-positive Helicobacter pylori infection may increase the risk of food allergy development. J Physiol Pharmacol, 1999.* ***50****: p. 827-831.*
14. *Zuckerman, M.J., et al., Intestinal permeability to [51Cr]EDTA in infectious diarrhea. Dig Dis Sci, 1993.* ***38****: p. 1651-1657.*
15. *Sampson, H.A., Food allergy. Part 1: immunopathogenesis and clinical disorders. J Allergy Clin Immunol, 1999.* ***103****(5 Pt 1): p. 717-28.*
16. *Kaczmarski, M. and B. Kurzatkowska, The contribution of some environmental factors to the development of cow's milk and gluten intolerance in children. Rocz Akad Med Bialymst, 1988.* ***33-34****(151): p. 65.*
17. *Chafen, J.J.S., et al., Diagnosing and Managing Common Food Allergies: A Systematic Review. JAMA, 2010.* ***303****(18): p. 1848-1856.*
18. *Venter, C., et al., Time trends in the prevalence of peanut allergy: three cohorts of children from the same geographical location in the UK. Allergy, 2010.* ***65****(1): p. 103-8.*

19. *Conseil fe´de´ ral suisse Ordonnance sur les denre´es alimentaires. 2002.*
20. *Sicherer, S.H. and H.A. Sampson, Food allergy. J Allergy Clin Immunol, 2006.* ***125****(2 Suppl 2): p. S116-25.*
21. *Rona, R.J., et al., The prevalence of food allergy: a meta-analysis. J Allergy Clin Immunol, 2007.* ***120****(3): p. 638-46.*
22. *Zuidmeer, L., et al., The prevalence of plant food allergies: a systematic review. J Allergy Clin Immunol, 2008.* ***121****(5): p. 1210-1218 e4.*
23. *Amlot, P.L., et al., Oral allergy syndrome (OAS): symptoms of IgE-mediated hypersensitivity to foods. Clin Allergy, 1987.* ***17****(1): p. 33-42.*
24. *Mari, A., B.K. Ballmer-Weber, and S. Vieths, The oral allergy syndrome: improved diagnostic and treatment methods. Curr Opin Allergy Clin Immunol, 2005.* ***5****(3): p. 267-73.*
25. *Asero, R., Plant food allergies: a suggested approach to allergen-resolved diagnosis in the clinical practice by identifying easily available sensitization markers. Int Arch Allergy Immunol, 2005.* ***138****(1): p. 1-11.*
26. *Urisu, A., et al., Japanese guideline for food allergy. Allergol Int, 2011.* ***60****(2): p. 221-36.*
27. *Jarvinen, K.M., et al., Use of multiple doses of epinephrine in food-induced anaphylaxis in children. J Allergy Clin Immunol, 2008.* ***122****(1): p. 133-8.*
28. *Oren, E., et al., Food-induced anaphylaxis and repeated epinephrine treatments. Ann Allergy Asthma Immunol, 2007.* ***99****(5): p. 429-32.*
29. *Asero, R., et al., IgE-mediated food allergy diagnosis: Current status and new perspectives. Mol Nutr Food Res, 2007.* ***51****(1): p. 135-47.*
30. *Bernstein, I.L., et al., Allergy diagnostic testing: an updated practice parameter. Ann Allergy Asthma Immunol, 2008.* ***100****(3 Suppl 3): p. S1-148.*
31. *Rance, F., et al., Correlations between skin prick tests using commercial extracts and fresh foods, specific IgE, and food challenges. Allergy, 1997.* ***52****(10): p. 1031-5.*
32. *Grimshaw, K.E., et al., Presentation of allergen in different food preparations affects the nature of the allergic reaction--a case series. Clin Exp Allergy, 2003.* ***33****(11): p. 1581-5.*
33. *Reider, N. and M. Ledochowski, Klinische Ernährungsmedizin. 2010: SpringerWienNewYork. 1028.*
34. *Shewry, P.R., et al., Plant protein families and their relationships to food allergy. Biochemi. Soc. Trans., 2002(30): p. 906.*
35. *Shewry, P.R., J.A. Napier, and A.S. Tatham, Seed storage proteins: structures and biosynthesis. Plant Cell, 1995.* ***7****(7): p. 945-56.*
36. *Monsalve, R.I., et al., In Plant Food Allergens. 2007.*
37. *Sathe, S.K., H.H. Kshirsagar, and K.H. Roux, Advances in Seed Protein Research: A Perspective on Seed Allergens. J. Food Sci, 2006(70).*
38. *Murtagh, G.J., et al., In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8 Clin. Exp. Allergy, 2003: p. 1147.*
39. *Gonzalez-Perez, S., et al., Physicochemical Properties of 2S Albumins and the Corresponding Protein Isolate from Sunflower (Helianthus annuus) (pages C98–C103). J. Food Sci, 2005.* ***70****: p. C98.*
40. *Moreno, F.J. and A. Clemente, 2S Albumin Storage Proteins: What Makes them Food Allergens? Open Biochem J, 2008.* ***2****: p. 16-28.*

41. *Aggizio, A.P., et al., The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells Plant Science, 2006.* ***171****(4): p. 515-522.*
42. *Mouecoucou, J., et al., Effects of gum arabic, low methoxy pectin and xylan on in vitro digestibility of peanut protein. Food Res. Int., 2004.* ***37****: p. 777.*
43. *Van Wijk, F., et al., The Effect of the Food Matrix on In Vivo Immune Responses to Purified Peanut Allergens. Toxicol. Sci. , 2005.* ***86****: p. 333.*
44. *Burnett, G.R., et al., Interaction between protein allergens and model gastric emulsions. Biochem. Soc. Trans. , 2002.* ***30****: p. 916.*
45. *Pastorello, E.A., et al., Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy Allergy Clin. Immunol, 1998(102): p. 1021.*
46. *Kortt, A.A. and J.B. Caldwell, Low molecular weight albumins from sunflower seed: identification of a methionine-rich albumin. Phytochemistry, 1990.* ***29****: p. 2805.*
47. *Pandya, M.J., et al., Structural characterization of a methionine-rich, emulsifying protein from sunflower seed. Proteins, 2000.* ***38****: p. 341.*
48. *Burnett, G.R., et al., Characterization of the Emulsification Properties of 2S Albumins from Sunflower Seed*
J. Colloid Interface Sci, 2002: p. 177-185.
49. *Moreno, F.J., et al., Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (Sesamum indicum L.). Biochim. Biophys. Acta, 2005.* ***1752****: p. 142.*
50. *Monsalve, R.I., M. Villalba, and R. Rodriguez, Allergy to Mustard Seeds: The Importance of 2S Albumins as Food Allergens. Internet Symp. Food Allergens, 2001: p. 57.*
51. *Jenkins, J.A., et al., Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: An in silico analysis. J. Allergy Clin Immunol, 2005.* ***115****: p. 163.*
52. *Kader, J.C., Lipid-Transfer Proteins in Plants. Annu Rev Plant Physiol Plant Mol Biol, 1996.* ***47****: p. 627-654.*
53. *Monlina, A., et al., Inhibition of bacterial and fungal plant pathogens by thionin of types I and II. Plant Sci, 1993.* ***92****: p. 169-177.*
54. *Pastorello, E.A. and A.M. Robino, Clinical role of lipid transfer proteins in food allergy. Mol Nutr Food Res, 2004.* ***48****(5): p. 356-62.*
55. *Hoffmann-Sommergruber, K., Pathogenesis-related (PR)-proteins identified as allergens. Biochem Soc Trans, 2002.* ***30****(Pt 6): p. 930-5.*
56. *Yagami, A., Anaphylaxis to lipid transfer protein from sunflower seeds. Allergy, 2010.* ***65****(10): p. 1340-1.*
57. *Skoric, D., et al., An analysis of heterotic potential for agronomically important traits in sunflower (Helianthus annuus L.) seed. J. Agric. Food Chem, 2007.* ***38****: p. 2103-2110.*
58. *Gueguen, J., et al., Functionality of the 2S albumin seed storage proteins from sunflower (Helianthus annuus L.) Agric. Food Chem. , 1996.* ***44****: p. 1184-1189.*
59. *Nordlee, J.A., et al., Identification of a Brazil-nut allergen in transgenic soybeans. N Engl J Med, 1996.* ***334****(11): p. 688-92.*

60. *Teuber, S.S., et al., Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (Juglans regia), a major food allergen. . J. Allergy Clin Immunol, 1998.* ***101****: p. 807-814.*
61. *Noyes, J.H., G.K. Boyd, and G.A. Settipane, Anaphylaxis to sunflower seed. J Allergy Clin Immunol, 1979.* ***63****(4): p. 242-4.*
62. *Kanny, G., et al., Food allergy to sunflower oil in a patient sensitized to mugwort pollen. Allergy, 1994.* ***49****(7): p. 561-4.*
63. *Birnbaum, J., et al., Allergy to sunflower honey associated with allergy to celery. Clin Exp Allergy, 1989.* ***19****(2): p. 229-30.*
64. *Bousquet, J., et al., Occupational allergy to sunflower pollen. J Allergy Clin Immunol, 1985.* ***75****(1 Pt 1): p. 70-4.*
65. *Jimenez, A., et al., Sensitization to sunflower pollen: only an occupational allergy? Int Arch Allergy Immunol, 1994.* ***105****(3): p. 297-307.*
66. *Vandenplas, O., T. Vander Borght, and J.P. Delwiche, Occupational asthma caused by sunflower-seed dust. Allergy, 1998.* ***53****(9): p. 907-8.*
67. *Axelsson, I.G., E. Ihre, and O. Zetterstrom, Anaphylactic reactions to sunflower seed. Allergy, 1994.* ***49****(7): p. 517-20.*
68. *Anisimova, I.N., et al., Genotypic variation and polymorphism of 2S albumins of sunflower. Euphytica, 1995.* ***83****(1): p. 15.*
69. *Berecz, B., et al., Structural stability and surface activity of sunflower 2S albumins and nonspecific lipid transfer protein. J Agric Food Chem, 2010.* ***58****(10): p. 6490-7.*
70. *Pantoja-Uceda, D., et al., Solution structure of a methionine-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties. Biochemistry, 2004.* ***43****(22): p. 6976-86.*
71. *Lottspeich, F. and J.W. Engels, Bioanalytik. 2006: Elsevier GmbH, München.*
72. *Sparham, S.J. and E.R. Huehns, The separation of human globin chains by ion-exchange chromatography on CM-Sepharose CL-6B. Hemoglobin, 1979.* ***3****(1): p. 13-20.*
73. *Hagel, L., et al., Properties, in theory and practice, of novel gel filtration media for standard liquid chromatography. J Chromatogr, 1989.* ***476****: p. 329-44.*
74. *Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970.* ***227****(5259): p. 680-5.*
75. *Meyer, T.S. and B.L. Lamberts, Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylaminde-gel strips. Biochim Biophys Acta, 1965.* ***107****: p. 144-145.*
76. *Diezel, W., G. Kopperschlager, and E. Hofmann, An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie Brilliant Blue. Anal Biochem, 1972.* ***48****(2): p. 617-20.*
77. *Smith, P.K., et al., Measurment of protein using bicinchoninic acid. Anal Biochem, 1985.* ***150****: p. 76-85.*
78. *Wiechelman, K.J., R.D. Braun, and J.D. Fitzpatrick, Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal Biochem, 1988.* ***175****(1): p. 231-7.*
79. *Berecz, B., et al., Structural stability and surface activity of sunflower 2S albumins and nonspecific lipid transfer protein. J Agric Food Chem.* ***58****(10): p. 6490-7.*

80. *Kean, D.E., et al., Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8. J Immunol, 2006.* ***177****(3): p. 1561-6.*
81. *Alcocer, M.J., et al., The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. J Mol Biol, 2002.* ***324****(1): p. 165-75.*
82. *Anisimova, I.N., et al., Polymorphism and inheritance of methionine-rich 2S albumins in sunflower. Euphytica, 2002.* ***129****: p. 99-107.*

Curriculum vitae

Name:	Claudia JELINEK
Date of Birth:	November 4th, 1988
Place of Birth:	Vienna, Austria
Nationality:	Austria
Address:	Aßmayergasse 44/5, 1120 Vienna, Austria
Contact:	claudia.jelinek@hotmail.com

EDUCATION

01/2005-07/2005	Exchange-semester in the United States, Michigan, USA
2007-2012	Studies in pharmacy, University of Vienna, Vienna, Austria
2008-2009	Tutor job (for 3 terms) at the Lab of Quantitative Chemistry, University of Vienna, Austria, under the direction of Prof. Dr. Mag. pharm. Urban
03/2012-current	Diploma thesis at the Department of Pathophysiology and Allergy Research, Medical University of Vienna, thesis on "*Characterisation of proteins with potential allergenic activities from sunflower seeds*".

PRACTICAL TRAINING:

Apotheke Aspern, 1220 Vienna, Austria
under the direction of Mag. Dr. Schlagintweit

Apotheke im Donauzentrum, 1220 Vienna, Austria
under the direction of Mag. pharm. Bittman

Apotheke in Neukagran, 1220 Vienna, Austria
under the direction of Mag. pharm. Margrit Weber-Hauer

Apotheke Altenmarkt, 5541 Salzburg, Austria
under the direction of Mag. Dr. Künßberg