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„Investigation of allergenic properties of birch juice“

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# Contents

## Abstract

IgE-mediated allergy to birch pollen is very frequent in Central and Northern Europe. Birch pollen contains a single major allergen, Bet v 1, which is recognized by IgE antibodies of more than 95% of birch pollen-allergic individuals. Birch juice is considered to be healthy and its consumption is very popular in Eastern Europe.

The aim of this project was to investigate whether birch juice contains Bet v 1 or other allergenic proteins. For this purpose, two commercially available and a freshly harvested birch juice were studied. Different methods to concentrate proteins were compared to enhance the initial low protein content. Thereafter, proteins were separated by SDS-PAGE. The profile of proteins in birch juice was analysed by mass spectrometry proteomics. Birch juice proteins were tested for IgE-reactivity in immunoblots. To test their potential allergenicity, basophil activation tests with cells from birch pollen-allergic individuals were performed.

In summary, these investigations demonstrated that birch juice has no allergenic potential. In contrast, several proteases were found in birch juice. After reconstitution of recombinant Bet v 1 in birch juice we found evidence for its proteolytic degradation. Moreover, Bet v 1 reconstituted in birch juice showed a clearly reduced capacity to activate basophils compared to Bet v 1 kept in PBS, indicative of the loss of its allergenicity. Because birch juice has been considered to promote the gastrointestinal uptake of proteins we tested whether it increases epithelial transport rates of Bet v 1 through Caco-2 monolayers. Our preliminary results did not provide evidence for an enhanced intestinal absorption of Bet v 1 by birch juice.

In summary, we conclude that birch juice does not contain allergens and therefore, will not induce allergic reactions when consumed by birch pollen-allergic individuals.

## Zusammenfassung

Birkenpollenallergie kommt in Zentral- und Nordeuropa häufig vor. Birkenpollen enthält ein Hauptallergen, Bet v 1, welches von IgE Antikörpern von mehr als 95% der Birkenpollenallergikern erkannt wird. In osteuropäischen Ländern wird Birkensaft und dessen Konsumation als sehr gesund angesehen.

Ziel des Projektes war zu untersuchen, ob Birkensaft Bet v 1 oder andere allergene Proteine enthält. Dafür wurden zwei im Handel erhältliche sowie frisch geernteter Birkensaft untersucht. Verschiedene Methoden zur Konzentrierung der Proteine wurden verglichen, um die anfangs niedrige Proteinkonzentration zu steigern. Danach wurden Proteine mittels SDS-PAGE aufgetrennt. Mittels Massenspektrometrie wurde das Profil der Proteine in Birkensaft analysiert. Proteine, welche im Birkensaft enthalten waren, wurden auf IgE Reaktivität in immunoblots getestet. Um eventuelle allergenität zu testen, wurden Basophilenaktivierungstests mit Zellen von Birkenpollen-Allergikern durchgeführt.

Zusammenfassend haben die Untersuchungen gezeigt, dass Birkensaft kein allergenes Potential hat. Stattdessen wurden einige Proteasen in Birkensaft gefunden. Durch Rekonstitution von rekombinantem Bet v 1 in Birkensaft konnte dessen proteolytische Degradation nachgewiesen werden. Weiters zeigte rekonstituiertes Bet v 1 eine deutlich niedrigere Basophilen Aktivierung verglichen mit Bet v 1 in PBS, welches den Verlust der Allergenität verdeutlicht. Da Birkensaft nachgesagt wird, die gastrointestinale Aufnahme von Proteinen zu fördern, wurde mittels einer Caco2-Zelllinie getestet, ob dieser den Epithelial-Transport von Bet v 1 verstärkt. Unsere vorläufigen Ergebnisse konnten keine Verbesserung der Darm-Aufnahme von Bet v 1 durch Birkensaft beweisen.

Zusammenfassend enthält Birkensaft keine Allergene und wird daher auch keine allergische Reaktion hervorrufen, wenn er von Birkenpollenallergikern konsumiert wird.

## Aim

Birch juice is said to have a lot of health benefits and is therefore consumed regularly in eastern European countries. It was not proven yet whether birch juice contains allergenic proteins. For now, the only available treatment of birch pollen allergy is the allergen-specific immunotherapy with birch pollen which unfortunately has limited benefits for birch-pollen related food allergies.

The aim of this study was to analyze and characterize proteins in birch juice and see if any proteins similar to birch pollen allergens could be detected. The detected proteins should be tested for IgE reactivity. A reactivity of birch pollen allergic donor's IgE to the proteins would prove allergic properties of those proteins. Thereby, a possible treatment method of birch pollen allergy by ingestion of birch juice to induce oral tolerance could be established.

# 1. Introduction

## 1.1. The immune system

The immune system provides protection against infectious microbes, non-infectious substances and products of damaged cells by reacting to molecules recognized as foreign, whereas it does not react against self-antigens and tissues. It is divided into two parts: the innate and the adaptive immune system. [1,2,3]

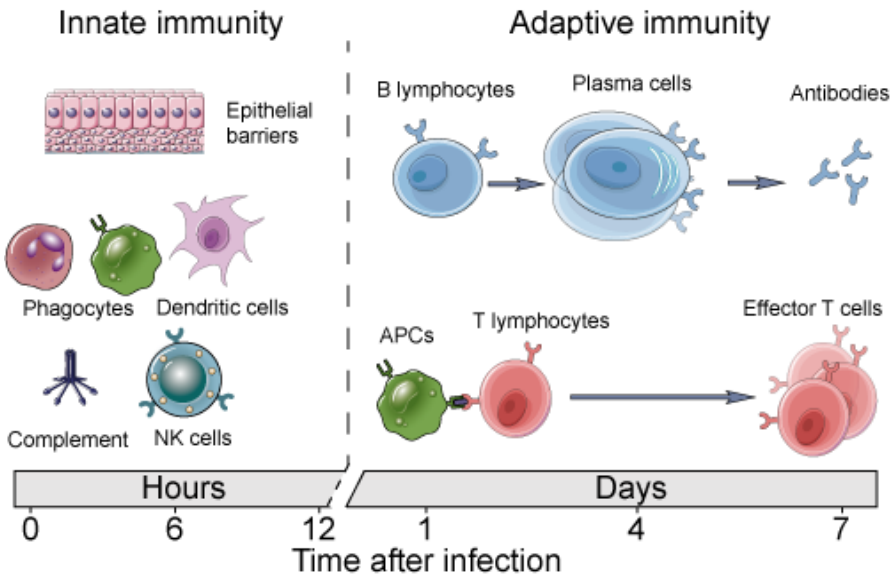



***Figure 1: Schematic overview about important immune system players and the time course of reactions during infection.***
*The primary source of protection is provided by the skin and the epithelial barrier and cells like phagocytes, which engulf the foreign substance or dendritic cells that present antigens to the T cells. After about 12 hours, the adaptive immune system becomes activated and produces antibodies and effector T cells to combat infection.*[4]

As depicted in Figure 1, a first protection comes from physical and chemical barriers like the epithelium and the antimicrobial chemicals produced on its surface which restrict entry into the host. Due to a constant circulation of lymphocytes and immune cells in the body the immune system is systemic, meaning that the response to a foreign molecule can be initiated at one site and protection carried out at distant sites. [1]

### 1.1.1. The innate immune system

Within the first hours after infection, the innate immune response becomes active. At the mucosal barrier, cells are located that constantly sample foreign molecules via surface bound and secreted pattern recognition receptors (PRRs). As soon as foreign molecules are detected, phagocytes and leukocytes are recruited to destroy microbes and initiate inflammation and tissue remodeling at the site of infection. Most of these cells are constantly present in tissue scanning for microbes [5,6]

Pattern recognition receptors are specific for microbe component structures and recognize either danger-associated molecular patterns (DAMPs) which are released upon host cell damage, during inflammation and immune response or pathogen-associated molecular patterns (PAMPs).

The innate immune system provides a rapid response by secreting early danger signals and stimulating the adaptive immune response with cytokines which in turn produces antibodies and effector T cells. [1,7]

### 1.1.2. The adaptive immune system

The adaptive immune system can recognize and react to large numbers of microbial and non-microbial substances. About 12 hours after exposure to an infectious agent, the adaptive immune system becomes stimulated. The intensity and ability of defense increases with each exposure to the same microbe. This process is known as the secondary immune response and works fast and specific due to memory cells specific for antigens that accumulate and react faster compared to naïve lymphocytes.

The reaction is mainly mediated by lymphocytes and their products which express receptors specifically recognizing various foreign substances. There are two main populations, the B lymphocytes producing antibodies and the T lymphocytes mediating cellular immunity.

The B lymphocytes recognize antigens by the B cell receptor, which is a membrane bound antibody, activating B cells. An antigen binding to the B-cell surface causes B cell division and maturation. The mature B cells secrete antibodies into the blood stream and lymphatic system.

Thereby, the infectivity of microbes can be neutralized and the ones to be eliminated by phagocytes can be targeted. This is the principle defense mechanism of the adaptive immune system. B lymphocytes develop in the bone marrow and after maturation are mostly found in secondary lymphoid tissues, lymphoid follicles and the bone marrow.

Antibodies are glycoproteins also known as Immunoglobulins (Ig) composed of two identical heavy and light chains. On each part of the chains there are constant and variable regions. The variable regions of the heavy and light chains on the N-terminus are antigen binding sites.

In mammals, there are five known Immunoglobulin isotypes: IgA, IgD, IgE, IgG and IgM. Immunoglobulins get activated by antigen recognition, supported by Th cell stimulation, leading to differentiation into antibody secreting plasma cells. Furthermore, irreversible class switching from one immunoglobulin isotype to another can be induced by various cytokines provided by Th cells. The class switch to IgE, which is promoted by interleukin 4 (IL-4), is important for allergic reactions. IgE recognizes specific allergens and interacts with its high-affinity receptor FcεRI which primes basophils and mast cells to release chemicals like histamine, leukotrienes and interleukins. Those in turn lead to allergy-related symptoms like asthma or eczema.[8]

## 1.2. Allergy

With more than 25% of individuals in industrialized countries affected and an increasing prevalence, allergy, also known as atopy, is a very common disorder. An undesired reaction of the immune system, also known as hypersensitivity, is induced which can be damaging or, in the worst case, fatal. Hypersensitivity reactions can be classified into four groups whereas allergies belong to the class I hypersensitivity.

Allergy is caused by an immune response to a non-microbial environmental antigen. The antigens that lead to hypersensitivity are called allergens that mostly are environmental proteins, animal products or chemicals which can modify self-proteins. The initial, primary response to an allergen is also known as sensitization.

Antigen presenting cells (APCs) take up the allergen, then prime allergen-specific T cells which help activating allergen-specific B cells. The Th2 cells and innate lymphoid cells produce the cytokines IL-4, -5 and -13 which in turn promote immunoglobulin class switching of B cells to IgE. [9]

The production of IgE antibodies is the major hallmark of an allergy. An allergic individual may develop IgE antibodies within the first years of birth due to early allergen contact at the mucosal surface. Atopic patients commonly develop strong IL-4 producing T cells and produce IgE when they are exposed to atopic substances. [10]

During the immediate phase reaction, IgE crosslinking ,caused by binding of antigens to IgE bound by FcεRI on mast cells, leads to degranulation, the release of inflammatory mediators like histamine or leukotrienes. Those in turn lead to an increase in vascular permeability, vasodilation and bronchial smooth muscle contraction. This causes disease symptoms like rhinitis, conjunctivitis and asthma. [11] During the vascular reaction, within minutes after the antigen challenge, immediate hypersensitivity, also known as Type I hypersensitivity, begins. During this phase of an allergic reaction, B cells become stimulated to produce IgE antibodies specific to the detected antigen. In the late phase reaction, the chronic manifestation of atopy takes place. This reaction is caused by activation of allergen-specific T cells after hours to days leading to a strong T cell infiltration as well as eosinophil, macrophage and neutrophil activation and accumulation. [9]

When the sensitization phase is over, an allergic patient produces IgE antibodies specific to the allergen and allergen-specific T cells are produced forming a pool of memory T cells responding to repeated allergen exposure.[12]

If many subsequent IgE and mast cell reactions take place, a chronic allergic disease can manifest which can include tissue damage and remodeling and is usually discernible by eczema, hay fever and allergic asthma.

An allergic response may be influenced by genetic background, allergen dose and contact. Environmental factors, like exposure to the allergens can influence the propensity to develop allergies which, in some people, can break the natural tolerant state and lead to allergic inflammation and allergen specific immune response maintained by host T and B lymphocytes.

### 1.2.1. Birch pollen allergy

Birch trees of the order *Fagales* and family *Betulaceae* are very prevalent in Europe. During flowering season, the trees release a lot of pollen, especially the major birch pollen allergen which is known as Bet v 1. In springtime, birch trees release large amounts of pollen which cause Type I allergies.

Birch pollen allergy is a dominant allergy in Europe and the major cause of symptoms like rhinoconjunctivitis and allergic asthma.

Once sensitized to birch pollen, one can easily develop pollen-related food allergies. This happens due to an IgE and T cell mediated cross-reaction between Bet v 1 and structurally related food proteins like the ones of apple (Mal d 1), cherry (Peru av 1) or hazelnut (Cor a 1)[13]. Those homologous proteins may activate Bet v 1 specific T cells to proliferate and produce cytokines. This cross reactivity can prolong the allergy symptoms for many patients and has also been known as “pollen food syndrome”.

### 1.2.2. Birch pollen allergens

The major birch pollen allergen Bet v 1 has a size of 17.4 kDa and belongs to the group of pathogenesis-related protein class 10 (PR-10) family. The major allergens in this group Mal d 1, Pru av 1 and Cor a 1 have a strong amino acid sequence identity. After ingestion of those plant-derived foods, patients often develop allergic symptoms which are caused by four cross-reactive structures present in birch pollen. Those allergens are also relevant for patients suffering from allergic rhinitis to pollen from birch and other trees. Since pollen from the family of PR-10 proteins share epitopes with Bet v 1 there is a high cross reactivity between proteins. [14]

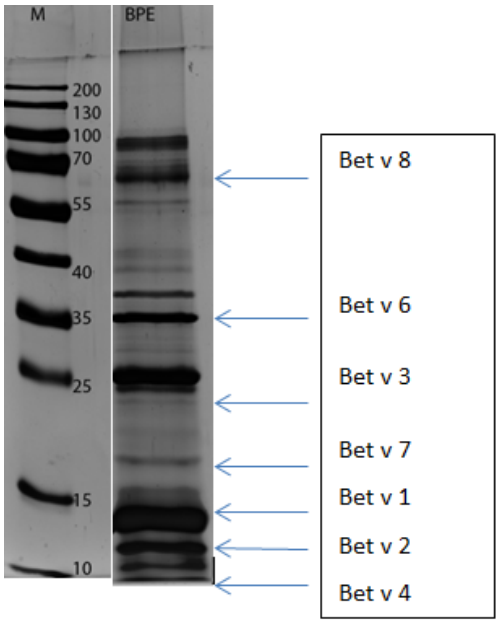



***Figure 2: 12% SDS PAGE of Birch pollen extract.*** *The diversity of proteins contained in birch pollen extract can clearly be seen on the gel.*

**Major allergen in birch pollen**

About 60% of birch pollen allergic patients react exclusively to Bet v 1. More than 90% of those display IgE antibodies to Bet v 1. The allergen has a molecular weight of around 17.4 kDa (Figure 2). [13]

There are many different isoforms of Bet v 1 some of which are known to have the ability to activate T cells and bind IgE. The most common isoform is Bet v 1.0101, which has been characterized in detail as well as expressed as recombinant protein. Already 13 isoforms have been identified which are classified into high, intermediate and low IgE binding. The isoforms vary in their molecular weight and isoelectric point. The abundance of the isoform present influences the allergic reaction a patient will have. There are some isoforms present which do not bind IgE in untreated patients but yet they show T-cell reactivity. Those could potentially be used in immunotherapy to prevent anaphylaxis. [15]

**Minor allergens in birch pollen**

Minor birch pollen allergens are recognized by less than 50% of patients with the respective allergy. At present, 6 minor allergens in birch pollen are known (Figure 2).

Around 15% of birch pollen-allergic individuals are sensitized to **Bet v 2**. The allergen is a 12-15 kDa sized protein that belongs to the profilin family, meaning that it is an actin binding protein. These proteins participate in regulation of division, growth and differentiation of cells. The pollen profilins may cross react with profilins in tomato, celery, carrot, peanuts, hazelnuts, bananas, apples and pineapples. The amino acid sequence of Bet v 2 contains 2 typical EF hand calcium binding domains, which is a helix-loop helix calcium binding domain.

The minor allergen **Bet v 3** has a molecular mass of 23.7 kDa. It is a calcium binding protein expressed in mature birch pollen that contains three calcium binding motifs. Binding of IgE requires Bet v 3 to be bound to $Ca^{2+}$. A sensitization is found in approximately 10% of allergic people.[16]

**Bet v 4** has a mass of 9.3 kDa and is an intracellular calcium binding protein with a two EF-hand structure, which are two helix loop helix calcium binding motifs. Bet v 4-specific IgE antibodies are specifically directed against these binding sites. 5-11% of birch pollen-allergic patients display Bet v 4-specific IgE. [17,18]

With a mass of 35 kDa, **Bet v 6** is a rather large minor allergen that has sequence homology (60-80%) with isoflavone reductase homologue proteins. Only 10% of patients are sensitized to Bet v 6. It is a highly cross-reactive allergen in plant foods.

**Bet v 7** is an allergen with a mass of 18 kDa. It has cis-trans isomerase activity meaning that it is a stress induced protein. Bet v 7 has a high homology with cyclophilins of some plants, the function of which is unknown but possible these proteins work as chaperones, folding catalysts or react with calcium binding proteins. [19,20]

**Bet v 8**, with a mass of 65.3 kDa, shows an end to end sequence homology to pectin esterases of plant species. These are ripening enzymes which cleave methoxylated pectins[21]

### 1.3. Immunotherapy

The current treatment for respiratory allergic disorders is allergen-specific immunotherapy (AIT). The treatment induces a shift in the immune response towards a Th1 type and allergen-specific regulatory T and B cells. These cells produce IL-10 which suppresses allergen-specific effector T cell subsets and thereby induces immune tolerance. AIT of patients also leads to a production of allergen-specific blocking IgG4 antibodies.

The disadvantage of AIT is that it is unable to treat food-related allergies. Therefore, the only possibility to evade the development of food allergies is to avoid ingesting the food a patient is sensitized to. [22]

#### 1.3.1. Oral tolerance induction

Oral tolerance is established when the humoral and or cellular immune response to an antigen is suppressed by administration of the same antigen via the oral route. This induces a state of antigen-specific immunologic hyporesonsiveness.[23]

Repa et al. proved in an animal model that oral tolerance induction by administration of recombinant Bet v 1 (rBet v 1) was possible. The study showed that Bet v 1-specific IgG1, IgG2a, IgA and IgE serum levels as well as cytokine production were reduced if the animals were given rBet v 1 intragastrically or intranasally prior to sensitization with Bet v 1. [24]

## 2. Materials and Methods

### 2.1. Birch sap and controls

#### 2.1.1. Birch sap

Birch juice, also known as birch sap, contains many different chemical elements, enzymes and amino acids. The tree sap is traditionally consumed in Baltic countries as well as Russia and China. Birch juice is said to decrease cavities and cholesterol, promote liver and kidney health by capturing toxic products and protect and replenish the skin. Nevertheless, the only scientifically proven health benefit is that xylitol, which can be manufactured from xylan-rich plants like birch, reduces *mutants streptococci* in plaque and saliva.

The adhesion of the bacterium to the teeth surface is avoided and their energy production process is disrupted. None of the other claimed health benefits have yet been proven in any scientific study. [25]

Birch sap is best consumed freshly or once opened stored in the refrigerator for maximum 48 hours. For long term storage, the fresh juice can also be frozen.

Three different types of birch juice were investigated in this study.

#### 2.1.1.1. *dm Bio Birkensaft (BS)*

The first birch juice examined was a conventional, store bought juice. It consisted of 99.8% birch juice and 0.02% some lemon juice concentrate was added to preserve the juice.
The measured pH was 5.32

The nutritional value per 100 mL according to the packaging were as follows:

| | |
|---|---|
| Energy | 14kCal |
| Fat | <0.5g |
| - saturated fat | <0.1g |
| Carbohydrates | 0.8g |
| - sugar | 0.8g |
| dietary fiber | <0.5g |
| Protein | <0.5g |
| Salt | < 0.01 g |

#### 2.1.1.2. *Vita7 Birkenwasser Natur (BSV)*

The second investigated birch sap was an online-bought juice which was claimed to be 100% birch juice. To conserve the juice, it was heated up to 120°C prior to bottling.

The measured pH of this juice was 6.52

The nutritional values per 100 mL according to package were listed as follows:

| | |
|---|---|
| Energy | 5kCal |
| Fat | 0.2g |
| - saturated fat | 0.08g |
| Carbohydrates | 0.8g |
| - sugar | 0.8g |
| Protein | 0 g |

#### 2.1.1.3. *Freshly harvested birch juice freshly (BSF)*

Since the birch juice was harvested from my colleague Ute, no information about the nutritional value can be provided.

The measured pH was 6.86

**Protocol**

To obtain fresh birch sap, 4 different trees with a diameter of minimal 20 cm were used. Into each tree, one hole with approximately 12-16 mm diameter was drilled using a drilling machine. The hole was around 1.5 to 2.5 cm deep. Into the hole, a hose with the same diameter as the drilled hole was placed. Underneath the hose, a bottle was placed and fixed with a rope. The sap was collected overnight and stored in the fridge for up to 48 hours.

The birch juice from four different trees was harvested, pooled, filtered and frozen at -20°C.

### 2.1.2. Allergens

Recombinant Bet v 1 and birch pollen extract (BPE) were used. Recombinant Bet v 1 is available since 1989, it represents the natural protein considering physiochemical and immunological factors. The recombinant protein shows the same binding capacities to anti-Bet v 1 antibodies and IgE as natural Bet v 1. Birch pollen extract contains all known allergens and was therefore used as positive control to compare molecular weights of proteins found in the examined birch sap. [26, 27,28]

## 2.2. Protein precipitation

### 2.2.1. Ammonium Sulfate Precipitation

Ammonium sulfate has a high solubility and can be used in solutions with very high ionic strength. Under low salt concentration, proteins containing positive and negative charged regions tend to self-aggregate. When a salt is present in solution, the cations and anions neutralize the charges on the protein's surface and thereby prevent aggregation. If the salt concentration is increased, the protein surface becomes charged again and competition of charged cations and anions of the salt with the proteins for polar water molecules takes place. Through this competition, water molecules get removed from the proteins which results in a decrease of their solubility and thereby in protein precipitation. This method is also known as salting out where proteins that easily aggregate are removed form very soluble ones.[29,30,31]

After this precipitation method is applied to the sample, it has a very high salt concentration that is best removed by dialysis.

**Protocol**

The precipitation method applied was adapted from the protocol of Exalpha Biologicals Inc. [32]

Prior to every protein precipitation, the required amount of Ammonium sulfate was calculated using the table depicted in Figure 3. To precipitate as many proteins as possible, precipitation was carried out in 80% $(NH_4)_2SO_4$, and, depending on the volume of birch sap used, the specific amount of ammonium sulfate was added. The sample was put into a beaker and left stirring at 4°C while ammonium sulfate was added gradually. The mixture was left shaking in the cold room to precipitate proteins overnight. The next day, the mixture was centrifuged for one hour at 4967 rpm at 4°C, the supernatant discarded, and the pellet resuspended in the lowest volume possible of 1x PBS.

| | Final percent saturation to be obtained | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 |
| Starting percent saturation | Amount of ammonium sulphate to add (grams) per liter of solution at +20 °C | | | | | | | | | | | | | | | | |
| 0 | 113 | 144 | 176 | 208 | 242 | 277 | 314 | 351 | 390 | 430 | 472 | 516 | 561 | 608 | 657 | 708 | 761 |
| 5 | 85 | 115 | 146 | 179 | 212 | 246 | 282 | 319 | 358 | 397 | 439 | 481 | 526 | 572 | 621 | 671 | 723 |
| 10 | 57 | 86 | 117 | 149 | 182 | 216 | 251 | 287 | 325 | 364 | 405 | 447 | 491 | 537 | 584 | 634 | 685 |
| 15 | 28 | 58 | 88 | 119 | 151 | 185 | 219 | 255 | 293 | 331 | 371 | 413 | 456 | 501 | 548 | 596 | 647 |
| 20 | 0 | 29 | 59 | 89 | 121 | 154 | 188 | 223 | 260 | 298 | 337 | 378 | 421 | 465 | 511 | 559 | 609 |
| 25 | | 0 | 29 | 60 | 91 | 123 | 157 | 191 | 228 | 265 | 304 | 344 | 386 | 429 | 475 | 522 | 571 |
| 30 | | | 0 | 30 | 61 | 92 | 125 | 160 | 195 | 232 | 270 | 309 | 351 | 393 | 438 | 485 | 533 |
| 35 | | | | 0 | 30 | 62 | 94 | 128 | 163 | 199 | 236 | 275 | 316 | 358 | 402 | 447 | 495 |
| 40 | | | | | 0 | 31 | 63 | 96 | 130 | 166 | 202 | 241 | 281 | 322 | 365 | 410 | 457 |
| 45 | | | | | | 0 | 31 | 64 | 98 | 132 | 169 | 206 | 245 | 286 | 329 | 373 | 419 |
| 50 | | | | | | | 0 | 32 | 65 | 99 | 135 | 172 | 210 | 250 | 292 | 335 | 381 |
| 55 | | | | | | | | 0 | 33 | 66 | 101 | 138 | 175 | 215 | 256 | 298 | 343 |
| 60 | | | | | | | | | 0 | 33 | 67 | 103 | 140 | 179 | 219 | 261 | 305 |
| 65 | | | | | | | | | | 0 | 34 | 69 | 105 | 143 | 183 | 224 | 267 |
| 70 | | | | | | | | | | | 0 | 34 | 70 | 107 | 146 | 186 | 228 |
| 75 | | | | | | | | | | | | 0 | 35 | 72 | 110 | 149 | 190 |
| 80 | | | | | | | | | | | | | 0 | 36 | 73 | 112 | 152 |
| 85 | | | | | | | | | | | | | | 0 | 37 | 75 | 114 |
| 90 | | | | | | | | | | | | | | | 0 | 37 | 76 |
| 95 | | | | | | | | | | | | | | | | 0 | 38 |

***Figure 3:*** *Overview of the amount of ammonium sulfate to be added to one liter of sample depending on the desired end concentration of ammonium sulfate in the sample.* [33]

### 2.2.2. Ethanol precipitation

This method is used to concentrate proteins but also to remove alcohol soluble impurities like salt and detergents from a protein sample. When placed in ethanol, proteins, in contrast to small molecules, are insoluble. In aqueous solutions, proteins tend to form hydrogen bonds that make them soluble. If ethanol is added to this solution, the environment becomes less polar, thereby reducing the solubility. This in turn leads to self-aggregation of proteins. The concentrated protein pellet can be precipitated by centrifugation. Ethanol precipitation is a very sensitive method with a minimal protein loss. [34,35]

**Protocol**

The working method was adapted from the protocol of Jiang et al.[36]

The 3.5-fold volume of absolute ethanol was added to the birch juice. The mixture was incubated at RT o/n shaking on the roller mixer. The next day, the mixture was centrifuged for one hour at 4967 rpm at 4°C, the supernatant was discarded, and the pellet was resuspended in the lowest volume possible of 1x PBS.

### 2.2.3. Trichloroacetic acid precipitation (TCA)

Trichloroacetic acid is an analog of acetic acid where the 3H atoms of methyl carbon are replaced with chlorine atoms. It is a weak acid and therefore cannot hydrolyze protein peptide bonds but can still maintain an acidic pH.

This method is used to concentrate protein samples and to remove unwanted contaminants like salts and detergents. If TCA is added to proteins in aqueous solution, it disrupts hydrogen bonded water molecules surrounding protein. Thereby, proteins are no longer soluble and lose their secondary structure. The insoluble proteins can be recovered by centrifugation. Because it is a denaturing assay, its effect on the biochemical activity of proteins must be determined.

The disadvantages of this methods are that it denatures the protein, a high starting concentration of protein (>5 µg/mL) is required and that it does not work for all proteins.[37,38,39]

**Protocol**

To carry out the precipitation reaction, 1 volume of 100% TCA was added to nine volumes of protein sample, and proteins were precipitated on ice for 30 minutes. Meanwhile, the micro centrifuge was pre-cooled to 4°C. All samples were centrifuged at 10 000 g 4°C for 10 minutes. The supernatant was aspirated carefully to not disrupt the pellet. The pellet was washed with ice cold acetone twice to remove any residual TCA. The samples were again centrifuged at 10 000 g 4°C for five minutes, the supernatant aspirated and the pellet let dry with an open lid. Each pellet was resuspended in 20µL 1x PBS.

## 2.3. Lyophilization (Freeze drying)

Lyophilization is a method to remove water from solid or liquid samples by sublimation. Prior to Lyophilization, the sample must be frozen. Then, the sample is put to a chamber with vacuum and low pressure to initiate sublimation of water molecules. Thereby, water molecules go from solid to gaseous phase instantly and the vapor produced turns into ice. What is left over in the end is a dry powder, the lyophilizate, that contains all non-volatile substances like proteins. The dry powder is resuspended in water or buffer. This method preserves the sample and keeps its initial structure. Due to the rapid transition from hydrated structure to almost completely dehydrated, porous matrix, the deterioration reaction is minimized and thereby protein denaturation is avoided. [40,41 42]

**Protocol**

The samples were frozen in liquid nitrogen for 30 minutes, taken out and holes were punched into the lids of the sample tubes to allow the sublimation reaction to take place later. While the samples froze, the device Lyo-Christ was warmed up. Therefore, the surface was fixed, all three valves on the sides were closed, the lid was put on and the device switched on. The program manual operation was chosen, selection Freeze- warm up- RUN mode and the device was warmed up for 35 minutes. Once the samples were ready, they were put onto the lyophilization stand and the lid was closed. The program manual- main drying was selected for a lyophilization over night with a pressure of 0.05 mbar and a temperature of -89°C. 10 minutes after the program was started, vacuum was controlled by checking the pressure. The samples were run over night or until a dry, fluffy pellet was observed. Then, the machine was put on standby, the valves were opened slowly to let the pressure increase until around 800 bar and then to atmospheric pressure. The cover was lifted, water valve opened, and the obtained peptide powder resuspended in the desired amount of 1x PBS.

## 2.4. Protein concentration with Vacuum: Speed Vac

The device used for this method is a centrifuge that can also produce a vacuum. The produced vacuum shifts the vapor-liquid equilibrium towards gas phase, the sample remains in the solid phase and thereby stays in the tube while solvent like water molecules on the surface can evaporate. This process does not harm the sample and results in a concentration of the sample. The sample is maintained in a liquid state at sub-ambient temperatures throughout the concentration process. The centrifugation force prevents bumping, boiling or physical loss of sample. Thereby, no loss of activity or damage to heat sensitive substances is obtained. Since the controlled vacuum lowers the boiling point of the solvent, the liquid vaporizes with minimum heat and no oxidation takes place during the drying process. The method is used to concentrate small volumes of samples whereas aqueous solutions are concentrated at 1 mL/100 min. [43,44,45]

**Protocol**

Before starting the run, the Univapo 150 ECH pump from UniEquip was warmed up for 10 minutes. The samples were put into 2 mL Eppendorf tubes with their lid open and assembled on the grid of the centrifuge. The program normal run was started at RT. After the run was started, the left valve was closed to stop letting air get in and the right valve was opened slowly to establish the vacuum. Every few hours, the run was stopped, and the obtained volume was checked using an Eppendorf tube filled with water to represent the desired volume. Once the desired volume was observed, the run was stopped, the right valve was opened the left one opened slowly, and the pumps were switched off.

## 2.5. Dialysis

Dialysis is used to remove small molecules like sugar and ions out of a sample by selective and passive diffusion. A semipermeable membrane with a specific pore diameter is used whereas molecules smaller than this diameter are excluded from the sample and larger ones are retained. The sample to be dialyzed is placed in a sealed membrane and immersed in buffer, which is the dialysate, and should be 200-500 times the volume of the sample. Small molecules then start equilibrating between sample and dialysate, and for every small molecule diffusing out of the membrane, solvent comes in. By repeatedly changing the dialysis buffer, more contaminants can diffuse into the dialysate and the small molecule concentration in the sample gets further reduced. [46, 47]

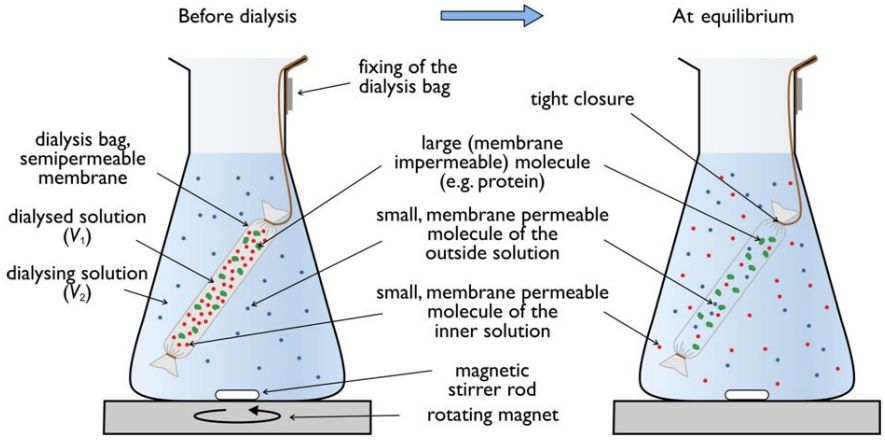



***Figure 4: Schematic overview about dialysis.*** *Large, impermeable molecules are retained inside the dialysis bag whereas small, membrane permeable, molecules may pass the membrane. For each molecule leaving the dialysis tube, one molecule of the outside solution enters the dialysis tube.* [48]

**Protocol**

**Dialysis tube preparation**

Make sure to wear gloves to avoid getting proteases on and in your tubes when preparing dialysis tubes. All solutions used for the preparation should be pre-heated to 60°C prior to usage. First, a bicarbonate/EDTA buffer had to be prepared. Into a 4 L Erlenmeyer flask filled with 2 L bicarbonate/EDTA buffer, a pack of Dialysis tubes was rolled. The flask was kept in a water bath at 60°C shaking slowly for two hours, then the buffer was changed, the tubes were shaken for two more hours. The buffer was exchanged another time and the dialysis tubes left in the water bath shaking o/n. On the next day, tubes were washed with 2 L Aqua bidest. for one hour at 60°C, this step was repeated until the water was clear. The dialysis tubes were slowly cooled down to 4°C and then stored at 4°C in Aqua bidest. with 1 mL $CHCl_3$/L.

**Dialysis**

A dialysis tube with a cutoff value of 3.5 kDa was used for all experiments. Molecules smaller than the pore size could diffuse through the membrane but larger ones were retained in the sample side of the membrane. Dialysis was performed in two ways, in 5L beakers for large volumes of samples and in 500mL beakers for small volumes.

***Dialysis with large sample volumes***

The dialysis was performed in 5L beakers containing 1x PBS. From the previously prepared dialysis tubes, a “U” shaped tube which fits into a 5L beaker without touching the bottom was cut out. The dialysis tube was cleaned with VE water inside and outside. Onto one end of the tube, two knots were made, and the end was fixed with a clip. Around 200 mL of sample were filled into the tube and one third of the tube was left empty, the air pushed out of the tube and the tube sealed by making two knots and adding a clip. To both ends of the dialysis tube, pieces of foam were added to prevent the dialysis tube from drowning. The beaker was placed on a magnetic stirrer at 4°C and the 1x PBS was exchanged every few hours. The more frequently the buffer was exchanged, the faster the dialysis proceeded.

Once the reaction was done, the buffer was discarded, the outside of the dialysis tube cleaned with VE water, the dialysis tube was cut open and the dialyzed sample collected.

***Dialysis with small sample volume***

The dialysis was performed in 500 mL beakers containing 1x PBS. The sample was placed in a 1.5 mL Eppendorf tube whose lid had previously been removed. A small piece of dialysis tube was cut open and put over the Eppendorf tube and sealed using a rubber ring. The sample was placed upside down into the beaker. To prevent drowning of the sample, it was fixed with a piece of foam to float on the surface of the liquid. The beaker was placed on a magnetic stirrer at 4°C and the 1x PBS was exchanged every few hours. The more frequently the buffer was exchanged, the faster the dialysis proceeded. Once the reaction was finished, the buffer was discarded, the sample taken out of the Eppendorf tube and further used.

## 2.6. Bicinchoninic acid assay (BCA)

This assay is used for colorimetric detection and quantification of proteins. When proteins are put in an alkaline medium, a reduction of $Cu^{+2}$ to $Cu^{+1}$, also known as biuret reaction, takes place. When two molecules of BCA chelate with one molecule of cuprous ion a purple-colored reaction product is made.

A reagent containing bicinchoninic acid is used together with the sensitive and selective colorimetric detection of the cuprous cation ($Cu^{+1}$). The purple reaction product has a strong absorbance at a wavelength of 562 nm. Using a standard of a common protein, in this case bovine serum albumin, protein concentrations are reported and determined. To obtain the standards, a serial dilution using known concentration of BSA is performed and assayed together with the samples whose concentration is unknown. The concentration to be determined is observed from the standard curve produced by the BSA standards.

Compared to the Bradford assay, BCA is susceptible to interference by chemicals present in protein samples. Nonetheless it is compatible with most protein samples and samples containing up to 5% detergent. The method is generally sensitive and responds more uniformly to different proteins. It is quite stable under alkaline conditions and has a broad dynamic range of protein concentrations from 0.5 µg/mL to 1.5 mg/mL.

The disadvantage of this method is that substances reducing copper will also produce purple color and can therefore interfere with the accuracy of protein quantification. [49,50]

**Protocol**

It was proceeded as written in the Thermo Scientific™ protocol (Microplate procedure, all samples prepared in duplicates). [51]

First, a serial dilution of bovine serum albumin standards was performed to later obtain a standard curve (Table 1).

***Table 1**: **Dilution Scheme for the production of BSA standards.** To later calculate the obtained concentrations of the unknown samples, a standard curve was made. This was done by measuring BSA samples of known concentration and correlating concentration and absorbance and thereof deduct the unknown sample concentrations.*

| Vial | Volume of Diluent (µL) | Volume and Source of BSA (µL) | Final BSA conc. (µg/mL) |
|---|---|---|---|
| A | 0 | 300 of Stock | 2000 |
| B | 125 | 375 of Stock | 1500 |
| C | 325 | 325 of Stock | 1000 |
| D | 175 | 175 of vial B dilution | 750 |
| E | 325 | 325 of vial C dilution | 500 |
| F | 325 | 325 of vial E dilution | 250 |
| G | 325 | 325 of vial F dilution | 125 |
| H | 400 | 100 of vial G dilution | 25 |
| I | 400 | 0 | 0 (=blank) |

Next, the working reagent was prepared using the formula provided by the kit: (#standard + #unknown) x (#replicates) x (volume of WR/sample) = total volume of WR required

**Microplate Procedure (sample to WR Ratio= 1:8)**

From each of the prepared standards and samples, 25 µL were pipetted into the microplate well. 200 µL of the freshly prepared working reagent were added to each well and the plate mixed on the plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes, cooled down to room temperature and the absorbance was measured near 562 nm on the plate reader.
The average absorbance of blanks was measured and subtracted from the measurements of all other standards and unknown samples.

A standard curve was prepared by plotting the average measurements for each BSA standard against its concentration in µg/mL. This curve was used to determine protein concentrations of each unknown sample.

## 2.7. Protein separation by SDS PAGE

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) is used for high resolution separation of protein mixtures according to their molecular weight. SDS is a detergent which denatures secondary and non-disulfide linked tertiary structures and coats proteins with negative charge correlating to their length. Thereby, all proteins become negatively charged and linear. In an electric field, they are separated according to their molecular weight. A Laemmli SDS PAGE consists of a discontinuous gel, an upper stacking and a lower resolving gel. These gels have different pH values and polyacrylamide concentrations. The upper stacking gel has a lower percentage of acrylamide where proteins can move through quickly and stack into tight bands. Then, they enter the separating gel. The polyacrylamide retains larger proteins stronger than smaller ones, thereby the larger proteins move slower towards the positively charged pole than smaller molecules which results in different distances travelled. The proteins can then be visualized by protein staining or western blot, therefore always a molecular weight marker must be loaded to estimate the size of the protein in the sample. The marker contains a mix of proteins with known molecular weights that become separated accordingly.[52,53]

**Protocol**

To separate proteins according to size, samples were denatured by addition of 4x sample buffer at 95°C for 10 minutes and then spun down quickly. Mercaptothenaol is a reducing agent that disrupts disulfide bonds within proteins. The reduction of the disulfide bonds is important to allow the protein to become completely unfolded and thereby migrate properly for its molecular weight.

The samples were separated according to their molecular weight using gels with various acrylamide concentrations.

***Table 2: Ingredients for SDS-PAGE.*** *Depending on the separation needed or which molecular weight range is investigated, different concentrations of acrylamide had to be used.*

| Separating Gel | 10% | 12% | 15% | 18% |
|---|---|---|---|---|
| Acrylamide | 4.4 mL | 5.3 mL | 6.6 mL | 7.92 mL |
| Lower Buffer | 3.3 mL | 3.3 mL | 3.3 mL | 3.3 mL |
| Aqua dest. | 5.5 mL | 4.6 mL | 3.3 mL | 1.98 mL |
| TEMED | 6.6 µL | 6.6 µL | 6.6 µL | 6.6 µL |
| APS (10%) | 40 µL | 40 µL | 40 µL | 40 µL |

| Stacking Gel |
|---|
| 0.7 mL |
| 1.1 mL (Upper Buffer) |
| 2.6 mL |
| 5 µL |
| 30 µL |

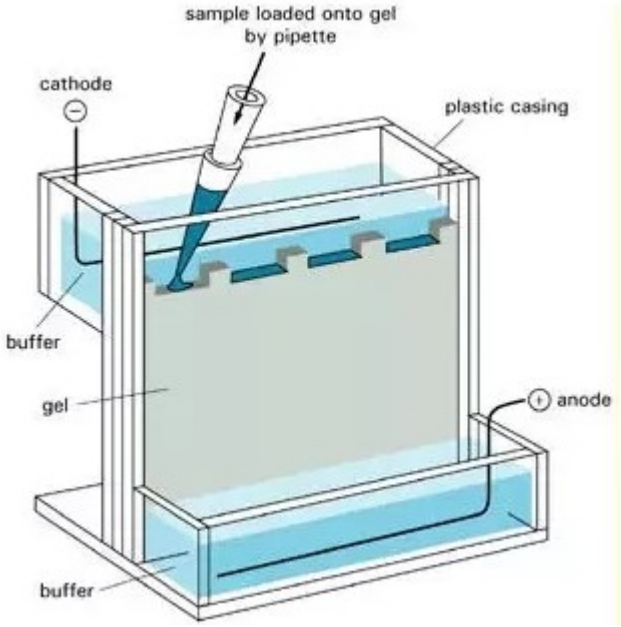



***Figure 5: Schematic depiction of an SDS PAGE set up.*** *The glass plates imbedding the gel are placed into the chamber, which was then filled with 1x SDS PAGE running buffer. Then samples were loaded into the wells, the chamber was connected to the current and proteins were first stacked in the stacking gel and then separated in the running gel. Larger proteins are stronger retained than smaller ones and therefore take longer to travel.* [52]

Using clips to hold them together and a rubber band in between, two glass plates were sealed. To prepare the gel for one run, the ingredients for the separating gel were combined in a beaker. Then, the gel was poured between two glass plates until about 5 cm before the end to leave space for the stacking gel.

150 µL of isobutanol were added to the separating gel to avoid bubbles on top of the gel and to ensure an even surface. The gel was left to polymerize for around 45 minutes, then the isobutanol was washed away with Aqua dest. All surfaces were completely dried and then the stacking gel was mixed and poured on top. The comb with the desired number of wells was inserted without letting any bubbles inside the gel. The gel was left for further polymerization for around 45 minutes. Once the gel hardened, the comb and the rubber band were removed, and the gel sealed within the glass plates was assembled in the chamber. The chamber was filled with 1x running buffer. For each run, 3 µL of the protein ladder Fermentas PAGE Ruler™ and 20 µL of sample (15 µL sample + 5 µL 4x sample buffer) were loaded. Prior to loading, the samples were mixed with sample buffer and boiled at 95°C for 10 minutes. After the heating, samples were spun down quickly, and each sample was pipetted into one well. The gels were run at 250 V and 0.02 A when running one gel or 0.04 A when running two gels simultaneously until the loading dye front reached the bottom of the gel and started running out.

### 2.7.1. Protein staining: Silver staining

This method is the most sensitive method to stain proteins in gels since it can detect protein concentrations < 1 ng/band. Separated proteins are first denatured and fixed in the gel with TCA using a silver nitrate solution. The silver ions are then added which leads to a selective reduction of silver ions to metallic silver evoked by formaldehyde. The reduction only takes place at initiation sites close to the protein molecules. Specific parts of the gel will be stained dark and thereby visualized. The reaction is stopped by addition of acetic acid. This method is simple, requires cheap equipment and chemicals and has a superior sensitivity.[54,55]

**Protocol**

After proteins were separated by SDS PAGE, the gel was stained using Silver stain. For each gel used, always 25 mL of every solution were taken (see Attachment for Solution composition). The gel was directly taken from the chamber, put into a dish, Solution 1 was added and left shaking for 5 minutes. Solution 1 was discarded; the gel was washed three times with Aqua dest. and subsequently incubated for 5 minutes with Aqua dest. Afterwards, the gel was washed three times with Aqua dest., then Solution 2 was added, and the dish left shaking for 5 minutes.

Solution 2 was discarded, and without a washing step in between, Solution 3 was added for a minute. Then, the gel was washed three times with Aqua dest. The freshly prepared Solution 4 was added, and the gel was left shaking for 8 minutes and then washed five times with Aqua dest. Next, Solution 5 was added for 30 seconds. It takes some time until the bands are clearly visible, but it is important not to wait too long which would result in a gel stained too dark. After 30 seconds, a solution containing 2% acetic acid was added which stops the reaction, but some slight color development will still proceed. After 30 more seconds, the solution mix was discarded, and the gel stored in Aqua dest. Pictures of the gel were taken using ChemiDoc™ Imaging System from BioRad.

### 2.7.2. Protein staining: Coomassie Brilliant Blue

Coomassie Brilliant Blue binds to proteins which leads to a shift in the absorption maximum of the dye. The protonated form of Coomassie blue is pale orange-red and the unprotonated form is blue. If Coomassie binds to a protein in acidic solution, the positive charges of Coomassie suppress protonation and a blue color develops. This binding causes a shift in the absorption maximum from 465 to 595nm and the increase in absorption is measured. The method is very rapid and reproducible and there is hardly any interference from cations or carbohydrates. [56]

The two Coomassie dyes available differ only by two methyl groups. Coomassie R-250 can already detect 0.1µg of protein per spot or band and is therefore the more commonly used dye. The sensitivity of this staining technique is 10- to 100-fold lower when compared to silver staining.

Coomassie G-250 in contrast, is faster and more convenient though less sensitive. It detects 0.5µg and due to the short protocol saves up to 11 hours to obtain results. [57]

**Protocol**

To send proteins for mass spectrometry, they have to be separated on a gel and stained with Coomassie Blue R-250.

All parts of the SDS PAGE apparatus were washed with ethanol and Aqua bidest. For preparing, loading and staining the gel gloves had to be worn.

Once the gel finished running, it was fixed in Fixing solution for 1 hour. Then, the gel was stained in freshly filtered Staining solution for 1 hour.

The gel was destained for 24 hours in Destaining solution. Then, protein bands were cut out, stored in 1% HAc and sent for mass spectrometry.

### 2.7.3. Glycoprotein gel staining

To stain glycoproteins in a gel, the Pro-Q-Emerald 300 Glycoprotein Gel and Blot Stain Kit was used. This method stains glycoproteins in gels or blots. The stain reacts with periodate-oxidized carbohydrate groups which creates a bright green fluorescent signal on glycoproteins. The method is very sensitive and can detect as little as 0.5 ng of glycoprotein per band. The green-fluorescent signal can be visualized using 300 nm UV illumination. A positive and negative control is provided by the CandyCane™ molecular weight standard which contains a mixture of glycosylated and non-glycosylated proteins.

**Protocol**

This protocol from Molecular Probes for the Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain Kit was used.

The experiment was implemented on a 12% SDS PAGE gel. Additional to the Page Ruler Marker, this time also the CandyCane™ molecular weight standard was loaded to compare glycosylated and non-glycosylated proteins. 0.5 µL of standards were mixed with 7.5µL of sample buffer and loaded onto the gel. While the gel was run and proteins were separated, all necessary stock solutions were prepared (see Appendix: Media and Buffers). After the gel was run, it was placed in a plastic dish and fixed in 100 mL Fix Solution with gentle agitation for 45 minutes. This step was repeated to ensure full washing out of SDS. The gel was then washed in 100 mL Wash Solution with gentle agitation for 15 minutes and this step was repeated once more. After the washing step, carbohydrates were oxidized by adding 25 mL of Oxidizing Solution to the gel and incubating it with gentle agitation for 30 minutes. The gel was then washed three more times as previously described. During the last washing step, fresh Staining Solution was prepared by diluting stock solution 50-fold into staining buffer. The gel was then stained in the dark in 25 mL of staining solution with gentle agitation for 100 minutes. After the staining, two more washing steps were executed and then the gel was examined with the transilluminator.

## 2.8. Blotting

This method is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Those antibodies identify target proteins amongst unrelated proteins. After separation by SDS PAGE, the proteins must be electrophoretically transferred to a nitrocellulose sheet. The transferred proteins bound to the surface of the membrane allow access for an immunodetection reagent. All non-specific sites on the sheet are blocked by putting the membrane in a solution containing protein or detergent blocking agent. First, the membrane is probed with primary antibody specific for the target, then washed and the antibody antigen complex is identified with a secondary antibody coupled to e.g. horseradish peroxidase if luminescent substrates are used to visualize enzymatic activity. [58,59,60]

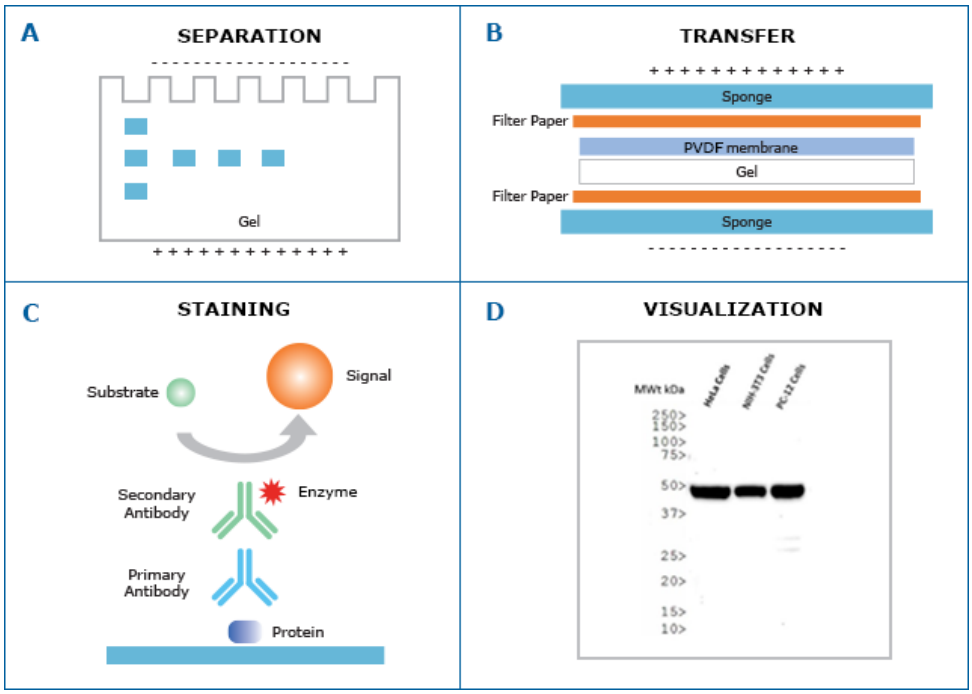



***Figure 6: Illustration of all the steps necessary to detect a specific protein in a mixture using the Western Blot technique.*** *The proteins are first electrophoretically separated by SDS PAGE and then transferred to a nitrocellulose or PVDF membrane via western blotting. The current passing through the setup depicted in B leads to proteins migrating from the gel onto the NC sheet. Once the proteins are transferred, non-binding surfaces are blocked and the nitrocellulose sheet is then incubated with antibodies, whereas the secondary antibody usually is coupled to an antibody which leads to the emission of a detectable signal.* [61]

**<u>Protocol</u>**

First, a nitrocellulose sheet with a pore diameter of 0.2 µm and two sheets of Whatman paper with the size of 6.5x8.5 cm were cut out.

Then, a blotting tray was filled with tap water, and the blot holder placed inside. On the lower and upper part of the blot holder, sponges were first soaked in water and then put on top of each blot holder. Furthermore, Whatman paper sheets were soaked and put on top of the sponges. The sheet of nitrocellulose was now put into the water, wettened and the gel was placed on top of it. In this step it is crucial to ensure proper placement of the gel on the nitrocellulose membrane as well as ensuring that there are no bubbles between the gel and the membrane. The gel and the nitrocellulose membrane were put between the Whatman paper sheets, the apparatus was closed, carefully removed from the water and a small tube was rolled over it several times to remove air bubbles. The blotting apparatus was put into a transfer chamber filled with transfer buffer in a way that the nitrocellulose was facing the positive side of the blotting apparatus because proteins are negatively charged and will therefore travel to the anode. The proteins were blotted for 1 hour at 0.25 A.

### 2.8.1. Immunoblot to detect human IgE antibodies

**Protocol**

A line 0.5cm beneath the top of the nitrocellulose paper was drawn using a pen. Then the paper was divided into strips (0.5 cm each) by marking and cutting them and the molecular weight marker was kept aside. In each furrow of the blotting chamber, 1 mL gold buffer (GB) was filled, one strip was placed and incubated with agitation for one hour at RT. Then, the gold buffer was discarded, 1 mL human serum 1:4 diluted in GB was filled into the chambers and the reaction was left shaking overnight at 4°C. On the next day, the stripes were washed three times with GB, then left in GB for one hour at room temperature and then washed again three times. Under the fume hood in the radioactive room, 1 mL anti-hu-IgE$^{125}$I diluted 1:10 in GB (Kit #DEILRE01 specific IgE RIA (RAST) von Demeditec) was added and left-over night at room temperature. To prevent the strips from drying out, blotting chambers were covered with cling film. On the next day, the strips were washed three times with GB, then incubated for an hour at room temperature and then washed again three times with GB. The strips were then taken out using forceps, let dry for 20 minutes, then put on a piece of paper and covered with cling film.

The paper was placed into an X-ray cassette in the dark room, a piece of X-ray film was placed exactly on top, the cassette was closed, and the strips were exposed at -70°C for at least three days. After the exposure, the X-ray film was developed using the device AGFA CP1000 film processor.

### 2.8.2. Immunoblot to detect mouse IgG antibodies

For this assay, the Bet v 1-specific, mouse monoclonal antibody, Bip-1 was used.

**Protocol**

A blotting chamber was filled with 6 mL of TBS/ 0.1% Tween/ 5% dry milk and the nitrocellulose sheet was placed inside using forceps. The blotting chamber was incubated with agitation and the nitrocellulose sheet saturated for one hour at room temperature or overnight at 4°C. The nitrocellulose was washed three times with TBS/ 0.1% Tween, incubated in the mixture for 30 minutes at room temperature and then washed three times using the same solution. Mouse antibody hybridoma supernatant was added (BIP-1) diluted 1:100 in TBS/ 0.1% Tween/ 1% dry milk and incubated for two hours at room temperature. The sheet was washed with TBS/ 0.1% Tween three times, incubated in TBS/ 0.1% Tween for 30 minutes at RT and washed again three times. The liquid on the sheet was sucked off using a paper towel. To detect chemiluminescence, 2mL of ECL Prime Western Blotting Detection Reagent were added to the nitrocellulose and incubated in the dark for five minutes at RT. The remaining liquid was sucked off the nitrocellulose using a paper towel and the nitrocellulose was put into clear film and pictures taken using the ChemiDoc Imager with the function Chemiluminescence.

### 2.8.3. Dot-Blot

The dot-blot is very similar to a western blot because both methods detect a protein immobilized to a membrane. The difference between the assays is that with dot-blot, the proteins are not separated electrophoretically on a gel but simply spotted on nitrocellulose. Since there is no protein separation taking place, neither a molecular weight determination of the proteins within the sample nor a differentiation between different protein forms is possible. Nevertheless, it is a quick assay to estimate the protein concentration or abundance in a sample or determine whether the proteins in the sample can be detected by antibodies or not. [62,63]

**Protocol**

The chamber was built by combining the reservoir with a 96-well support and a 96-well latex layer. A piece of Whatman paper and a sheet of nitrocellulose membrane were pre-wet in western blot transfer buffer. The Whatman paper was put on the Dot Blot chamber first and then layered with the sheet of nitrocellulose. The chamber was closed with the cover and 100µL of transfer buffer were added into each well. Here, it was important to avoid bubbles as they would prevent the fluid passing through the membrane. The chamber was connected to a vacuum and the buffer aspirated. Once all the liquid was sucked through, the chamber was disconnected from the vacuum and samples were pipetted into the wells. The sample always had to be at least 200µL to not have a sample transfer problem. The chamber was again connected to the vacuum and all liquid aspirated. Once this was done, the chamber was disassembled, and the nitrocellulose was transferred into a plate, covered with Ponceau S for a few seconds and then washed with $H_2O$. This step was necessary to see if there were any proteins transferred. Then, immunoblots were carried out on the nitrocellulose sheets.

## 2.9. Mass Spectrometry

Mass spectrometry is a very sensitive instrumental analytical technique used to determine the molecular mass or the structure of proteins. Because biological samples usually contain a lot of different proteins, proteins are first separated from a protein mixture and then analyzed. The analysis is done by a measurement of the mass-to-charge ratio of ions. Therefore, the sample is ionized by bombarding it with electrons which leads to either charged fragments or a charged sample that did not become fragmented. The ions are then accelerated and subjected to an electric or magnetic field. Ions of the same mass-to-charge ratio undergo the same amount of deflection. The ions are then detected by an apparatus like an electron multiplier which can detect charged particles. As a result, a mass spectrum is displayed which is a plot of the ion signal intensity as a function of the mass-to-charge ratio. To identify samples, proteins with known masses are correlated to the detected masses or fragmentation patterns are compared against standard databases.

**Protocol**

Acrylamide gels of different percentage were prepared, and samples were loaded to separate proteins of the mixture. The gels were fixed in Fixing Solution for 1h and then stained in filtered Coomassie R-250 solution for one hour. After that, the gels were destained in Destaining Solution for 24 hours. The next day, duplicates of the protein bands of interest were cut out using a scalpel. The cut-out bands were stored in a clean Eppendorf tube in 1% HAc, sealed and sent for analysis.

### 2.9.1. Proteomics

A proteome is the complete set of proteins expressed by an organism. [64]

In contrast to the genome, which is determined by heredity only, the proteome is additionally influenced by the environment. Proteomics analyses the protein complement of the cell and can thereby identify proteins, modification of proteins or interaction between proteins. This is done by mass spectrometry, liquid chromatography or antibodies that enable protein identification. The usual approach is to separate protein mixtures, and then do a mass spectrometry to gather information about the individual proteins. The data are then assembled and analyzed by use of bioinformatics.[14,65,66]

## 2.10. Basophil activation test (BAT)

Basophils are leukocytes which are circulating white blood cells expressing the high-affinity IgE receptor. They secrete cytokines and inflammatory mediators upon stimulation. Human basophils show secretory granules competent to secrete cytokines, chemokines and lipid mediators. Upon cross-linking of the high-affinity IgE receptor by allergens, basophils become activated. The basophil activation test is a flow-cytometry based assay in which the number of activated cells upon stimulation is determined. The 53kDa glycoprotein CD63 is located on the membrane of intracellular secretory granules of basophils. By Knol et al.[67] it was demonstrated that the surface marker CD63 is upregulated on basophils concomitantly to basophil degranulation due to a fusion of the granules with the plasma membrane. To identify basophils, one can look at surface markers like the chemokine receptor CCR3 or CD123, which is a subunit of the IL-3 receptor that is expressed on basophils. [68]

**Protocol**

Before the experiment was started, all solutions were prepared (see Appendix: Media and Buffers) and blood was taken from a birch pollen allergic donor. All samples were diluted in HCB+IL-3. As positive control, fMLP and anti-IgE were used. 20 µL of each allergen sample were put into a 96-well Micronics plate and 100 µL of whole blood were added to each well. The samples were vortexed and incubated for 15 minutes at 37°C. Afterwards, 10 µL of stop buffer were added, and incubated for 5 minutes at RT. Then, 20 µL antibody dilution were added to each well, and incubated in the dark for 15 minutes. Then, 850 µL of Erythrocyte lysis buffer were added to each sample, mixed up and down 6 times and incubated for 7 minutes in the dark. The plate was then centrifuged for 5 minutes at 300 x g and the supernatant discarded. The erythrocyte lysis was repeated once more and after removing the supernatant, the pellet was resuspended in 200 µL of FACS buffer. The samples were centrifuged again for 5 minutes 300 x g, the supernatant discarded, and the cells resuspended in 200 µL FACS buffer. Then, the samples were transferred into 96-well microtiter plates and measured on the flow cytometer. It was gated for $CD123^+/CCR3^+$ cells, and 1000 cells were acquired.

## 2.11. Caco-2 cell line

When grown under standard conditions on semipermeable membranes, fully differentiated Caco-2 cells express morphological and biochemical characteristics of small intestinal enterocytes. These cells grow in a monolayer and show a cylindrical polarized morphology with microvilli on the apical side and tight junctions between adjacent cells. This epithelial cell monolayer is the gold standard used *in vitro* to predict oral drug absorption in humans. [69]

Since for this experiment, Caco-2 cells were treated with birch juice, the toxicity of birch juice on Caco-2 cells had to be evaluated. Therefore, a neutral red uptake viability assay was established.

Neutral red has a net charge close to zero at physiological pH which lets it penetrate cellular membranes. Once it enters the lysosome, a lower pH is encountered, the dye becomes protonated and thereby trapped inside the lysosomes. The cationic form of the neutral red dye cannot cross membranes anymore and gets enriched in the acidic compartments of the cell, a process also known as ion trapping.

Upon cell death or change of pH, the dye is no longer held in the lysosomes. Thereby, cellular viability can easily be quantified by determining the amount of retained dye which is proportional to the number of living cells. [70]

**Protocol**

A defined number of cells were grown in 96-well plates for 48 hours. Cells were washed with 150 µL of PBS and 200 µL of the stimuli whose toxicity was to be determined were added to the cells for 4 hours. After the incubation, cells were washed with 150 µL PBS and 200 µL of neutral red solution (4 mg/mL stock 1:100 diluted) was added and incubated for further 4 hours. 200 µL of lysis buffer was added to each well and the plates put for 20 minutes, 450 rpm, 37°C on the shaker. The observed color change was quantified by measuring the OD value at 540 nm using lysis buffer as blank.

Once the neutral red viability assay was established, cells were grown on cell culture filter inserts with a pore size of 0.3µm. Therefore, 1.5 x $10^5$ cells/mL were harvested from the previously grown cell culture, and 300 µL plated onto the apical side of each insert. The basolateral side was filled with 700 µL of Caco-2 medium. Cells were grown for 21 days and the medium changed every 2 days. From day 9 on, TEER measurement was performed every second day to assure the monolayer integrity.

The monolayer integrity of tight junction dynamics of the cell line was determined by transepithelial/ transendothelial electrical resistance (TEER) measurement. This non-invasive measurement determines electrical resistance across a monolayer and is possible without causing cellular damage (Figure 7).[71]
The TEER was measured using the EVOM2 epithelial voltmeter.

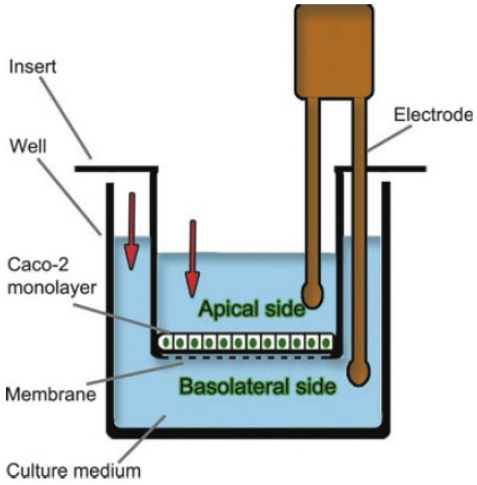



***Figure 7: Depiction of TEER measurement.*** *The electrical resistance across a monolayer is measured using an electrode.* [72]

Once the TEER measurement reached a constant value, different stimuli were added to the cells and phenol red absorption was measured.

This method measures the passage of phenol red in a culture medium from the apical side of a well to the basolateral side filled with phenol-red free medium. This method is a non-destructive measurement to determine confluency of monolayers and measures the percentage diffusion of this substance. [73]

**Protocol**

The culture medium of the cells was removed, and the cells were washed once with phenol-red free medium. 300 µL of phenol-red containing medium were pipetted onto the apical side of cell culture inserts and 700 µL of phenol-red free medium into the basolateral chamber. Cells were incubated for 4 hours and then triplicates of each basolateral fraction were measured at 559 nm. As controls, cell culture inserts without cells grown on them were placed into wells. The 100% passage control contained phenol-red medium on the apical and phenol-red containing medium on the basolateral well were pipetted. A 0% passage control was measured where phenol-red free medium was placed in both the apical and the basolateral chamber.

## 2.12. FITC labelling of Bet v 1

Fluorescein isothiocyanate (FITC) is a derivative of fluorescein, a fluorescent tracer. The molecule has excitation and emission and emission maxima at 495 and 519 nm.

**Protocol**

1 mL of a 1 mg/mL Bet v 1 was taken and 100 µL of carbonate buffer pH 8.7 were added. The mixture was separated into two aliquots of 550 µL each and 25 µL of FITC (1 mg/mL in DMSO) were added to each of the aliquots. This mixture was incubated at RT for 2h. After incubation, MicroBio-Spin Chromatography columns from BioRad were used for purification. Those columns are packed with a special polyacrylamide that produces a size separation. The columns were inverted several times to resuspend the gel. The lid was removed, and the columns placed in 2mL Eppendorf tubes. The caps of the column were taken off and the buffer allowed to drain for about 2 minutes. The buffer was discarded, the column placed again in the microcentrifuge tube and then centrifuged at 1000 x g for 2 minutes to remove remaining buffer. To have the FITC-labelled Bet v 1 in PBS buffer, a buffer exchange was carried out. Therefore, 500µL of PBS was added to each microcentrifugation tube and centrifuged at 1000 x g for 1 minute. This step was repeated 4 times to obtain a 99.9% buffer exchange. Then, each column was placed in a clean 1.5 mL microcentrifuge tube and 50 µL of sample were carefully applied to the gel bed of each column. Columns were centrifuged for 4 minutes at 1000 x g and purified samples recovered.

To examine the efficacy of the purification, the protein concentration was determined by Nanodrop measurement.

# 3. Results

Birch sap is known to contain low amount of protein. To increase the protein concentration in the samples, different protein concentration methods were tested. Thereby, a workflow could be established. Initially, birch juices available in the supermarket were tested due to a high availability and low cost. Once a protocol was established, fresh birch juice was harvested and analyzed according to the established protocol.

## 3.1. Investigation of allergens in different birch juices

### 3.1.1. Birch sap dm (BSdm)

The first examined juice was natural birch juice with 0.02% lemon concentrate added.

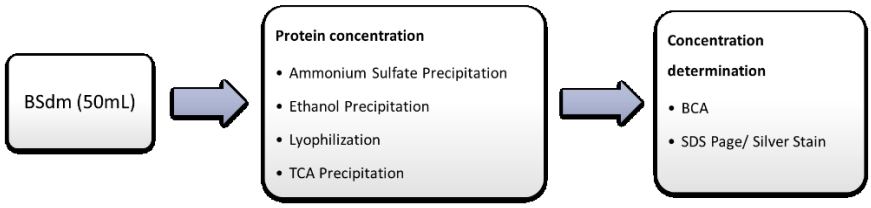



***Figure 8: Workflow for protein concentration using BSdm.*** *50mL of juice were taken for each approach, proteins were concentrated with the respective methods and the obtained concentration measured with a BCA assay. The concentrated proteins were then visualized and analyzed with SDS PAGE and Silver Stain.*

As seen in Figure 8, 50 mL of birch juice were taken, and each of the precipitation methods depicted carried out according to the protocols described in Methods. All methods were carried out simultaneously. Once the proteins were precipitated, the protein content of the fractions was investigated with a BCA assay. Pure, untreated, birch juice was measured as well to compare the effectivity of the respective methods.

***Table 3: Protein concentration of birch sap dm.*** *The absorbance of 25 µL sample in various dilutions was measured and an average protein concentration determined by a BCA assay. Concentration is given in g/100 mL*
*n.d.: not detected*

| Sample | Concentration |
|---|---|
| Pure BSdm | 1.32 |
| Lyophilization | 8.21 |
| TCA | n.d. |
| EtOH | 0.63 |
| $NH_4$ | 0.33 |

The measured protein concentration of the pure birch juice and lyophilized birch juice (Table 3) was higher than indicated on the birch sap package. These values could be due to confounding factors in the juice like lipids, sucrose, antioxidants or phenol groups. Those substances can interfere with the BCA measurement and therefore lead to false high values.

The protein concentration after TCA protein precipitation was too low to be determined with the BCA assay. (Table 3) This is probably due to TCA being a denaturing agent. Even after repeating the experiment with more washing steps in between to remove all TCA, the protein concentration was still too low, possibly due to protein denaturation, and therefore it was decided to not further use this method for protein precipitation.

Proteins precipitated with ethanol or ammonium sulfate showed concentrations that were similar to the ones written on the birch sap package. (Table 3) Some possible contamination could still not be yet excluded and therefore these methods had to be further adapted.

After determining the protein concentrations, the samples were loaded on a gel which was stained using the silver stain method. This staining method was chosen due to a higher sensitivity than Coomassie staining and a low protein concentration in the investigated samples. To have the same concentration of protein in each lane, 13.2µg were loaded into each slot.

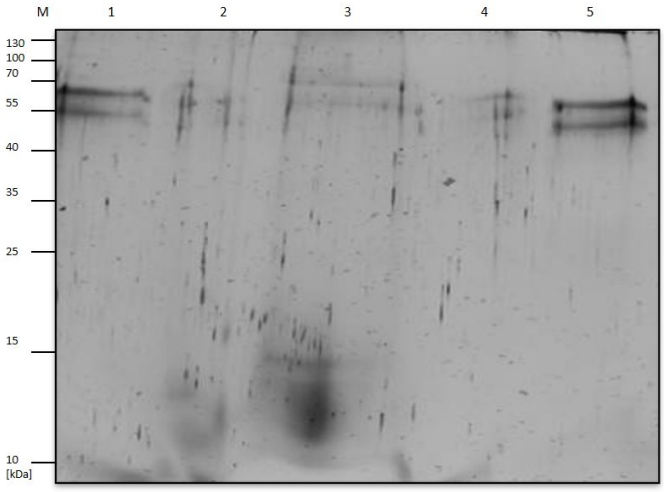



**Figure 9: Detection of proteins in birch sap dm.**

Birch sap dm was differently treated: pure birch juice dm (1), ethanol precipitated (2), $NH_4$ precipitated (3), TCA precipitated (4), lyophilized (5).

40 µL of sample were loaded, proteins were separated on a 15% gel and stained with Silver Stain

M: Page Ruler Marker (3 µL)

When loading pure birch juice, no protein band could be detected. (Figure 9) Some slight protein smears can be seen after Ethanol precipitation and a slightly stronger smear when using Ammonium sulfate precipitation. Both smears were located in the area between 10 to 15 kDa. After TCA precipitation, the protein concentration was below the sensitivity limit of the BCA assay. Once loaded onto the gel, no protein bands could be visualized. The lyophilized sample measured a high protein concentration in the BCA assay. Nonetheless, no protein bands could be visualized on a gel. Therefore, it was decided to not further use these two methods. In all the loaded samples nevertheless, some protein bands between 55 and 70 kDa were visible. This could probably be sugar moieties in the birch juice like xylitol or also indigested proteins.

Since none of the methods lead to clearly visible protein bands on the gel, the methods were further adapted. It was expected that there were still some interfering substances in the sample which inhibit correct concentration measurement and protein detection. Therefore, the effect of dialysis of the birch juice samples prior to protein precipitation on the samples was tested. This was supposed to eliminate unwanted contaminants like sugars or other interfering substances.

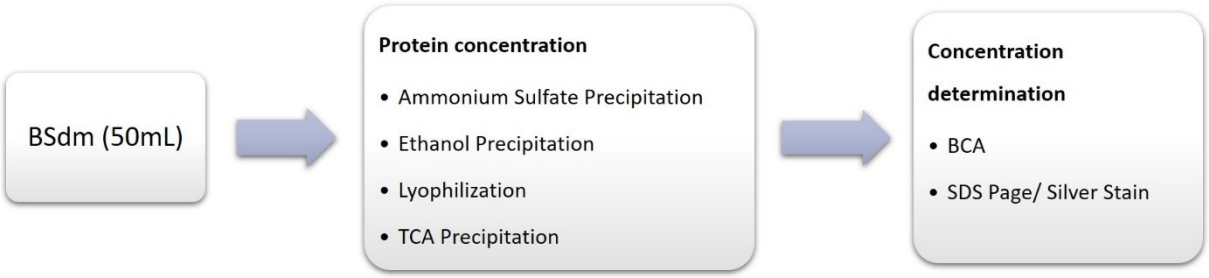



***Figure 10: Workflow to concentrate proteins in BSdm.*** *200 mL of birch juice dm were dialyzed against PBS overnight and then subjected to concentration with $NH_4$ and EtOH.*

As indicated in Figure 10, for each dialysis, 200 mL of birch juice were dialyzed against PBS for 52 hours at 4°C. Even though the buffer was exchanged regularly, no increase in sample volume was detected after dialysis. After dialyzing, the birch juice was taken, and proteins were precipitated with the respective methods. The protein concentration was measured by the BCA assay.

***Table 4: Protein concentration of dialyzed birch sap dm.*** *The absorbance of 25 µL sample in various dilutions was measured and an average protein concentration determined by a BCA assay.*
*n.d.: not detected*

| Sample | Concentration |
|---|---|
| Pure BSdm, dialyzed | n.d. |
| BSdm, dialyzed, EtOH prec. | n.d. |
| BSdm, dialyzed, $NH_4$ prec. | n.d. |

When previously dialyzing the samples, the protein concentration for each sample was too low to be detected by the BCA assay. (Table 4) A possible explanation for that is that the protein stability decreased over the time since the dialysis run for 52 hours in total. Therefore, the decision was made to only do dialysis overnight from now on with buffer exchanges in between to eliminate the risk of protein degradation due to instability.

Nonetheless, the samples were loaded onto an acrylamide gel. Since no protein concentration could be determined, 20 µL of each sample were loaded. This time, a 12% gel was used to obtain a better protein separation. Recombinant Bet v 1 was loaded as a control to compare possible detected protein bands and check whether the detected proteins are located at a similar position as Bet v 1.

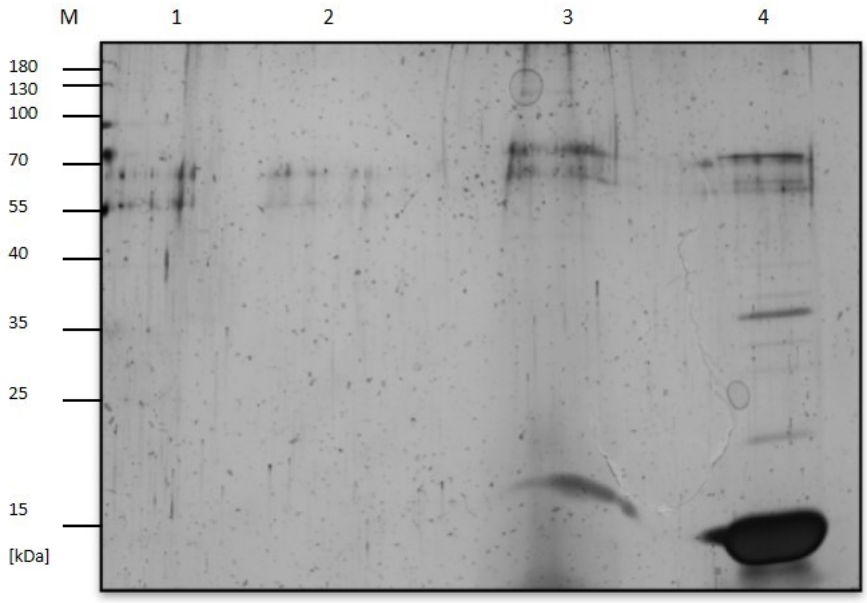



***Figure 11: Detection of proteins in dialyzed birch sap dm.***
*Birch sap dm was dialyzed and then differently treated: pure BSdm (1), ethanol precipitated (2), $NH_4$ precipitated (3). 1 µg Bet v 1 was loaded as control (4)*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain. M: Page Ruler Marker (3 µL)*

With dialysis prior to protein precipitation, no protein concentration could be determined (Table 4), and no protein bands visualized (Figure 11). When proteins were precipitated with $NH_4$, one could see a slight band.

When the gel was re-loaded the next day, nothing was visible. This could be due to protein decay or the visible band could possibly be part of a protein that had flown over from another lane due to a pipetting mistake.

Because there were no proteins detected when dialyzing samples prior to concentrating proteins, a dialysis over the weekend prior to precipitating the proteins was not considered a suitable method to concentrate and visualize proteins.

To investigate whether the low protein concentration might have been a problem arising from the birch juice used and not the method itself, a differently treated birch juice was taken, and the same protocol was tried out.

### 3.1.2. Vita7 Birkenwasser Natur (BSV)

To compare the effects of dialysis on BSdm, another purchased birch juice, the Vita7 Birkenwasser Natur (BSV) was investigated. This time, the birch juice was dialyzed, proteins precipitated with the respective methods and then loaded on a gel without previously measuring the obtained concentration. (Figure 12)

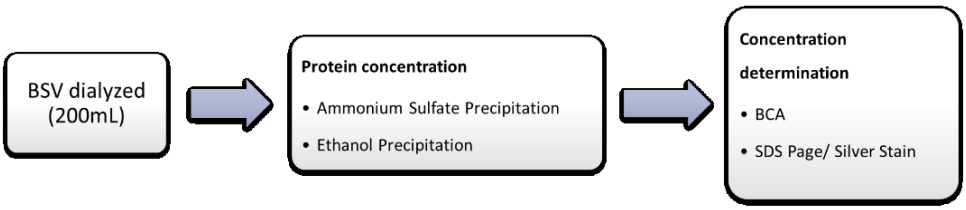



***Figure 12: Workflow for Vita 7 Birkenwasser Natur.*** *The previously established work flow was taken and tried out with BSV.*

The results are shown in Figure 13.

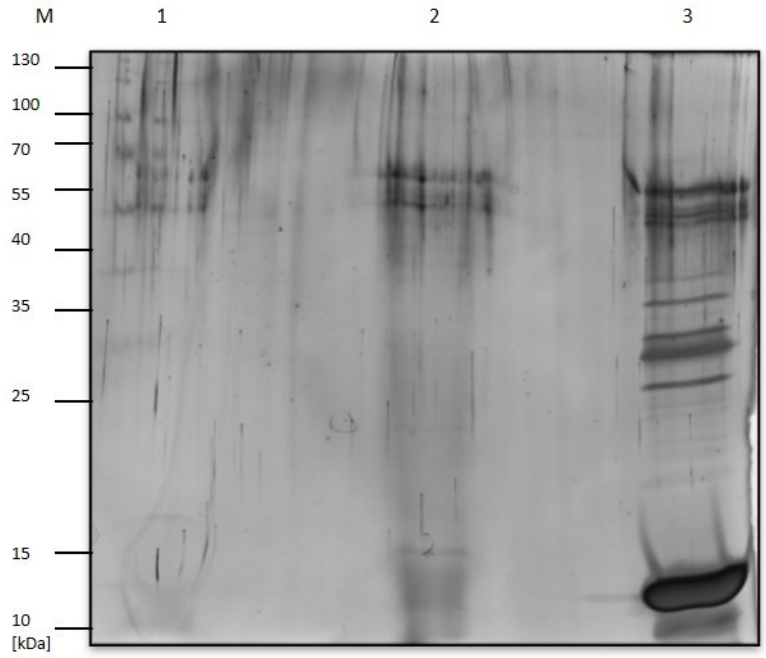



***Figure 13: Detection of proteins in Vita 7 birch juice.***
*Vita 7 birch sap was dialyzed and differently treated: ethanol (1) and $NH_4$ precipitated (2). 20 µg BPE were loaded as control (3)*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain. M: Page Ruler Marker (3 µL)*

After dialysis, no protein bands could be observed. Only a slight smear at around 13 kDa was visible after $NH_4$ precipitation. (Figure 13)

Since the gel sometimes curved slightly when loading $NH_4$ precipitated samples, it was assumed that the sample contained salt which could possibly interfere with protein migration. For the ethanol precipitation method, it was assumed that there might be some ethanol residue left which inhibits the protein migration.

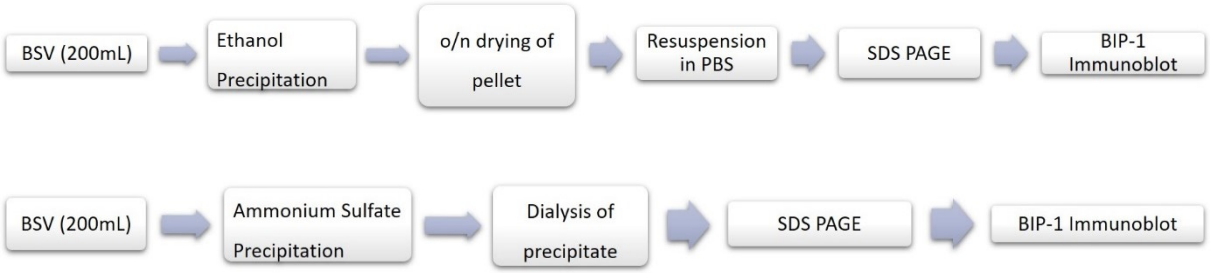



***Figure 14: Newly established workflow to concentrate proteins in BSV.*** *200 mL of birch juice were used for each method. When ethanol precipitation was carried out, the pellets were dried overnight under the fume hood to evaporate any remaining ethanol and then resuspended in 1x PBS and proteins analyzed with SDS PAGE. After ammonium sulfate precipitation, the precipitate was dialyzed against 1x PBS and after dialysis 10-fold concentrated in the SpeedVac then analyzed by SDS PAGE and Silver Stain.*

A few different combinations of working steps were tried out until a new workflow was designed. (Figure 14) The juice samples were precipitated overnight with the respective methods.

Afterwards, the ethanol precipitated pellet was dried overnight under the fume hood to evaporate any ethanol residue, the pellet was then resuspended in PBS and analyzed with SDS PAGE. The $NH_4$ precipitated protein pellets were taken up in PBS and then dialyzed against 1x PBS overnight. The ammonium sulfate precipitated, dialyzed samples were also concentrated in the SpeedVac centrifuge. Because the previously run gel showed a protein smear more intense with ethanol precipitation, it was decided not to do a vacuum concentration with those samples. Later all samples were analyzed with SDS PAGE.

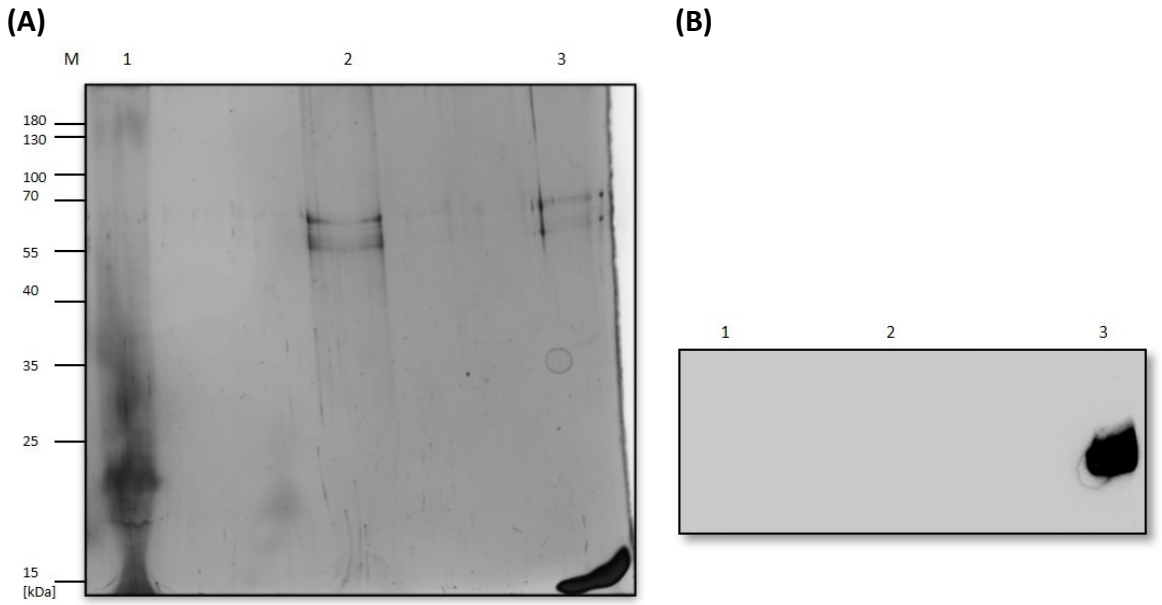



***Figure 15: Detection of proteins in Vita 7 birch juice***

*Vita 7 birch sap was differently treated: ethanol (1) and $NH_4$ precipitated (2). 1 µg Bet v 1 was loaded as control (3)*

***(A)*** *20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.*

***(B)*** *20 µL of sample were loaded, proteins were separated and used for western blots. The blots were incubated with mouse monoclonal antibody BIP-1*

*M: Page Ruler Marker (3 µL)*

On the gel, in the lane of the ethanol precipitated sample, an intense smear could be detected (Figure 15, A). This was now more intense than in previously loaded gels but nonetheless; no clear protein band could be detected. The ammonium sulfate precipitation, even though the method had been adapted, did not show any protein bands on the gel. Because there was a strongly visible protein smear in the lane where the ethanol precipitated sample was loaded, where a protein with a size similar to Bet v 1 could possibly be located, an immunoblot with BIP-1 as primary antibody was started. This mouse monoclonal antibody binds to Bet v 1.

No protein binding in the precipitated BSV samples could be visualized by this immunoblot (Figure 15, B). Only the control Bet v 1 in the loaded birch pollen extract was detected. Therefore, it was assumed that there was no Bet v 1 in the investigated specimen. The possibility arose that maybe, due to previous treatment, the commercially available birch juice does not contain Bet v 1 or any proteins at all, but only the freshly tapped one does. Therefore, fresh birch juice was harvested and investigated.

### 3.1.3. Fresh birch juice (BSF)

A total of 16L of fresh birch juice were harvested from 4 different trees from Styria. The different juices were filtered and pooled and analysis was carried out following the protocol shown in Figure 14.

This time, 1 L of birch juice pool was used for each precipitation method.

After concentrating the ammonium sulfate precipitated sample in the SpeedVac, it separated into supernatant and pellet and therefore both fractions were loaded separately to detect possible differences.

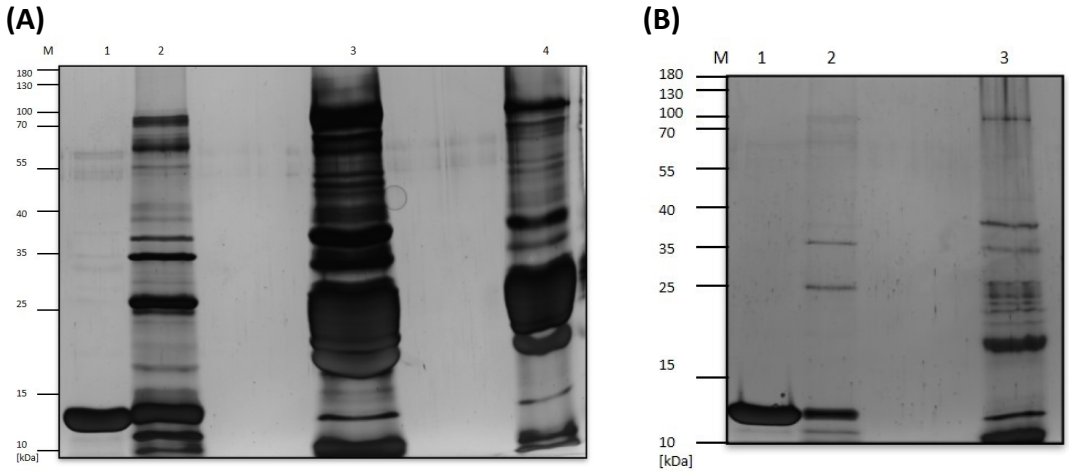



**Figure 16:** ***Detection of proteins in fresh birch juice***
***(A)*** *Fresh birch juice was ammonium sulfate precipitated: supernatant, 1:4 diluted (3) and pellet, 1:4 diluted (2). 1 µg Bet v 1 (1) and 25 µg BPE (2) were loaded as control*
*20 µL of sample were loaded, proteins were separated on a 12% gel and Stained with Silver Stain.*
*M: Page Ruler Marker (3 µL)*
**(B)** *Fresh birch juice was ethanol precipitate (3) 1 µg Bet v 1 (1) and 25 µg BPE (2) were loaded as control*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.*
*M: Page Ruler Marker (3 µL)*

When loading 20µL of ammonium sulfate precipitated birch juice without previous dilution, a very intense protein smear was detected. Therefore, the samples were diluted 1:4 in 1x PBS.

A variety of protein bands were detected in both the supernatant and the pellet samples (Figure 16, A). Many of the protein bands were on similar levels as the proteins found in the birch pollen extract.

After protein precipitation with ethanol, some proteins were found in the fresh birch juice, but not as many as after ammonium sulfate precipitation method (Figure 16, B).

Here, fewer proteins were located at similar molecular sizes as the proteins found in birch pollen extract. Since different dilutions were loaded on the gels, a BCA assay was performed, and protein concentrations of the samples were determined to further load uniform protein concentrations on the gel.

**(A)**

| Sample | Concentration |
|---|---|
| Birch pollen extract | 22.1 µg/µL |
| BS, EtOH prec. | 31.31 µg/µL |
| BS, $NH_4$ prec., dialyzed | 1.255 µg/µL |

**(B)**

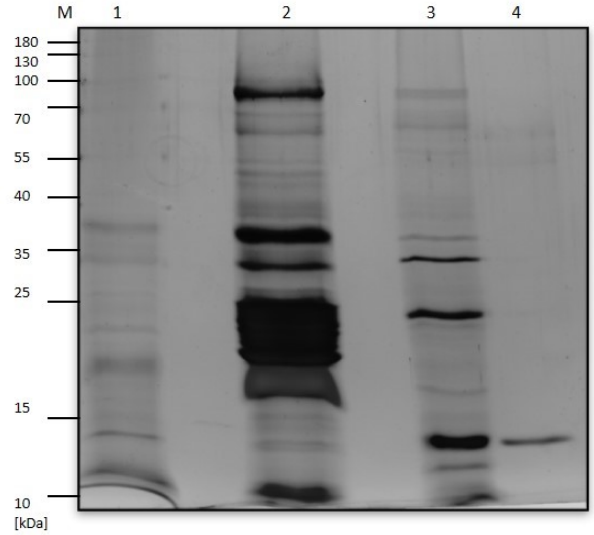



***Figure 17: Protein concentration of fresh birch sap***
***(A)*** *The absorbance of 25 µL sample in various dilutions was measured and an average protein concentration determined by a BCA assay.*
***(B)*** *Fresh birch juice was differently treated: ethanol (1) and $NH_4$ precipitated (2). 30 µg BPE (3) and 1 µg Bet v 1 (4) were loaded as control*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain. M: Page Ruler Marker (3 µL)*

The BCA assay measured a high protein concentration for the ethanol precipitated sample (Figure 17, A). This result was rather unexpected compared to indicated protein concentrations on birch sap packages. This measurement confirmed that the BCA is not a reliable method to measure the protein concentration of birch juice.

When loading equal amounts of both precipitated samples, ammonium sulfate precipitation led to a higher protein diversity and intensity of bands visualized on the gel in comparison to ethanol precipitation (Figure 17, B).

To investigate the effect of mercaptoethanol on protein separation in the different samples, two acrylamide gels were prepared with different sample buffers whereas one contained mercaptoethanol and one did not.

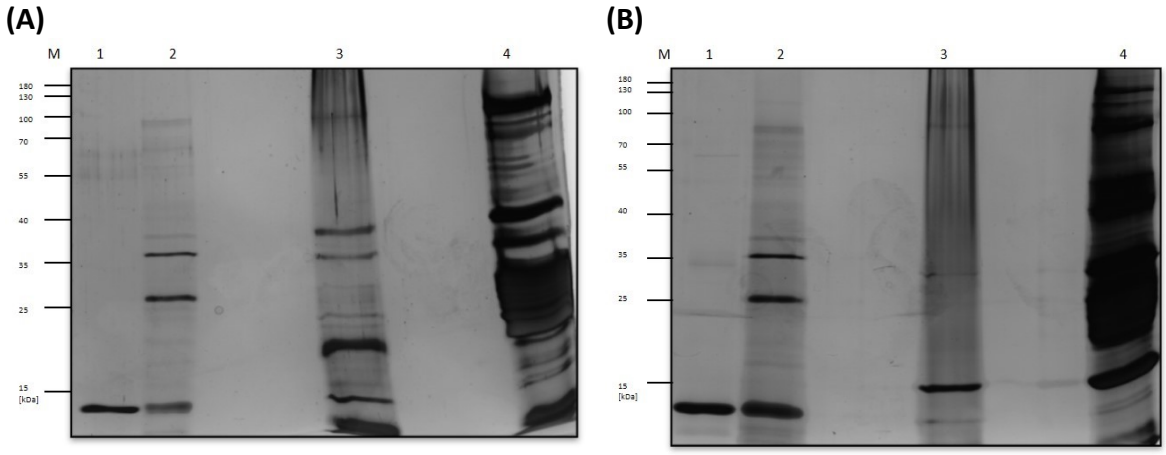



***Figure 18: Detection of proteins in fresh birch juice***
*Fresh birch juice was differently treated: ethanol (2) and $NH_4$ precipitated (3). Samples were diluted 1:4 in 1xPBS prior to loading. 1 µg Bet v 1 (1) and 25 µg BPE (2) were loaded as control.*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.*
***(A)** Sample buffer with mercaptoethanol was used*
***(B)** Sample buffer without mercaptoethanol was used*
*M: Page Ruler Marker (3 µL)*

When using a sample buffer with mercaptoethanol, proteins get properly separated (Figure 18, A). The ethanol precipitated sample showed only one properly visible band when using a sample buffer without mercaptoethanol and otherwise just a protein smear (Figure 18, B). Since mercaptoethanol breaks disulfide bonds of proteins and a sample buffer without cannot do this, proteins did not get separated properly and therefore could not travel to their proper position.

Due to the high amount of proteins visible on the gels, an IgE immunoblot on 300µg/ NC sheet of birch pollen extract, ethanol or ammonium sulfate precipitated proteins was performed. Therefore, patient sera reacting to Bet v 1,2,6 and 8 were used for each precipitation method, and birch pollen extract as positive control. As negative control, just buffer or a non-allergic donor sample which should not show any reactivity were added.

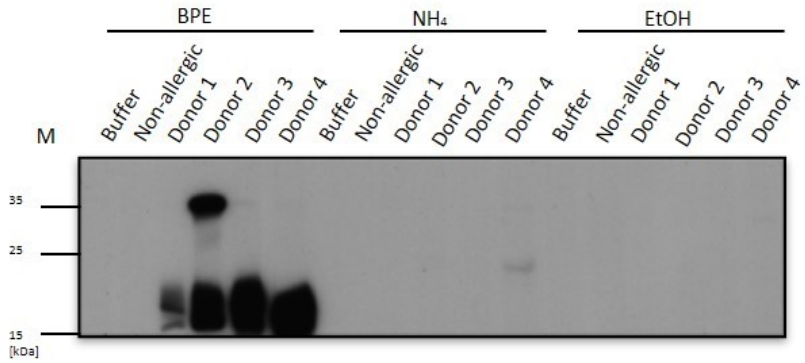



***Figure 19: Detection of IgE binding to birch pollen allergens.***
*Fresh birch juice was ammonium sulfate or ethanol precipitated. BPE were loaded as control. Strips were always loaded with buffer, a non-allergic donor and different, allergic donors. The donors produced IgE against following allergens: Donor 1 to Bet v 1,8; Donor 2: Bet v 1,6; Donor 3: Bet v 1,2; Donor 4: Bet v 1,6,8. The blot was exposed for 96 hours.*
*30 µL of sample were loaded per strip, proteins separated and used for western blots. The blots were incubated with anti-human IgE antibodies.*
*M: Page Ruler Marker (3 µL)*

When testing single patients in an IgE immunoblot, patients showed IgE reactivity to proteins in BPE. No IgE binding could be visualized to birch sap proteins precipitated with either $NH_4$ or ethanol (Figure 19). Thereby, it could be concluded that proteins precipitated in birch juice do not possess allergenic properties.

To see if donors would react to proteins in birch juice without any precipitation method, pure birch juice was used for an IgE immunoblot. Figure 20 shows that all 3 donors produced IgE reactivity to birch pollen protein but not to birch juice.


***Figure 20: Detection of IgE binding to birch pollen allergens.***
*Strips were always loaded with buffer, a non-allergic donor, or different donors. The donors produced IgE against following allergens: Donor 1 to Bet v 1,8; Donor 2: Bet v 1,6; Donor 3: Bet v 1,2; Donor 4: Bet v 1,6,8. The blot was exposed for 300 hours.*
*30 µL of sample were loaded per strip, proteins separated and used for western blots. The blots were incubated with anti-human IgE antibodies.*
*M: Page Ruler Marker (3 µL)*

To rule out that the precipitation methods harmed the birch proteins, birch pollen extract was precipitated with the ethanol and ammonium sulfate. The precipitates were loaded on a gel and analyzed.

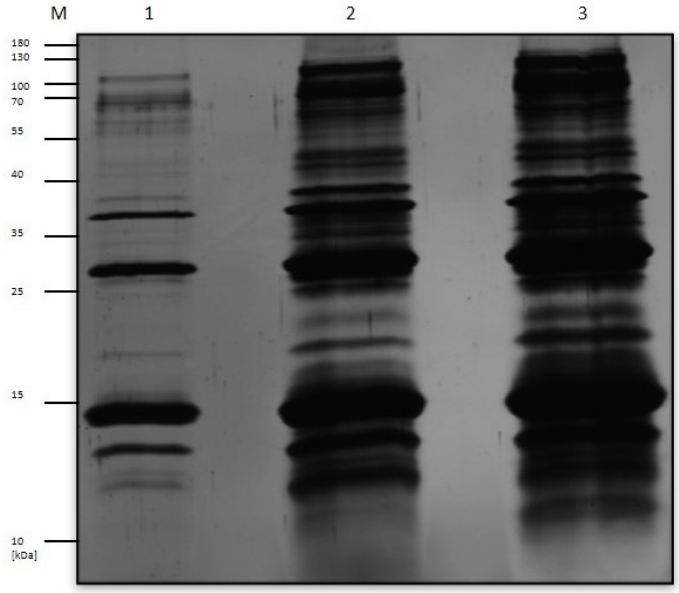



***Figure 21: Detection of proteins in fresh birch juice***
*Birch pollen extract was differently treated: ethanol (2) and $NH_4$ precipitated (3). 25 µg BPE (1) were loaded as control.*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.*
*M: Page Ruler Marker (3 µL)*

As seen in Figure 21, there was no negative effect on birch pollen extract when proteins were precipitated with the two respective methods. Bet v 1 remained stable and both protein precipitation methods did not harm the allergens contained in birch pollen extract. Rather it revealed a greater variety of proteins and more intense bands. The stability of Bet v 1 especially was not affected by any of the two methods, thereby it was concluded that the precipitation did not have negative effects on allergens in birch pollen extract.

Possible sides of glycosylation in the proteins found in birch juice were investigated to see similarities to glycosylated proteins in birch pollen extract.

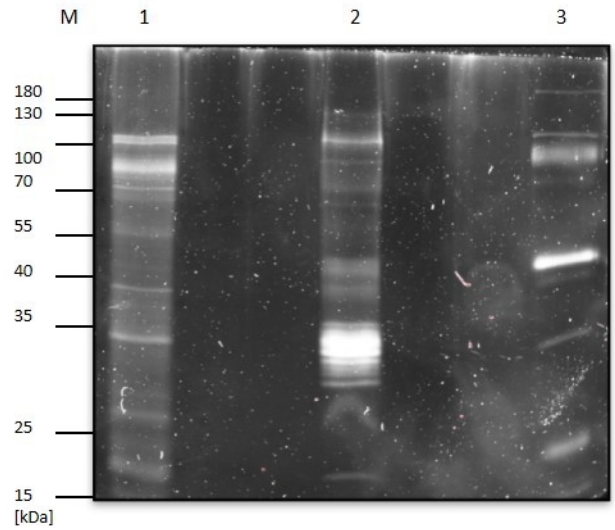



***Figure 22: Detection of glycosylated proteins in fresh birch juice.***
*Fresh birch juice (1), BPE (2) and Candy Cane glycosylation marker (3) were loaded and stained with the glycosylation detection kit.*
*20µL of sample were loaded into each lane and 3µL of Page Ruler Marker were loaded to estimate obtained protein sizes.*

A variety of glycosylated proteins could be detected in the fresh birch juice as well as in the birch pollen extract (Figure 22). In the fresh birch juice, glycosylated proteins at 20, 25, 35, 55, 70, 85, 100 kDa could be visualized. When comparing the glycosylated proteins, only two similarities were found between the birch juice and birch pollen extract: one at around 15kDa and one around 35kDa.

### *3.1.4. Analysis of proteins in fresh birch juice*

To identify different proteins observed with each precipitation methods, mass spectrometric analysis was performed. Therefore, gels with different acrylamide concentrations were prepared to separate proteins of all sizes precisely. As a rule of thumb, the smaller the protein of interest is, the higher the percentage of the gel needs to be. Therefore, gels with 10 and 18% of acrylamide were produced and run for different times to observe protein separation in distinct sections. The gels were then stained with Coomassie Blue, and protein bands numbered, cut out, stored in 1%HAc and sent for Mass Spectrometry.

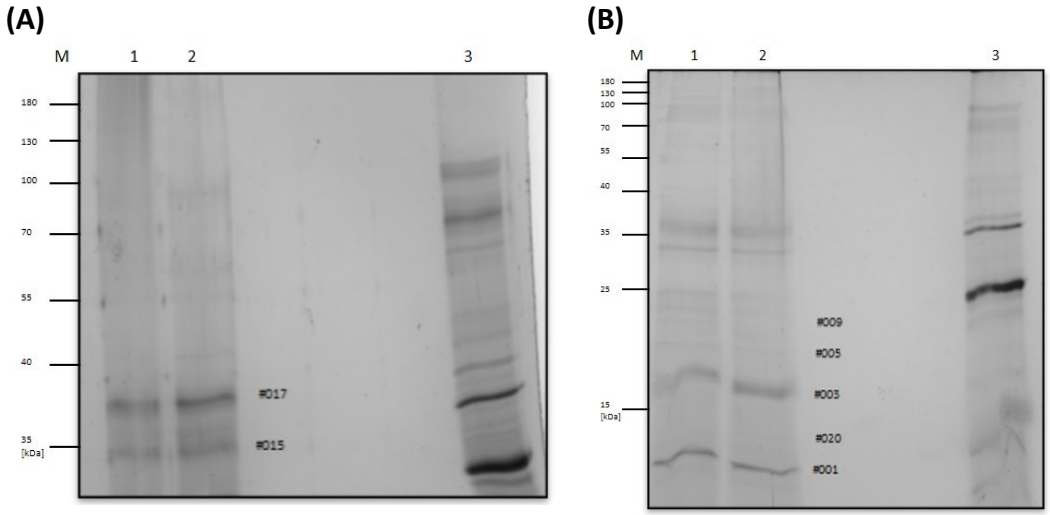



***Figure 23: Detection of proteins in fresh birch juice***
*Fresh birch juice was ethanol precipitated (1), (2). 25 µg BPE were loaded as control (3)*
*40 µL of sample were loaded, proteins separated on 10* ***(A)*** *and 18%* ***(B)*** *acrylamide gels and stained with Coomassie Blue.*
*M: Page Ruler Marker (3 µL)*

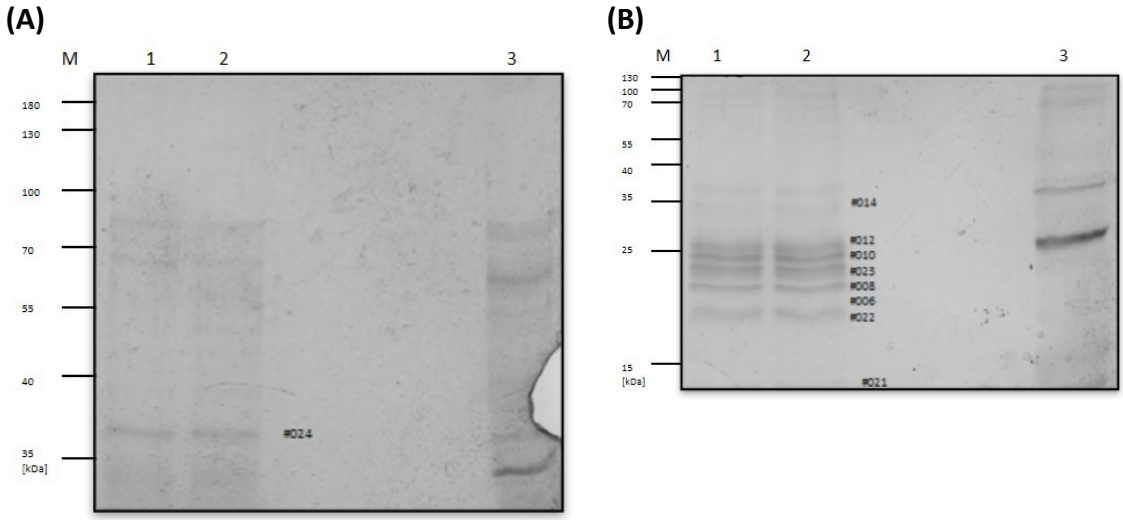



***Figure 24: Detection of proteins in fresh birch juice***
*Fresh birch juice was ammonium sulfate precipitated (1), (2). 25 µg BPE were loaded as control (3)*
*40 µL of sample were loaded, proteins separated on 10* ***(A)*** *and 18%* ***(B)*** *acrylamide gels and stained with Coomassie Blue.*
*M: Page Ruler Marker (3 µL)*

Figure 23 shows the protein bands observed using different percentages of polyacrylamide gels when loading ethanol precipitated samples, Figure 24 those when ammonium sulfate precipitated samples were loaded. The bands appear slightly fainter than in the previous figures because here, protein bands were stained with the less sensitive Coomassie blue stain.

After protein bands were visualized, they were cut out with a scalpel and sent for analysis to Peter Briza. Additionally, a proteome of birch juice was made by this lab. (see: Appendix)

***Table 5**: **Proteins in birch sap detected by mass spectrometry.** Through sequence comparison, possible functions of the different proteins could be determined.*
***(A)** Prediction of the function of the proteins bands cut out of after ethanol precipitation of birch juice samples*
***(B)** Prediction of the function of the proteins bands cut out of after ammonium sulfate precipitated birch juice samples*

**(A)**

| Sample | Size [kDa] | Number | Possible function |
|---|---|---|---|
| **EtOH** | 13 | #001 | LTP2-domain containing protein |
| | 15 | #020 | too little material |
| | 17 | #003 | thaumatin-like proteins |
| | 20 | #005 | too little material |
| | 24 | #009 | too little material |
| | 35 | #015 | peroxidase-like protein |
| | 37 | #017 | Aspartyl-protease-like protein |

**(B)**

| Sample | Size [kDa] | Number | Possible function |
|---|---|---|---|
| **NH4** | 17 | #004 | Thaumatin-like protein |
| | 20 | #006 | basic secretory protease |
| | 22 | #008 | basic secretory protease |
| | 24 | #010 | basic secretory protease |
| | 25 | #012 | basic secretory protease |
| | 28 | #014 | Mix of peroxidase and protease |
| | 17 | #021 | Thaumatin-like protein |
| | 19 | #022 | basic secretory protease |
| | 23 | #023 | basic secretory protease |
| | 27 | #024 | basic secretory protease |

Unfortunately, some of the analyzed samples had too little material to detect any proteins. Nonetheless, a diversity of proteins were found as seen in Table 3: a LTP2-domain containing protein which is a 11 kDa, potential phospholipid transfer protein, non-specific lipid transfer protein with several highly conserved cysteines. Some thaumatin-like proteins that are 20–30 kDa of size, have a very stable 3D structure maintained by six disulphide bridges and have been characterized as minor allergens in some fruit and pollen. A lot of proteases were identified with a size of 15-50 kDa and catalyze the cleavage of peptide bonds in other proteins. A peroxidase-like protein was also identified which is around 30-55 kDa in size, and its primary function is oxidizing hydrogen donors at the expense of peroxides. Additionally, an aspartyl-protease-like protein was found which has a molecular weight of around 30-45 kDa, and is a proteolytic enzyme with an aspartate residue in its active site.

The established proteome revealed a diversity of proteins belonging to different protein families found in birch juice (see: Appendix). Furthermore, the proteomic analysis could confirm the proteins already identified by mass spectrometry. All of the proteins that had previously been identified were also found in the proteomic analysis with 1 member of LTP2-domain containing proteins, 38 peroxidases, 1 aspartyl protease, 1 basic secretory protease, 6 thaumatin-like proteins. Except for thaumatin-like proteins, no known allergens were identified.

### 3.1.5. Bet v 1 in fresh birch juice

#### *3.1.5.1. Stability of Bet v 1 in fresh birch juice*

Mass spectrometric analysis detected the presence of proteases in birch juice. (Table 5) To investigate whether these proteases had an effect on the stability of the allergens, we reconstituted different concentrations of recombinant Bet v 1 and birch pollen extract in either PBS, BSdm or BSF and incubated them at RT on the rolling mixer overnight. The next day, the samples were dotted on a nitrocellulose sheet and the presence of Bet v 1 was detected using the monoclonal antibody BIP-1.

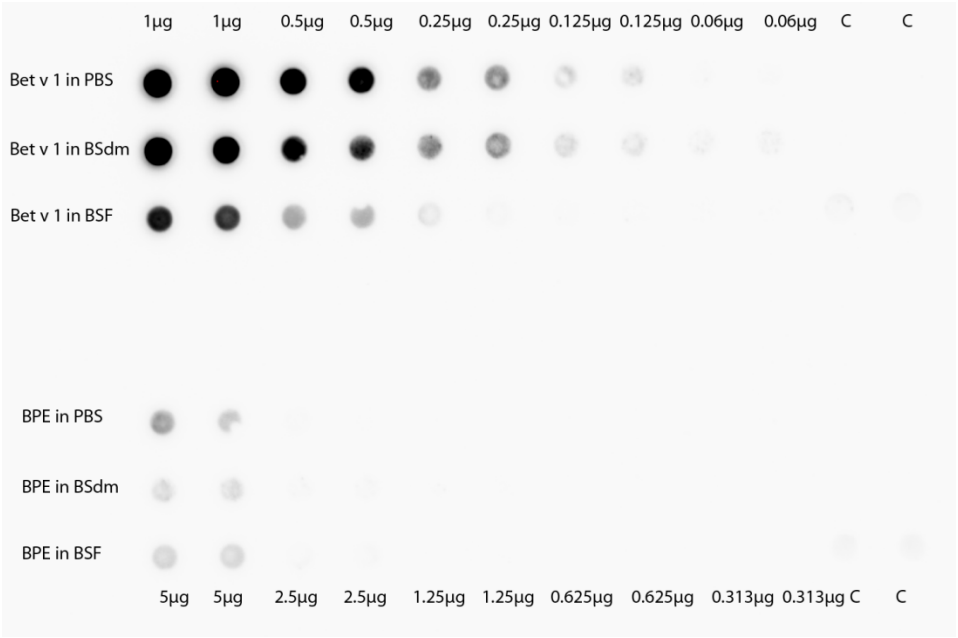



***Figure 25: Integrity of Bet v 1 in birch sap.*** *rBet v 1 and BPE were reconstituted in PBS, BSdm or BSF and dotted in duplicates in decreasing concentrations onto nitrocellulose. Bet v 1 was detected using the antibody BIP-1*
*C: Medium in which the allergen was reconstituted*

For birch pollen extract, the starting concentration was too low. Therefore, an effect of the different reconstitution media on the stability of proteins in birch pollen extract could not be determined by this experiment. When recombinant Bet v 1 was reconstituted in the different media, one could strongly see that it was as stable in BSdm and in PBS but less stable in BSF. (Figure 25) The intensity of the protein band indicated that approximately 50% of Bet v 1 were degraded in BSF.

To evaluate whether the pH of the different birch juices affected protein stability, the pH values of all used birch juices were measured.

BSdm: pH 5.32

BS Vita7: pH 6.52

BSF: pH 5.86

To test the effect of pH on Bet v 1, rBet v 1 was also reconstituted in PBS with a pH of approximately 6 which was close to the pH of fresh birch juice. Moreover, fresh birch juice was heated for 10 minutes at 95°C prior to reconstituting rBet v 1 or BPE in the cooled down juice. The heat treatment degrades any proteases contained in the birch juice. This time, higher starting concentrations of BPE were used. The mixtures were again left overnight at RT on the rolling mixer and the next day dotted onto nitrocellulose and Bet v 1 presence detected with BIP-1. (Figure 26)

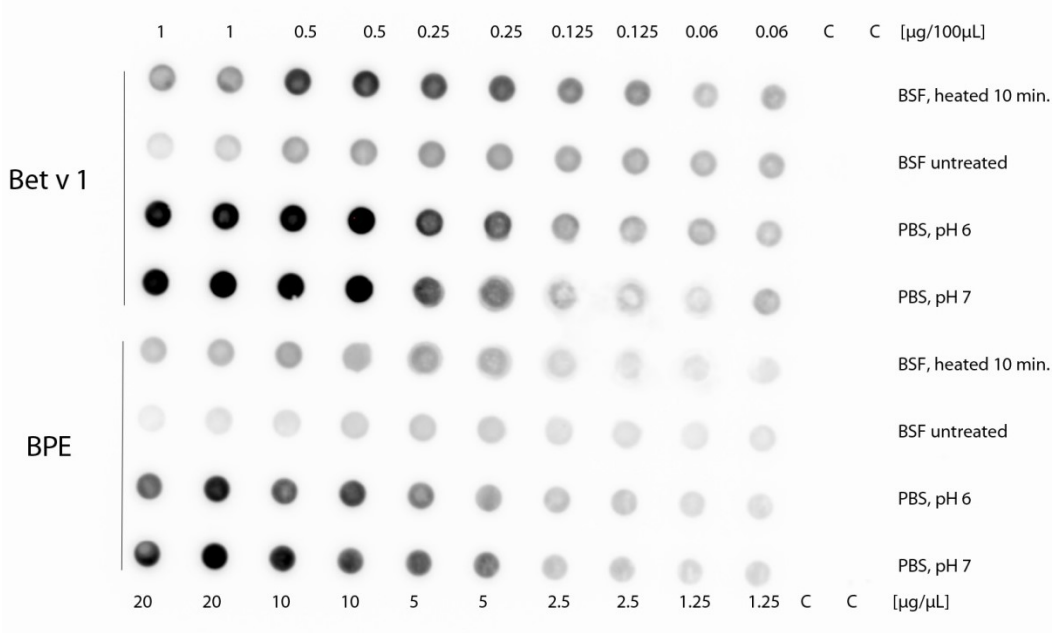



***Figure 26: Integrity of Bet v 1 in birch sap.*** *rBet v 1 and BPE were reconstituted in BSF, heated BSF and PBS pH 6 and 7 and dotted in duplicates in decreasing concentrations onto nitrocellulose. Bet v 1 was detected using the antibody BIP-1*
*C: Medium in which the allergen was reconstituted*

The lower pH influencing the stability of Bet v 1 can be excluded as a possibility since there was no observable change when the allergen was reconstituted in PBS with either pH 6 or pH 7 as seen in Figure 26. The heat treatment nevertheless did influence the Bet v 1 stability.

When pre-treating the birch juice for 10 minutes at 95°C, Bet v 1 showed a similar stability in heated, fresh birch juice as it did when reconstituted in PBS.
Bet v 1 showed an increased stability in heated birch juice compared to fresh, untreated birch juice. The stability of Bet v 1 was not affected by a pH change.

To evaluate whether the heating affects the proteins in birch juice, an SDS PAGE was performed where heat treated (BSFh) and untreated birch juice were separated on 12% acrylamide.

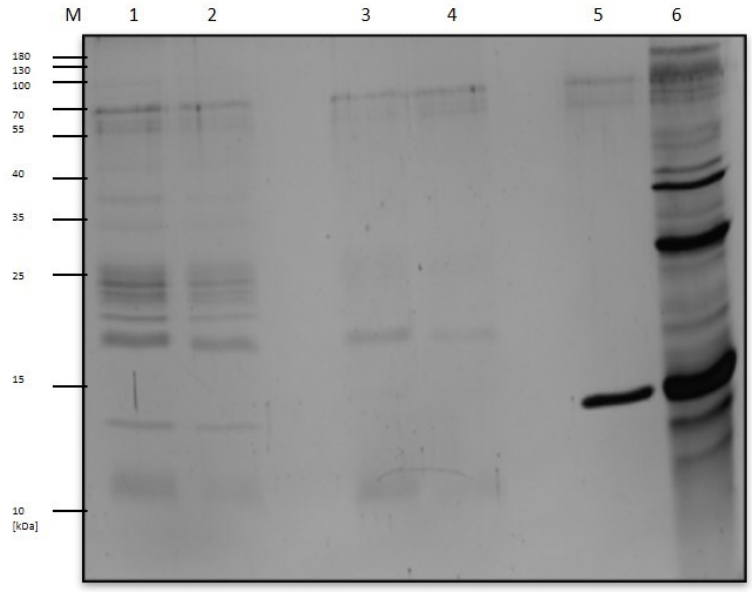



***Figure 27: Effect of heat treatment on fresh birch juice***
*Birch juice was differently treated: pure birch juice (1), BSF 1:2 diluted in PBS (2), BSF, heat treated for 10 minutes (3) BSF, heat treated for 10 minutes, 1:2 diluted in PBS (4). 1 µg Bet v 1 (5) and 20 µg BPE (6) were loaded as controls.*
*20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.*
*M: Page Ruler Marker (3 µL)*

Figure 27 shows that after heating the birch juice for 10 minutes, some proteins got degraded as less protein bands were visible in comparison to the untreated, natural birch sap. The protein bands at 12, 20-25, 30 and 35 kDa that cannot be seen after heat treatment correspond to the protease-like proteins detected by mass spectrometry. Those were probably heat sensitive proteases that had been degraded by heat treatment.

To evaluate whether heat treatment also stopped the degradation of Bet v 1 or BPE respectively, those were reconstituted in fresh or heat-treated birch sap.

Additionally, the samples were also reconstituted in PBS with different pH values to see if the pH also affected the stability of the allergens which could not be shown by the Dotblot.

The samples were left shaking overnight at 4°C and the next day loaded on a gel and analyzed.

**(A)** **(B)**

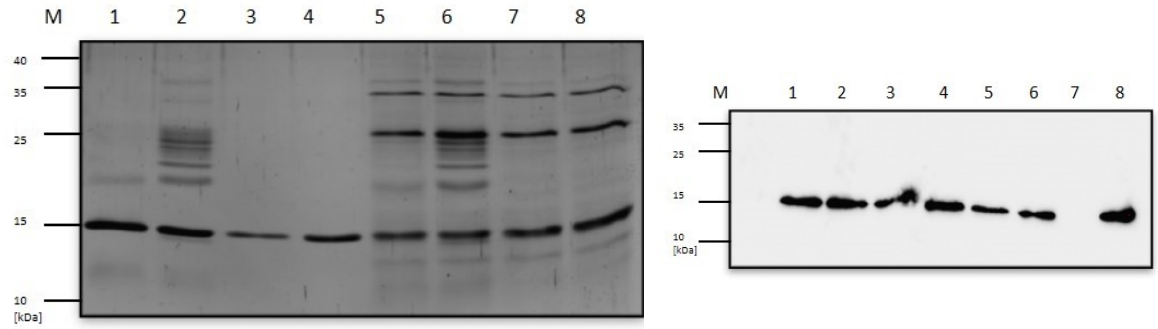



***Figure 28: Effect of heat treatment on Bet v 1 stability in fresh birch juice***
*1 µg of Bet v 1 was reconstituted in different media: heated BSF (1), BSF (2), PBS pH 6 (3), PBS pH 7 (4) 20 µg BPE were reconstituted in different media: in BSFh (5), BSF (6), PBS pH 6 (7), PBS pH 7 (8)* ***(A)*** *20 µL of sample were loaded, proteins were separated on a 12% gel and stained with Silver Stain.* ***(B)*** *20 µL of sample were loaded, proteins were separated and used for western blots. The blots were incubated with mouse monoclonal antibody BIP-1*
*M: Page Ruler Marker (3 µL)*

In Figure 28 A, it was visualized that heat treatment of birch sap does not influence the stability of neither Bet v 1 nor BPE therefore it can be aid that these proteases found were heat sensitive ones but not degradative towards Bet v 1. When reconstituting Bet v 1 in PBS pH 6 overnight there was a slight decrease of band intensity, but this was not seen when reconstituting BPE.

Also, on the immunoblot, no effect of the different media could be observed (Figure 28, B). The signal maintained the same intensity in each loaded well. In lane 7, no signal was detected which was possibly due to a pipetting mistake of detection solution since there was a band observed in the gel.

It was concluded that neither pH nor heat treatment had a negative effect of the Bet v 1 stability.

To investigate if basophils become activated when Bet v 1 or BPE are reconstituted in birch sap, a basophil activation test was performed. This is a flow cytometry-based

assay which measures the activation of basophils after stimulation with allergen. This time, also protease inhibitor was also added to the birch juice to compare the effect of the inhibitor to those of heat treatment.

$CD63^+$ basophils were analyzed by flow cytometry. The gating strategy for CD63 positive cells can be found in Figure 29. For each run of the assay, the same gating strategy was applied.

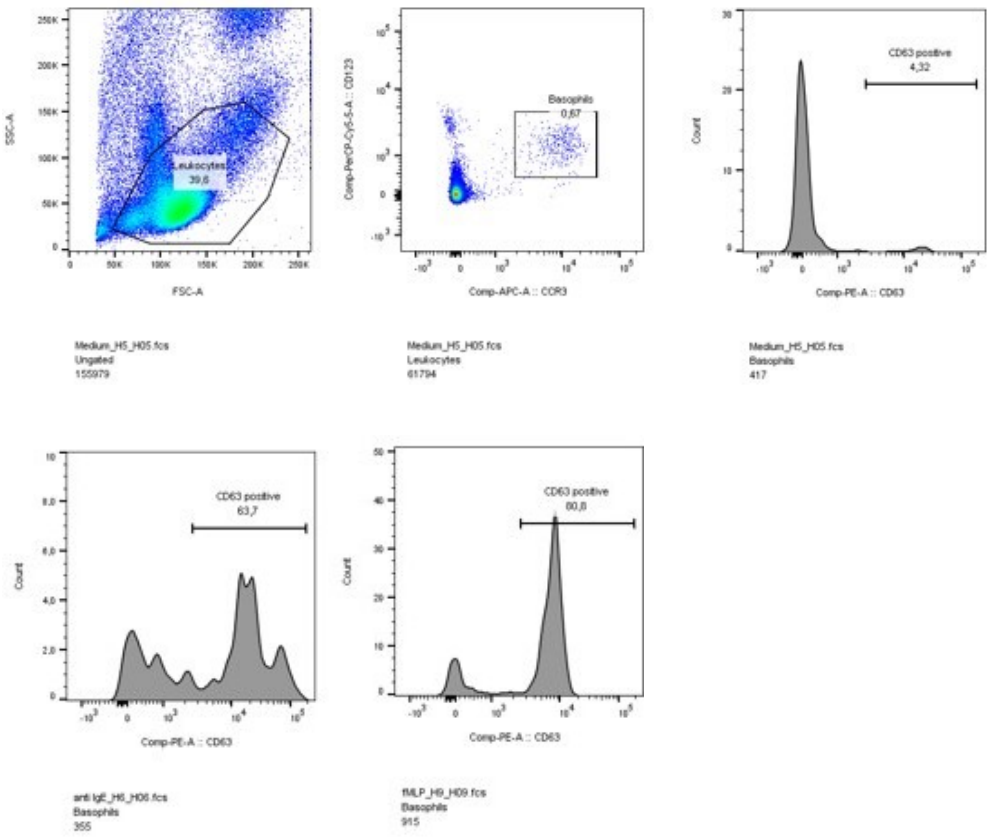



***Figure 29: Gating strategy for activated basophils.*** *Primarily, leukocytes were selected in the FSC/SSC. Then, $CCR3^+/CD123^+$ cells were selected and identified as basophils. In the next step, the percentage of CD63+ basophils were identified. The lower two histograms depict the positive controls for this experiment. The anti-IgE control was used to see if IgE was bound on the surface and to monitor the CD63 expression. The fMLP control was used to prove that basophils could be activated.*

The medium control, which was used in Figure 29 to show the gating strategy, showed a very low amount of CD63+ cells which is as expected since there should not be any basophils activated. The positive controls used for this assay were anti-IgE and fMLP. Anti-IgE cross-links membrane bound IgE and helps monitoring CD63 expression. Formyl-methionyl-leucyl-phenylalanine (fMLP) is a non-IgE mediated positive control. It acts via the G-protein-coupled receptor, activating MAPK pathways and phospholipase C instead of signaling downstream of the IgE receptor FcεRI.

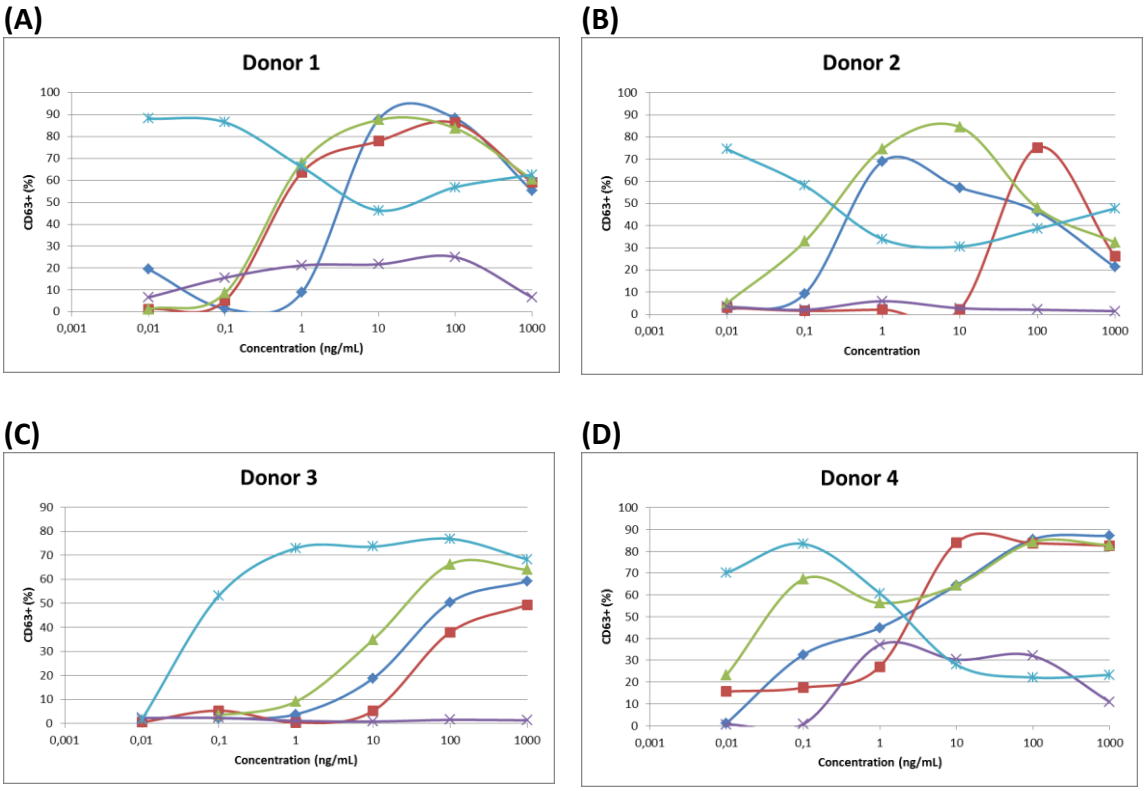



***Figure 19: Amount of CD63+ basophils in 4 different donors.*** *Light blue: Bet v 1 in PBS, purple: BSF, green: Bet v 1 in BSF, heated; red: Bet v 1 in BSF; dark blue: Bet v 1 in BSF with protease inhibitor added.*

Birch juice alone did not activate basophils in 2 out of 4 donors as demonstrated in Figure 30. In donors 1 and 3, up to 30% of basophils were activated solely by birch juice. Comparing this to the number of activated basophils when the blood was stimulated with Bet v 1, the number is rather low. When Bet v 1 was reconstituted in treated birch juice, a 4-fold shift of basophil reactivity could be observed in heat and protease inhibitor treated birch juice. As expected from previous experiments (Figure 22-25), a similar reactivity as with Bet v 1 in PBS could not be detected. Additionally, there was a very high donor-to-donor variability observed which made it difficult to generalize about the basophil activating properties of the different birch juice samples.

### *3.1.5.2. Transepithelial transport of Bet v 1*

Throughout the previous experiments, the IgE reactivity of Bet v 1 was reduced when reconstituted in birch sap (Figure 25+26). Nonetheless, rBet v 1 was not degraded in any of the tested environments. To establish oral tolerance, rBet v 1 reconstituted in birch sap would have to pass the intestinal epithelia.

Therefore, a CaCo2 cell line was established to determine whether birch juice enhances or inhibits the transport of Bet v 1 through the intestinal barrier. Caco2 cells are heterogeneous human epithelial colorectal adenocarcinoma cells that, when grown and differentiated, resemble enterocytes lining the small intestine.

Cells were grown at 37°C, 5% $CO_2$ and split whenever the flask reached full confluency.

### a) Viability

To find out whether birch juice itself was toxic to the cell line, a neutral red viability experiment was established (see: Methods). Cells were grown for 24 to 48h, then treated with different stimuli like birch juice, water, triton or PBS for 2 or 4 hours. The cells were then incubated with different concentrations of Neutral Red solutions for 2 to 4 hours. Then, cells were lysed and the absorbance of released neutral red incorporated into the cell's lysosomes was measured at 540nm measuring either the plate with all samples or just the harvested supernatant.

After several experiments, a reliable and reproducible protocol could be established (see: Methods). Caco2 cells were grown for 48 hours and then treated with either fresh or concentrated birch juice, water, PBS, or DMEM for 4 hours. As seen in Figure 31, the neutral red assay confirmed that birch juice, when diluted in medium, was not harmful to Caco2 cells.

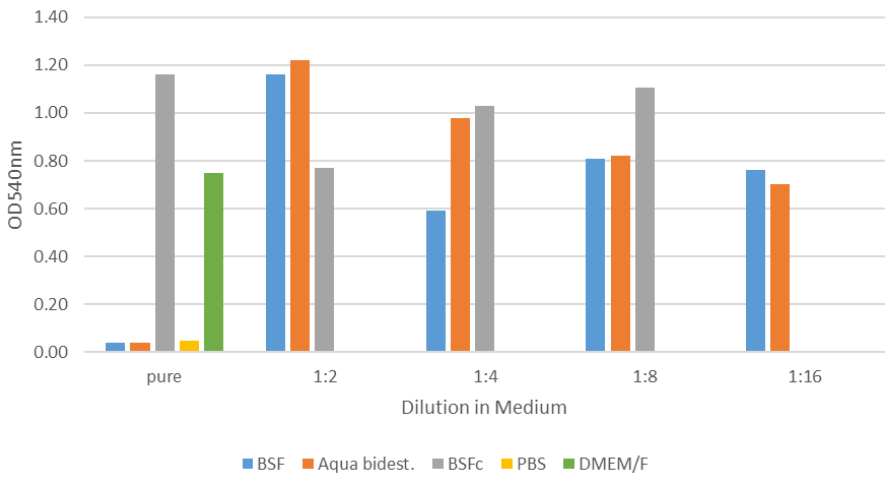



***Figure 31: Viability of Caco2 cells determined by Neutral Red assay.*** *Cells were grown for 48 hours and treated with different stimuli to investigate possible harming of cells.*

**b) Transport**

Once this was established, 1.5x $10^5$ cells/well were grown on cell culture inserts in 24 well plates. To validate monolayer formation and integrity of it, TEER was measured from day 9 on every second day until a plateau value was reached and therefore the cell monolayer completely established.

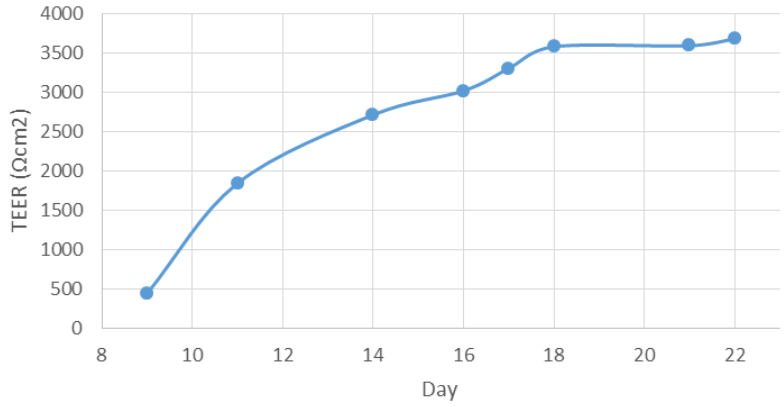



***Figure 32: Kinetics of TEER.*** *An increase in TEER value was measured until day 22.*

After 21 days of growth, a proper monolayer was established (Figure 32). Bet v 1 was labelled with FITC (see: Methods) and then applied to cells reconstituted in different media. Cells were treated in quadruplicates either with medium only, medium + 25µg Bet v 1 FITC/well, birch juice 1:2 diluted in medium+ 25µg Bet v 1 FITC/well, heated birch juice 1:2 diluted in medium + 25µg Bet v 1 FITC/well for 4 hours. TEER values were measured before and after stimulation. Furthermore, phenol red absorption after stimulation was determined and FITC was detected in apical and basolateral fractions. Apical and basolateral fractions of each well were collected and blotted onto nitrocellulose, and BIP-1 was used to detect Bet v 1.

***Table 6: Summary of results of application of reconstituted Bet v 1 to Caco2 monolayer.***
*The following test substances were added to the described wells: A: Medium only, B: Medium + FITC labelled Bet v 1, C: BSF 1:2 diluted in Medium + FITC labelled Bet v 1, D: BSF, heated 1:2 diluted in Medium + FITC labelled Bet v 1; a: apical compartment of the monolayer, b: basolateral compartment TEER was measured before and after cell stimulation, Phenol red in the basolateral compartment after stimulation, FITC in apical and basolateral fractions after stimulation.*

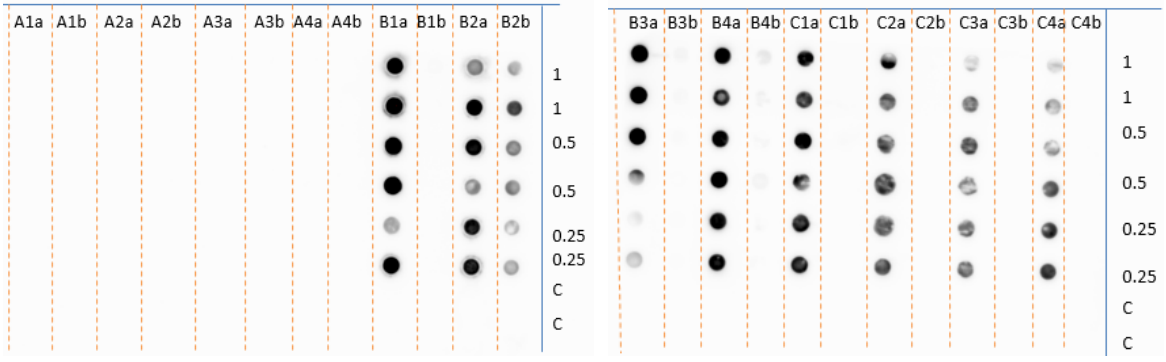
| Well | TEER before | TEER after | Phenol Red | FITC | DotBlot signal? |
|---|---|---|---|---|---|
| **A1a** | 3509 | 3474 | | 399 | No |
| **A1b** | | | 6% | 114 | No |
| **A2a** | 1981 | 1666 | | 387 | No |
| **A2b** | | | 4% | 123 | No |
| **A3a** | 1094 | 997 | | 363 | No |
| **A3b** | | | 6% | 118 | No |
| **A4a** | 2639 | 2314 | | 412 | No |
| **A4b** | | | 5% | 107 | No |
| Average | **2306** | **2113** | **5%** | **a: 390**<br>**b: 116** | |
| **B1a** | 3802 | 3429 | | 11206 | Yes |
| **B1b** | | | 7% | 128 | No |
| **B2a** | 1441 | 1341 | | 11098 | Yes |
| **B2b** | | | 8% | 266 | Yes |
| **B3a** | 3922 | 3664 | | 10837 | Yes |
| **B3b** | | | 4% | 126 | No |
| **B4a** | 3329 | 2464 | | 10382 | Yes |
| **B4b** | | | 3% | 125 | No |
| Average | **3124** | **2725** | **6%** | **a: 10881**<br>**b: 161** | |
| **C1a** | 4319 | 5404 | | 10096 | Yes |
| **C1b** | | | 4% | 128 | No |
| **C2a** | 2294 | 1858 | | 10314 | Yes |
| **C2b** | | | 5% | 127 | No |
| **C3a** | 3619 | 4414 | | 10319 | Weak |
| **C3b** | | | 6% | 126 | No |
| **C4a** | 3289 | 4144 | | 10810 | Weak |
| **C4b** | | | 6% | 120 | No |
| Average | **3380** | **3955** | **5%** | **a: 10385**<br>**b: 125** | |
| **D1a** | 3537 | 4494 | | 9962 | Yes |
| **D1b** | | | 6% | 166 | Very weak |
| **D2a** | 2959 | 3697 | | 11253 | Yes |
| **D2b** | | | 7% | 149 | No |
| **D3a** | 4707 | 7267 | | 12618 | Yes |
| **D3b** | | | 4% | 145 | Very weak |
| **D4a** | 1004 | 1119 | | 7654 | Yes |
| **D4b** | | | 19% | 1851 | Yes |
| Average | **3052** | **4144** | **5%** | **a: 10371**<br>**b: 115** | |

The measured TEER values before and after treatment remained stable in most of the wells. This shows that the integrity of the cell layer was maintained independent of the treatment applied. (Table 6)

The average phenol red absorption did not increase significantly after application of different birch juices to the Caco2 cells compared to medium only application as seen in Table 6. The measured FITC signal on the apical and basolateral side of wells A was low since there was medium only added and no FITC labelled Bet v 1. In all other wells, the signal measured on the apical side was 100-fold higher than on the basolateral side. This again proves that the birch juice did not disrupt the monolayer.

In well B2, the phenol red absorption measured 8% and on the dot blot, both the apical and the basolateral fractions gave signals. In well D4, the phenol red absorption measured 19%, a value way higher than any other measurements.
When looking at Figure 33, both the apical and the basolateral fractions of D4 loaded onto nitrocellulose gave a signal. The TEER measurement of both wells did not show any striking changes. Most probably the cell layers in wells B2 and D4 did not form properly or was somehow disrupted throughout the measurement.

For the Dotblot, 100µL of each sample taken, apical samples diluted from a Bet v 1 concentration of 25µg/300µL to 1µg/100µL with PBS, then further diluted until 0.25µg/100µL. Basolateral samples were diluted 1:2 in PBS and then further diluted in two-fold dilutions. 100µL of each sample applied to nitrocellulose in duplicates.



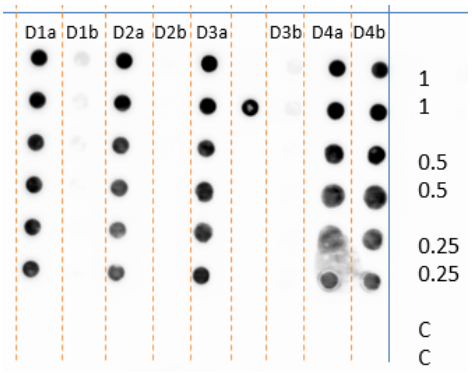



***Figure 203: Detection of Bet v 1 in apical and basolateral samples.***
*Supernatant from cells after treatment were pipetted onto nitrocellulose: Medium (A1-A4), Medium + 25µg Bet v 1/well (B1-B4), Birch juice 1:2 in medium + 25µg FITC labelled Bet v 1/well (C1-C4), Birch juice, heated 1:2 in medium + 25µg FITC Bet v 1/well (D1-D4)*
*Bet v 1 was detected with BIP-1.*
*a… apical, b… basolateral, C… Control (just PBS)*

All in all, it can be concluded that the integrity of the monolayer was not affected by application of any of the birch juice samples to Caco2 cells.

Still this experiment shows that one should always use different methods to verify an outcome because if only TEER would have been measured, wells B2 and D4 ( Table 6) would not be identified as instable ones.

The transport of recombinant Bet v 1 across the monolayer was similar when reconstituted in medium, pure or heated birch sap. Therefore, it can be said that birch sap can act as reconstitution medium for rBet v 1 without negatively affecting its transepithelial transport.

# 4. Discussion

The aim of this master thesis was to investigate allergens in birch sap. We purchased and freshly collected birch sap. The purchased juices were either heat treated or contained lemon juice concentrate to extend shelf life. Fresh birch juice was harvested from four different trees, pooled and filtered before usage. Birch juice is known to have a low initial protein concentration; therefore, different protein concentration methods were tested and the effects of those on enriching the protein quantity were compared.

*Investigation of allergens in different birch juices*

## *4.1. Birch sap dm*

The initially investigated birch juice was the birch juice dm, which had lemon juice concentrate added and measured a pH of 5.32.

Four protein concentration methods were tested: lyophilization, TCA, ammonium sulfate and ethanol precipitation. After application of these methods, protein concentration was measured using BCA assay and samples were loaded on a gel to detect protein bands.

Pure and lyophilized birch juice measured protein concentrations higher than expected comparing to the nutritional values depicted on the package of the juice. Nonetheless, when loaded on a gel, no protein bands could be visualized. The high measured protein concentration could possibly be due to cofounding factors in the birch juice like lipids or sucrose which were also detected by the BCA assay.

Since the pure birch juice as well as the lyophilized birch juice measured protein concentrations higher than expected, the birch juice was dialyzed prior to protein concentration to eliminate unwanted molecules. The dialysis was performed for around 48 hours and the protein concentration was too low to be detected by BCA assay for either method. Furthermore, when loading all dialyzed and precipitated fractions on a gel, no protein bands could be visualized. Proteins contained in the birch juice could have degraded throughout dialysis due to a short fridge stability of the sap.

The birch juice was then treated with TCA, resulting in a protein concentration too low to be detected. In addition to that, the TCA-treated birch juice was loaded on a gel, where no proteins could be visualized.

TCA precipitation is known to be very harsh and protein degrading. Therefore, we can assume that no detectable protein concentration was measured, and no protein bands visualized due to the effects of the acid on birch juice proteins.
Ethanol and ammonium sulfate precipitations resulted in protein concentrations similar to the ones depicted on the birch sap package. Nonetheless, no protein bands could be visualized upon loading on a gel, only slight smears.

Due to incompatibility with the BCA measurement and no detected protein bands, the concentration methods lyophilization and TCA precipitation were not further used with other birch saps.

The protein concentration depicted on the package of birch sap dm already stated a very low protein concentration. Despite the usage of four different concentration methods, no proteins could be detected in this birch sap. Since this birch juice contained lemon juice concentrate, it is possible that the sour compartment led to degradation of proteins contained in the birch sap. Furthermore, the birch sap could have undergone additional treatments like heat, which was not stated on the package, that could have caused protein degradation. Lastly, the 200mL volume used for protein concentration could have been too low.
To put it in a nutshell, the birch juice dm did not contain any proteins with allergenic properties.

### *4.2. Birch sap Vita 7*

This was 100% birch juice at a pH of 6.52, heated to 120°C for preservation.
When proteins of the dialyzed juice were precipitated with ethanol, not even a protein smear was detected, whereas ammonium sulfate precipitation could visualize a small smear.
A BIP-1 immunoblot was negative for both ammonium sulfate and ethanol precipitated samples.

The heat treatment of this birch juice possibly degraded most of the originally contained proteins and therefore no protein bands could be visualized or IgE reactivity detected.

Another possibility for the outcome could be the low volume of birch juice (250 mL) taken to concentrate the proteins, since the protein concentration depicted on the juice package is already very low.
Nonetheless, trying out different protein concentration methods resulted in establishment of a protein precipitation protocol. However, no so far known allergens could be detected in Birch sap Vita 7.

Ammonium sulfate and ethanol precipitation of birch pollen extract visualized a variety of protein bands rather than harming the allergens. Thereby the established protocol could be confirmed not to harm birch pollen allergens.

### *4.3. Fresh birch sap*

Fresh birch juice was harvested from four trees, pooled and filtered. The pH measured 5.86.
In the freshly harvested birch juice, a variety of proteins could be detected. Interestingly, even though the ethanol precipitated sample measured a higher protein concentration, less diversity and intensity of protein bands could be visualized compared to ammonium sulfate precipitated samples. This again showed how unreliable the BCA assay was to properly determine the protein concentration of birch juice.
Furthermore, we discovered that fresh birch juice could also be used without prior protein concentration.

After mass spectrometric analysis, several proteins were found in fresh birch sap: 1 member of LTP2-domain containing proteins, 38 peroxidases, 1 aspartyl protease, 1 basic secretory protease and 6 thaumatin-like proteins were identified. However, no Bet v 1 could be detected in fresh birch juice. Moreover, the mass spectrometric analysis of the birch juice did not detect so far known birch pollen allergens except for thaumatin-like proteins.
Due to the detection of a variety of proteins, fresh birch sap was considered the best sample to continue with immunological assays.

With the antibodies BIP-1 and anti-IgE, binding of allergic donor's IgE to Bet v 1 or other birch pollen allergens was investigated. Neither of the antibodies could detect IgE binding to allergens.

To further test the potential allergenicity, BAT assays with cells from 4 birch pollen allergic donors were performed. Two donors showed a low number of activated basophils (<30%) when challenged with birch juice only. When rBet v 1 was reconstituted in fresh birch juice, the number of activated basophils went up for all donors.

Recombinant Bet v 1, reconstituted in heat or protease inhibitor treated birch sap, showed a higher number of activated basophils compared to rBet v 1 in untreated birch sap.

Nonetheless, the number of activated basophils was around 4-fold lower than when reconstituting rBet v 1 in PBS. This showed the loss of allergenicity of rBet v 1 upon reconstitution in birch sap.

***Stability of rBet v 1 in fresh birch juice***

After mass spectrometric analysis, no Bet v 1 but several proteases were found in birch juice. Recombinant Bet v 1 was reconstituted in birch sap to analyze whether it gets proteolytically degraded or not.

Since it is physiochemically and immunologically identical to the natural protein, recombinant Bet v 1 was used for this experiment. Furthermore, the binding capacity to anti- Bet v 1 and IgE antibodies are the same. Recombinant Bet v 1 can be manufactured in bulk with no batch-to-batch variability.[27,74]

When fresh birch sap was heat treated prior to reconstitution of rBet v 1, an enhanced IgE reactivity could be visualized in various blots. The heat treatment possibly led to degradation of the contained proteases and thereby improved the stability of rBet v 1.

Furthermore, a change of pH value of the milieu of rBet v 1 was tested and found to not affect the stability of the allergen.

***Transepithelial transport of Bet v 1***

Birch sap is known to support gastrointestinal uptake of nutrients. To address this, a transepithelial approach using Caco2 cells was established. This cell line expresses morphological and biochemical characteristics of enterocytes lining the small intestine.

With a neutral red uptake assay, fresh birch juice was confirmed not to be harmful to the established cell layer.

FITC labelled rBet v 1 in medium, pure or heat-treated birch juice as well as a medium control were applied to the Caco2 cell layers. TEER, phenol red absorption and FITC absorbance were measured to evaluate the effects of the different media applied. The transport of rBet v 1 across the monolayer was similar when reconstituted in medium, pure or heated birch sap.
This showed that the cell layer was not harmed by birch juice application and the sap is therefore safe to be ingested. Nonetheless, no enhanced intestinal absorption of rBet v 1 by birch juice could be proven and thereby birch juice was not considered to increase epithelial transport rates of rBet v 1.

Since no so far known allergens could be detected in any of the investigated birch juices, it was concluded that no allergic reaction will be triggered when consumed by birch-pollen allergic individuals.

# Appendix

## Abbreviations

| | |
|---|---|
| Aqua (bi)dest. | (Bi)-distilled water |
| BS | Birch juice/sap |
| BSdm | Birch juice dm |
| BSF | Freshly harvested birch juice |
| BSFh | Freshly harvested birch juice, heated |
| BSV | Birch juice Vita 7 |
| BSA | Bovine serum albumin |
| CLR | C-type lectin receptors |
| DAMP | Danger associated molecular pattern |
| EDTA | Ethylenediaminetetraacetic acid |
| EtOH | Ethanol |
| Ig | Immunoglobulin |
| IL | Interleukin |
| kDa | kilodalton |
| Na | Sodium |
| $NH_4$ | Ammonium |
| NLR | nucleotide binding domain/leucine-rich repeat receptors |
| MHC | Major histocompatibility complex |
| o/n | overnight |
| PAGE | Polyacrylamide gel electrophoresis |
| PAMP | Pathogen associated molecular pattern |
| PBS | Phosphate buffered saline |
| PRR | Pattern recognition receptors |
| RT | Room temperature |
| SDS | Sodium dodecyl sulfate |
| TCA | Trichloroacetic acid |
| TCR | T-cell receptor |
| TLR | Toll-like receptor |

## Media and Buffers

**10x PBS**

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $Na_2HPO_4$ | 14.4 g |
| $KH_2PO_4$ | 2.4 g |

ad 1L Aqua bidest.

pH in 1x PBS= 7.4 (adjust using NaOH or HCl)

**Dialysis**

***Bicarbonate/EDTA buffer***

| | |
|---|---|
| $NaHCO_3$ | 33.604 g |
| $Na_2EDTA$ | 14.8896 g |

ad Aqua bidest. 4 L

**SDS PAGE**

***10x running buffer***

| | |
|---|---|
| Tris base | 30.0 g |
| Glycine | 144.0 g |
| SDS | 10.0 g |

Dissolve ingredients in 1 L of $H_2O$. The pH should be 8.3.

Store running buffer at RT and dilute to 1x before use.

***4x Sample loading buffer***

| | |
|---|---|
| 1M Tris-HCl | 2.0 mL |
| SDS | 0.8 g |
| 100% glycerol | 4.0 mL |
| 14.7M ß-mercaptoethanol | 0.4 mL |
| 0.5M EDTA | 1.0 mL |
| Bromophenol Blue | 8 mg |

Dissolve ingredients and adjust to 10 mL using $H_2O$.

Store at -20°C until further use and dilute before usage.

**Buffers for gel preparation**

***Upper buffer***

| | |
|---|---|
| Tris/HCl 6.8 | 0.5 M |
| Tris | 30.3 g |
| 10% SDS | 20 mL |

***Lower buffer***

| | |
|---|---|
| Tris/HCl pH 8.8 | 1.5 M |
| Tris | 90.85 g |
| 10% SDS | 20 mL |

**Silver stain solutions**

***Solution 1***

| | |
|---|---|
| 20% TCA | 20.8 mL |
| 37% Formaldehyde | 208.3 µL |

ad 500 mL with 50% Acetone

***Solution 2***

50% Acetone

***Solution 3***

10% Sodium thiosulfate pentahydrate 830 µL

ad 500 ml with Aqua dest.

***Solution 4***

| | |
|---|---|
| 37% Formaldehyde | 4.8 mL |

ad 500 mL Aqua dest.

→ take out 25 mL and freshly add 0.33 mL 20% Silver Nitrate Solution per gel

***20% Silver Nitrate Solution***

0.2 g Silver Nitrate ($AgNO_3$) → weighed in and fresh solution 4 prepared every time a gel was stained

ad 1 mL Aqua dest.

***Solution 5***

| | |
|---|---|
| 10% Sodium thiosulfate pentahydrate | 208.3 µL |
| 37% Formaldehyde | 208.3µL |
| Sodium Carbonate ($Na_2CO_3$) | 10 g |

ad 500 mL with Aqua dest.

***Stop Solution***

2% Acetic Acid

**Western Blot**

***Transfer buffer***

| | |
|---|---|
| Glycine (0.2 M) | 36.0 g |
| Tris Base (25 mM) | 7.57 g |
| Methanol | 500 mL |

ad 2.5 L with Aqua dest.

**Immunoblot**

***Gold buffer***

| | |
|---|---|
| $Na_2HPO_4$* $2H_2O$ | 37.5g |
| $NaH_2PO_4$*$2H_2O$ | 5g |
| BSA | 25 g |
| Sodium acide | 2.5 g |
| Tween | 25 mL |

adjust pH to 7.5 with $H_3PO_4$, fill to 5L with $H_2O$.

***10x TBS***

| | |
|---|---|
| 24.2 g | Tris |
| 80 g | NaCl |

Fill up to 1L with $H_2O$, adjust pH to 7.6 with HCl.

**Coomassie Brilliant Blue staining for Mass Spectrometry**

***Fixing Solution***

46% MeOH

7% HAc

***Staining Solution***

46% MeOH

7%HAc

0.1% Coomassie Brilliant Blue R-250

Filter this solution before use.

***Destaining Solution***

5% MeOH

7.5% HAc

***Storage Solution***

2% HAc

**Glycoprotein Gel stain**

***Pro-Q-Emerald 300 Stock solution***

Add 6 mL DMSO to the vial containing Pro-Q-Emerald 300 reagent and mix gently to dissolve the components. Store the stock solution at -20°C.

***Fix Solution***

50% Methanol

5% Acetic acid

fill up with $dH_2O$

200 mL fix solution per gel required

***Wash Solution***

3% glacial acetic acid

fill up with $dH_2O$

1L of wash solution per gel required

***Oxidizing solution***

Take the provided bottle with periodic acid and add 250 mL of 3% acetic acid and mix until completely dissolved.

**Basophil Activation Test**

***Incubation buffer: Hepes Calcium Buffer (HCB)***

| | |
|---|---|
| Hepes | 20 mM |
| NaCl | 133 mM |
| KCl | 5 mM |
| $CaCl_2$ | 7 mM |
| $MgCl_2$ | 3.5 mM |
| BSA | 1 mg/mL |

pH 7.4

IL-3 (2 ng/mL) add shortly before use

***Stop buffer: Hepes/EDTA buffer***

| | |
|---|---|
| Hepes | 20 mM |
| NaCl | 133 mM |
| KCl | 5 mM |
| EDTA | 20 mM |

pH 7.4

***Erythrocyte lysis buffer 10x concentrated***

| | |
|---|---|
| $NH_4Cl$ | 1.5 M |
| $KHCO_3$ | 100 mM |
| EDTA | 1 mM |

pH 7.3

Store at 4°C, dilute shortly before using

***FACS buffer***

PBS

0.1% BSA

0.1% $NaN_3$

***Antibody dilution: 20µL per sample***

| | |
|---|---|
| FACS buffer | 17.2 µL |
| anti-CD63-PE | 0.1 µL |
| anti-CD123-FITC | 1 µL |
| anti-CCR3-APC | 1.5 µL |

**Caco2 cell line**

***Medium***

DMEM High Glucose

| | |
|---|---|
| FCS | 15% |
| HEPES | 15mM |
| Genta | 0.2% |

***Neutral Red (NR) Stock Solution (3.3mg)***

3.3mg NR dye powder

dissolve in 100 mL $H_2O$

This stock is stored in the dark at RT for up to two months.

To obtain NR medium, the 1mL of stock solution is diluted in 99mL of Routine Culture Medium (37°C) to reach a final concentration of 33µg NR dye/mL

***Neutral red (4mg)***

4mg NR dye powder

Dissolve in 100 mL $H_2O$.

This stock is stored in the dark at RT for up to two months.

To obtain NR medium, the 1mL of stock solution is diluted in 99mL of Routine Culture Medium (37°C) to reach a final concentration of 40µg NR dye/mL

## Proteomics

| *Accession Nr.* | -10lgP | Peptides | Unique | Avg. Mass | Description |
|---|---|---|---|---|---|
| ***Acidic class III chitinase (n=1)*** | | | | | |
| *I3RVA1\|I3RVA1_9ROSI* | 92.77 | 4 | 2 | 30787 | Acidic class III chitinase OS=Citrullus colocynthis OX=252529 PE=3 SV=1 |
| ***Acidic endochitinase (n=2)*** | | | | | |
| *A0A392QTU5\|A0A392QTU5_9FABA* | 64.68 | 2 | 1 | 16386 | Acidic endochitinase (Fragment) OS=Trifolium medium OX=97028 PE=4 SV=1 |
| *A0A151UD79\|A0A151UD79_CAJCA* | 65.55 | 2 | 1 | 26308 | Acidic endochitinase OS=Cajanus cajan OX=3821 GN=KK1_021466 PE=4 SV=1 |
| ***Alpha-amylase isozyme 2A (n=1)*** | | | | | |
| *A0A2G2VBX5\|A0A2G2VBX5_CAPBA* | 77.66 | 4 | 2 | 41003 | Alpha-amylase isozyme 2A OS=Capsicum baccatum OX=33114 GN=CQW23_29959 PE=4 SV=1 |
| ***Alpha-amylase (n=5)*** | | | | | |
| *A0A059BSW8\|A0A059BSW8_EUCGR* | 70.98 | 2 | 1 | 47601 | Alpha-amylase OS=Eucalyptus grandis OX=71139 GN=EUGRSUZ_F02347 PE=3 SV=1 |
| *A0A067KBX0\|A0A067KBX0_JATCU* | 79.24 | 3 | 2 | 44690 | Alpha-amylase OS=Jatropha curcas OX=180498 GN=JCGZ_18655 PE=3 SV=1 |
| *A0A200QJ61\|A0A200QJ61_9MAGN* | 61.6 | 2 | 2 | 38932 | Alpha-amylase OS=Macleaya cordata OX=56857 GN=BVC80_8983g49 PE=3 SV=1 |
| *Q8LJQ6\|Q8LJQ6_MUSAC* | 67.73 | 2 | 1 | 46798 | Alpha-amylase OS=Musa acuminata OX=4641 PE=2 SV=1 |
| *A0A438FMB4\|A0A438FMB4_VITVI* | 79.25 | 3 | 2 | 43141 | Alpha-amylase OS=Vitis vinifera OX=29760 GN=AMY1.1_1 PE=4 SV=1 |
| ***Alpha-galactosidase (n=2)*** | | | | | |
| *A0A1J7H3I1\|A0A1J7H3I1_LUPAN* | 99.55 | 4 | 2 | 118728 | Alpha-galactosidase OS=Lupinus angustifolius OX=3871 GN=TanjilG_19281 PE=3 SV=1 |
| *A0A2R6Q087\|A0A2R6Q087_ACTCH* | 99.01 | 4 | 2 | 101637 | Alpha-glucosidase OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc23878 PE=3 SV=1 |
| ***Alpha-mannosidase (n=6)*** | | | | | |
| *A0A151SJN7\|A0A151SJN7_CAJCA* | 68.02 | 2 | 2 | 109087 | Alpha-mannosidase OS=Cajanus cajan OX=3821 GN=KK1_001226 PE=3 SV=1 |

| | | | | | |
|---|---|---|---|---|---|
| A0A1Q3AUV8\|A0A1Q3AUV8_CEPFO | 60.64 | 2 | 1 | 115849 | Alpha-mannosidase OS=Cephalotus follicularis OX=3775 GN=CFOL_v3_03042 PE=3 SV=1 |
| A0A2C9VQB3\|A0A2C9VQB3_MANES | 62.72 | 2 | 1 | 113520 | Alpha-mannosidase OS=Manihot esculenta OX=3983 GN=MANES_06G126300 PE=3 SV=1 |
| G7LB83\|G7LB83_MEDTR | 73.82 | 3 | 2 | 114189 | Alpha-mannosidase OS=Medicago truncatula OX=3880 GN=MTR_8g075330 PE=3 SV=1 |
| A0A251Q9K0\|A0A251Q9K0_PRUPE | 60.97 | 2 | 1 | 79748 | Alpha-mannosidase OS=Prunus persica OX=3760 GN=PRUPE_3G313300 PE=3 SV=1 |
| A0A218WFX8\|A0A218WFX8_PUNGR | 64.74 | 2 | 1 | 101627 | Alpha-mannosidase OS=Punica granatum OX=22663 GN=CDL15_Pgr005576 PE=3 SV=1 |
| ***Aspartic proteinase Asp1 (n=1)*** | | | | | |
| A0A314Y7J0\|A0A314Y7J0_PRUYE | 66.03 | 2 | 1 | 43726 | Aspartic proteinase Asp1 OS=Prunus yedoensis var. nudiflora OX=2094558 GN=Pyn_34438 PE=3 SV=1 |
| A0A2I4HQQ6\|A0A2I4HQQ6_JUGRE | 68.35 | 2 | 2 | 46474 | aspartic proteinase Asp1-like isoform X2 OS=Juglans regia OX=51240 GN=LOC109020466 PE=3 SV=1 |
| ***Aspartic proteinase nepentesin-1 (n=1)*** | | | | | |
| W9R8P6\|W9R8P6_9ROSA | 64.69 | 2 | 1 | 46433 | Aspartic proteinase nepenthesin-1 OS=Morus notabilis OX=981085 GN=L484_023982 PE=3 SV=1 |
| ***Aspartyl protease AED3-like (n=2)*** | | | | | |
| A0A1S2YWI2\|A0A1S2YWI2_CICAR | 68.81 | 2 | 1 | 46787 | aspartyl protease AED3-like OS=Cicer arietinum OX=3827 GN=LOC101491094 PE=3 SV=1 |
| A0A2I4EFL0\|A0A2I4EFL0_JUGRE | 125.5 | 7 | 4 | 47138 | aspartyl protease AED3-like OS=Juglans regia OX=51240 GN=LOC108989124 PE=3 SV=1 |
| ***B-1,3-glucanase (n=1)*** | | | | | |
| Q9LKN3\|Q9LKN3_CASSA | 88.17 | 3 | 3 | 17681 | B-1,3-glucanase (Fragment) OS=Castanea sativa OX=21020 PE=2 SV=1 |
| ***Basic blue copper protein (n=1)*** | | | | | |
| Q9ZRV5\|Q9ZRV5_CICAR | 61.6 | 2 | 1 | 12928 | Basic blue copper protein OS=Cicer arietinum OX=3827 GN=plantacyanin PE=2 SV=1 |
| ***Basic blue protein-like (n=2)*** | | | | | |
| A0A2I4E077\|A0A2I4E077_JUGRE | 74.21 | 2 | 2 | 13466 | basic blue protein-like OS=Juglans regia OX=51240 GN=LOC108985094 PE=4 SV=1 |
| A0A2I4GDW2\|A0A2I4GDW2_JUGRE | 89.65 | 4 | 3 | 12992 | basic blue protein-like OS=Juglans regia OX=51240 GN=LOC109007045 PE=4 SV=1 |
| ***Beta-galactosidase (n=2)*** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| A0A1U8JJH0\|A0A1U8JJH0_GOSHI | 61.49 | 1 | 1 | 82130 | Beta-galactosidase OS=Gossypium hirsutum OX=3635 GN=LOC107906099 PE=3 SV=1 |
| A0A0E0GJW0\|A0A0E0GJW0_ORYNI | 86.99 | 4 | 2 | 92385 | Beta-galactosidase OS=Oryza nivara OX=4536 PE=3 SV=1 |
| ***Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain (n=1)*** | | | | | |
| A0A200R4V6\|A0A200R4V6_9MAGN | 129.1 | 7 | 5 | 12668 | Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain OS=Macleaya cordata OX=56857 GN=BVC80_1835g129 PE=4 SV=1 |
| ***Canabidiolic acid synthase-like 1 (n=1)*** | | | | | |
| A0A1U7ZD10\|A0A1U7ZD10_NELNU | 86.21 | 2 | 2 | 60196 | cannabidiolic acid synthase-like 1 OS=Nelumbo nucifera OX=4432 GN=LOC104592826 PE=3 SV=1 |
| ***Carboxypeptidase (n=3)*** | | | | | |
| A0A445LAP6\|A0A445LAP6_GLYSO | 72.07 | 2 | 1 | 51548 | Carboxypeptidase OS=Glycine soja OX=3848 GN=D0Y65_006983 PE=3 SV=1 |
| A0A251N635\|A0A251N635_PRUPE | 62 | 1 | 1 | 58799 | Carboxypeptidase OS=Prunus persica OX=3760 GN=PRUPE_7G030500 PE=3 SV=1 |
| A0A2K3MW63\|A0A2K3MW63_TRIPR | 74.3 | 2 | 1 | 48128 | Carboxypeptidase OS=Trifolium pratense OX=57577 GN=L195_g018217 PE=3 SV=1 |
| ***Chitinase (n=1)*** | | | | | |
| Q42428\|Q42428_CASSA | 86.7 | 3 | 2 | 33946 | Chitinase Ib OS=Castanea sativa OX=21020 PE=2 SV=1 |
| ***CO(2)-response secreted protease (n=1)*** | | | | | |
| A0A371FE51\|A0A371FE51_MUCPR | 112.9 | 7 | 5 | 161387 | CO(2)-response secreted protease (Fragment) OS=Mucuna pruriens OX=157652 GN=CRSP PE=4 SV=1 |
| ***CO(2)-response secreted protease-like (n=1)*** | | | | | |
| A0A445KUT2\|A0A445KUT2_GLYSO | 107.7 | 7 | 4 | 201577 | CO(2)-response secreted protease isoform C OS=Glycine soja OX=3848 GN=D0Y65_008541 PE=4 SV=1 |
| ***CO(2)-response secreted protease-like (n=1)*** | | | | | |
| A0A2I4GSE3\|A0A2I4GSE3_JUGRE | 167.8 | 14 | 10 | 83990 | CO(2)-response secreted protease-like OS=Juglans regia OX=51240 GN=LOC109010434 PE=4 SV=1 |
| ***DUF26 domain-containing protein 2 (n=2)*** | | | | | |
| B9T5Q2\|B9T5Q2_RICCO | 83.67 | 4 | 3 | 27004 | DUF26 domain-containing protein 2, putative OS=Ricinus communis OX=3988 GN=RCOM_0366110 PE=4 SV=1 |
| ***Endochitinase (n=1)*** | | | | | |
| W9RHR6\|W9RHR6_9ROSA | 62.1 | 2 | 2 | 30331 | Endochitinase OS=Morus notabilis OX=981085 GN=L484_018124 PE=4 SV=1 |
| ***Endoglucanase 4 (n=1)*** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| A0A3G3C2Q5\|A0A3G3C2Q5_ACTDE | 70.09 | 2 | 2 | 37754 | Endoglucanase 4 OS=Actinidia deliciosa OX=3627 GN=EG4 PE=2 SV=1 |
| ***Endoglucanase (n=1)*** | | | | | |
| A0A2R6REV0\|A0A2R6REV0_ACTCH | 73.24 | 2 | 1 | 62545 | Endoglucanase OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc03362 PE=3 SV=1 |
| ***Expansin-like protein A2 (n=1)*** | | | | | |
| A0A3S4PMF2\|A0A3S4PMF2_9MAGN | 73.85 | 2 | 1 | 28364 | Expansin-like protein A2 OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_02162100 PE=3 SV=1 |
| ***Galactose mutarotase-like domain containing protein (n=2)*** | | | | | |
| A0A3S3NB60\|A0A3S3NB60_9MAGN | 84.56 | 4 | 2 | 192586 | Galactose mutarotase-like domain-containing protein OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_01774700 PE=4 SV=1 |
| A0A103Y5I0\|A0A103Y5I0_CYNCS | 84.3 | 2 | 1 | 93676 | Galactose mutarotase-like domain-containing protein OS=Cynara cardunculus var. scolymus OX=59895 GN=Ccrd_018800 PE=4 SV=1 |
| ***Germin-like protein 2 (n=1)*** | | | | | |
| sp\|P85352\|GLP2_BETPN | 75.19 | 2 | 2 | 1606 | Germin-like protein 2 (Fragment) OS=Betula pendula OX=3505 PE=1 SV=1 |
| ***germin-like protein subfamily 2 member 1 (n=1)*** | | | | | |
| A0A2I4G258\|A0A2I4G258_JUGRE | 67.59 | 3 | 2 | 22698 | germin-like protein subfamily 2 member 1 OS=Juglans regia OX=51240 GN=LOC109004037 PE=3 SV=1 |
| ***glucan 1,3-beta-glucosidase A-like (n=1)*** | | | | | |
| A0A2I4H3U8\|A0A2I4H3U8_JUGRE | 158 | 11 | 7 | 56043 | glucan 1,3-beta-glucosidase A-like OS=Juglans regia OX=51240 GN=LOC109013245 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-D-glucosidase (n=1)*** | | | | | |
| A0A3S3M9G7\|A0A3S3M9G7_9MAGN | 67.97 | 2 | 1 | 49112 | Glucan endo-1,3-beta-D-glucosidase OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_00058100 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-glucosidase 4-like (n=1)*** | | | | | |
| A0A2I4FSD4\|A0A2I4FSD4_JUGRE | 68.75 | 3 | 2 | 53715 | glucan endo-1,3-beta-glucosidase 4-like OS=Juglans regia OX=51240 GN=LOC109001653 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-glucosidase 7 (n=1)*** | | | | | |
| A0A2I0VL61\|A0A2I0VL61_9ASPA | 62.17 | 2 | 1 | 45670 | Glucan endo-1,3-beta-glucosidase 7 OS=Dendrobium catenatum OX=906689 GN=MA16_Dca025763 PE=3 SV=1 |
| ***glucan endo-1,3-beta-glucosidase 7-like (n=1)*** | | | | | |
| A0A1S4C4C9\|A0A1S4C4C9_TOBAC | 64.04 | 2 | 1 | 49508 | glucan endo-1,3-beta-glucosidase 7-like OS=Nicotiana tabacum OX=4097 GN=LOC107814940 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-glucosidase (n=1)*** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| A0A2R6QXV7\|A0A2R6QXV7_ACTCH | 80.76 | 2 | 1 | 49854 | Glucan endo-1,3-beta-glucosidase OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc11993 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-glucosidase (n=2)*** | | | | | |
| W9R6M6\|W9R6M6_9ROSA | 75.41 | 2 | 2 | 38274 | Glucan endo-1,3-beta-glucosidase, basic isoform OS=Morus notabilis OX=981085 GN=L484_016574 PE=3 SV=1 |
| B9T6M9\|B9T6M9_RICCO | 149.8 | 7 | 5 | 28816 | Glucan endo-1,3-beta-glucosidase, basic isoform, putative OS=Ricinus communis OX=3988 GN=RCOM_1277490 PE=3 SV=1 |
| ***glucan endo-1,3-beta-glucosidase, basic isoform-like (n=1)*** | | | | | |
| A0A2I4G6V0\|A0A2I4G6V0_JUGRE | 70.74 | 2 | 1 | 37543 | glucan endo-1,3-beta-glucosidase, basic isoform-like OS=Juglans regia OX=51240 GN=LOC109005246 PE=3 SV=1 |
| ***Glucan endo-1,3-beta-glucosidase-like protein (n=2)*** | | | | | |
| G7JG52\|G7JG52_MEDTR | 80.06 | 2 | 1 | 49372 | Glucan endo-1,3-beta-glucosidase-like protein OS=Medicago truncatula OX=3880 GN=11438652 PE=3 SV=1 |
| A0A072VE71\|A0A072VE71_MEDTR | 77.76 | 2 | 1 | 44689 | Glucan endo-1,3-beta-glucosidase-like protein OS=Medicago truncatula OX=3880 GN=25482127 PE=3 SV=1 |
| ***glycerophosphodiester phsphodiesterase GDPDL1-like (n=1)*** | | | | | |
| A0A2I4DZF1\|A0A2I4DZF1_JUGRE | 66.89 | 2 | 1 | 74547 | glycerophosphodiester phosphodiesterase GDPDL1-like OS=Juglans regia OX=51240 GN=LOC108984882 PE=4 SV=1 |
| ***Glyco_hydro_17 domain-containing protein/X8 domain-containing protein (n=1)*** | | | | | |
| A0A1Q3B6L1\|A0A1Q3B6L1_CEPFO | 71.24 | 2 | 2 | 53848 | Glyco_hydro_17 domain-containing protein/X8 domain-containing protein OS=Cephalotus follicularis OX=3775 GN=CFOL_v3_07156 PE=3 SV=1 |
| ***Glycoside hydrolase family 5 protein (n=1)*** | | | | | |
| G7IYK2\|G7IYK2_MEDTR | 67.14 | 2 | 1 | 56160 | Glycoside hydrolase family 5 protein OS=Medicago truncatula OX=3880 GN=11422986 PE=3 SV=1 |
| ***Glycoside hydrolase (n=2)*** | | | | | |
| A0A443N381\|A0A443N381_9MAGN | 65.61 | 2 | 2 | 43573 | Glycoside hydrolase OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_00123600 PE=4 SV=1 |
| A0A2P5ABK4\|A0A2P5ABK4_PARAD | 110.7 | 4 | 2 | 50410 | Glycoside hydrolase OS=Parasponia andersonii OX=3476 GN=PanWU01x14_348570 PE=3 SV=1 |
| ***Glycoside hydrolase, catalytic domain-containing protein (n=1)*** | | | | | |
| A0A103Y291\|A0A103Y291_CYNCS | 68.95 | 2 | 1 | 49279 | Glycoside hydrolase, catalytic domain-containing protein OS=Cynara cardunculus var. scolymus OX=59895 GN=Ccrd_020513 PE=3 SV=1 |
| ***heparanase-like protein 1 (n=1)*** | | | | | |
| A0A2I4FW47\|A0A2I4FW47_JUGRE | 85 | 2 | 2 | 54461 | heparanase-like protein 1 OS=Juglans regia OX=51240 GN=LOC109002527 PE=4 SV=1 |

| | | | | | |
|---|---|---|---|---|---|
| ***heparanase-like protein 3 isoform X3 (n=1)*** | | | | | |
| A0A2I4G7S1\|A0A2I4G7S1_JUGRE | 64.16 | 3 | 3 | 59376 | heparanase-like protein 3 isoform X3 OS=Juglans regia OX=51240 GN=LOC109005442 PE=4 SV=1 |
| ***Hevamine-A, putative (n=1)*** | | | | | |
| B9SY59\|B9SY59_RICCO | 170.9 | 9 | 2 | 31484 | Hevamine-A, putative OS=Ricinus communis OX=3988 GN=RCOM_0481320 PE=3 SV=1 |
| ***Hevamine A-like (n=4)*** | | | | | |
| A0A2I4ECP0\|A0A2I4ECP0_JUGRE | 125.2 | 8 | 4 | 32226 | hevamine-A-like OS=Juglans regia OX=51240 GN=LOC108988371 PE=4 SV=1 |
| A0A2I4FWR3\|A0A2I4FWR3_JUGRE | 172.6 | 9 | 0 | 31817 | hevamine-A-like OS=Juglans regia OX=51240 GN=LOC109002689 PE=3 SV=1 |
| A0A2I4FWQ7\|A0A2I4FWQ7_JUGRE | 75.45 | 3 | 2 | 32095 | hevamine-A-like OS=Juglans regia OX=51240 GN=LOC109002690 PE=3 SV=1 |
| A0A2I4HTP0\|A0A2I4HTP0_JUGRE | 83.13 | 3 | 2 | 33014 | hevamine-A-like OS=Juglans regia OX=51240 GN=LOC109021353 PE=3 SV=1 |
| ***Laccase 3 (n=1)*** | | | | | |
| A0A2U1NTV5\|A0A2U1NTV5_ARTAN | 71.6 | 2 | 2 | 17161 | Laccase 3 OS=Artemisia annua OX=35608 GN=CTI12_AA227560 PE=4 SV=1 |
| ***Laccase (n=4)*** | | | | | |
| A0A0B2P696\|A0A0B2P696_GLYSO | 75.61 | 2 | 1 | 61147 | Laccase OS=Glycine soja OX=3848 GN=D0Y65_037036 PE=3 SV=1 |
| A0A067KJC4\|A0A067KJC4_JATCU | 81.4 | 4 | 2 | 63736 | Laccase OS=Jatropha curcas OX=180498 GN=JCGZ_11126 PE=3 SV=1 |
| I1R5C0\|I1R5C0_ORYGL | 62.61 | 2 | 1 | 63492 | Laccase OS=Oryza glaberrima OX=4538 PE=3 SV=1 |
| A0A2K1YGR3\|A0A2K1YGR3_POPTR | 66.17 | 2 | 1 | 63553 | Laccase OS=Populus trichocarpa OX=3694 GN=POPTR_011G071100 PE=3 SV=2 |
| ***L-ascorbate oxidase like (n=1)*** | | | | | |
| A0A2H9ZQQ9\|A0A2H9ZQQ9_9ASPA | 62.3 | 2 | 1 | 60708 | L-ascorbate oxidase like OS=Apostasia shenzhenica OX=1088818 GN=Bp10 PE=4 SV=1 |
| ***leucine-rich repeat receptor-like protein kinase PXC2 (n=1)*** | | | | | |
| A0A2I4FRC3\|A0A2I4FRC3_JUGRE | 85.73 | 4 | 3 | 52752 | leucine-rich repeat receptor-like protein kinase PXC2 OS=Juglans regia OX=51240 GN=LOC109001404 PE=4 SV=1 |
| ***Lipid transfer protein (n=1)*** | | | | | |
| G7L5L7\|G7L5L7_MEDTR | 64.11 | 2 | 2 | 12210 | Lipid transfer protein OS=Medicago truncatula OX=3880 GN=11407869 PE=2 SV=1 |
| ***LTP_2 domain containing protein (n=1)*** | | | | | |

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|---|---|---|---|---|---|
| A0A1Q3AM55\|A0A1Q3AM55_CEPFO | 127.5 | 11 | 5 | 12337 | LTP_2 domain-containing protein (Fragment) OS=Cephalotus follicularis OX=3775 GN=CFOL_v3_00373 PE=4 SV=1 |
| ***Matrix metalloproteinase-9 (n=1)*** | | | | | |
| A0A0K9PY38\|A0A0K9PY38_ZOSMR | 106.9 | 5 | 5 | 34240 | Matrix metalloproteinase-9 OS=Zostera marina OX=29655 GN=ZOSMA_153G00140 PE=3 SV=1 |
| ***Mavicyanin-like (n=2)*** | | | | | |
| A0A2K3M5Z8\|A0A2K3M5Z8_TRIPR | 63.68 | 2 | 1 | 13606 | Mavicyanin-like (Fragment) OS=Trifolium pratense OX=57577 GN=L195_g042276 PE=4 SV=1 |
| A0A2I4G993\|A0A2I4G993_JUGRE | 67.18 | 2 | 1 | 19523 | mavicyanin-like OS=Juglans regia OX=51240 GN=LOC109005859 PE=4 SV=1 |
| ***Metalloendoproteinase 2-MMP (n=1)*** | | | | | |
| A0A371GJD2\|A0A371GJD2_MUCPR | 75.8 | 3 | 2 | 42022 | Metalloendoproteinase 2-MMP (Fragment) OS=Mucuna pruriens OX=157652 GN=2MMP PE=3 SV=1 |
| ***metalloendoproteinase 3-MMP-like (n=1)*** | | | | | |
| A0A2I4DLH9\|A0A2I4DLH9_JUGRE | 69.57 | 3 | 2 | 34531 | metalloendoproteinase 3-MMP-like OS=Juglans regia OX=51240 GN=LOC108981328 PE=3 SV=1 |
| ***Metalloendoproteinase (n=1)*** | | | | | |
| A0A2R6RUN1\|A0A2R6RUN1_ACTCH | 101.7 | 5 | 4 | 35463 | Metalloendoproteinase OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc04138 PE=3 SV=1 |
| ***Metallopeptidase, catalytic domain-containing protein (n=2)*** | | | | | |
| A0A118JVA5\|A0A118JVA5_CYNCS | 70.8 | 1 | 1 | 33911 | Metallopeptidase, catalytic domain-containing protein OS=Cynara cardunculus var. scolymus OX=59895 GN=Ccrd_005189 PE=3 SV=1 |
| A0A118DD10\|A0A118DD10_CYNCS | 137 | 11 | 11 | 30481 | Metallopeptidase, catalytic domain-containing protein OS=Cynara cardunculus var. scolymus OX=59895 GN=Ccrd_024319 PE=3 SV=1 |
| ***Non-specific lipid-transfer protein (n=1)*** | | | | | |
| M5W0S9\|M5W0S9_PRUPE | 64.17 | 1 | 1 | 12474 | Non-specific lipid-transfer protein OS=Prunus persica OX=3760 GN=PRUPE_6G293000 PE=3 SV=1 |
| ***Non-specific lipid-transfer protein-like protein (n=1)*** | | | | | |
| W9RLU9\|W9RLU9_9ROSA | 60.42 | 2 | 2 | 18113 | Non-specific lipid-transfer protein-like protein OS=Morus notabilis OX=981085 GN=L484_024132 PE=3 SV=1 |
| ***NtPRp27 (n=1)*** | | | | | |
| A0A443NE20\|A0A443NE20_9MAGN | 173.4 | 19 | 8 | 26779 | NtPRp27 OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_00521300 PE=4 SV=1 |
| ***NTPRp27-like protein (n=1)*** | | | | | |
| A0A0A0K4M0\|A0A0A0K4M0_CUCSA | 126.5 | 10 | 6 | 25225 | NtPRp27-like protein OS=Cucumis sativus OX=3659 GN=Csa_7G072810 PE=4 SV=1 |

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|---|---|---|---|---|---|
| ***Osmotin-like protein (n=3)*** | | | | | |
| *Q9SBT2\|Q9SBT2_FRAAN* | 104.2 | 4 | 3 | 24237 | Osmotin-like protein OS=Fragaria ananassa OX=3747 GN=olp PE=4 SV=1 |
| *Q2MJK2\|Q2MJK2_FRAAN* | 115.9 | 9 | 5 | 24569 | Osmotin-like protein OS=Fragaria ananassa OX=3747 GN=olp2 PE=4 SV=1 |
| *P93621\|P93621_VITVI* | 146 | 10 | 7 | 23875 | Osmotin-like protein OS=Vitis vinifera OX=29760 GN=OSM1 PE=2 SV=1 |
| ***Pathogenesis related protein-5 (n=3)*** | | | | | |
| *E5FPA0\|E5FPA0_POPTO* | 62.09 | 3 | 2 | 24608 | Pathogenesis related protein-5 OS=Populus tomentosa OX=118781 PE=2 SV=1 |
| *A0A0K1L9H1\|A0A0K1L9H1_ALLSA* | 84.43 | 4 | 2 | 23530 | Pathogenesis-related protein 5 OS=Allium sativum OX=4682 GN=PR5 PE=2 SV=1 |
| *C9E2I5\|C9E2I5_PANGI* | 188.9 | 24 | 12 | 27541 | Pathogenesis-related protein 5 OS=Panax ginseng OX=4054 GN=PR5 PE=2 SV=1 |
| ***Peptidase A1 (n=1)*** | | | | | |
| *A0A1R3J768\|A0A1R3J768_9ROSI* | 66.79 | 2 | 2 | 47262 | Peptidase A1 OS=Corchorus olitorius OX=93759 GN=COLO4_18985 PE=3 SV=1 |
| ***Peptidase M10, metallopeptidase (n=3)*** | | | | | |
| *A0A1R3GPA8\|A0A1R3GPA8_COCAP* | 128.8 | 5 | 3 | 34250 | Peptidase M10, metallopeptidase OS=Corchorus capsularis OX=210143 GN=CCACVL1_24541 PE=3 SV=1 |
| *A0A1R3GP75\|A0A1R3GP75_COCAP* | 104.5 | 5 | 5 | 34482 | Peptidase M10, metallopeptidase OS=Corchorus capsularis OX=210143 GN=CCACVL1_24542 PE=3 SV=1 |
| *A0A1R3J3H8\|A0A1R3J3H8_9ROSI* | 86.68 | 2 | 1 | 35635 | Peptidase M10, metallopeptidase OS=Corchorus olitorius OX=93759 GN=COLO4_19787 PE=3 SV=1 |
| ***Peptidase_S8 domain-containing protein/PA domain-containing protein/Inhibitor_I9 domain-containing protein (n=1)*** | | | | | |
| *A0A1Q3ALS8\|A0A1Q3ALS8_CEPFO* | 67.87 | 2 | 1 | 74424 | Peptidase_S8 domain-containing protein/PA domain-containing protein/Inhibitor_I9 domain-containing protein (Fragment) OS=Cephalotus follicularis OX=3775 GN=CFOL_v3_00249 PE=4 SV=1 |
| - | | | | | |
| *A0A1J6IB52\|A0A1J6IB52_NICAT* | 61.39 | 2 | 1 | 69745 | Peptide-n4-(N-acetyl-beta-glucosaminyl)asparagine amidase a OS=Nicotiana attenuata OX=49451 GN=PNAA_4 PE=4 SV=1 |
| ***Peroxidase (n=38)*** | | | | | |
| *A0A199VLG6\|A0A199VLG6_ANACO* | 99.43 | 5 | 4 | 38431 | Peroxidase (Fragment) OS=Ananas comosus OX=4615 GN=ACMD2_21775 PE=3 SV=1 |

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|---|---|---|---|---|---|
| W1NG99\|W1NG99_AMBTC | 60.54 | 2 | 1 | 35686 | Peroxidase OS=Amborella trichopoda OX=13333 GN=AMTR_s00010p00198480 PE=3 SV=1 |
| W1PQG6\|W1PQG6_AMBTC | 102.8 | 6 | 3 | 35532 | Peroxidase OS=Amborella trichopoda OX=13333 GN=AMTR_s00034p00235030 PE=3 SV=1 |
| A0A2G5DMC2\|A0A2G5DMC2_AQUCA | 66.51 | 3 | 2 | 35634 | Peroxidase OS=Aquilegia coerulea OX=218851 GN=AQUCO_01700327v1 PE=3 SV=1 |
| A0A0H4CPS8\|A0A0H4CPS8_BETPL | 212.9 | 32 | 24 | 34829 | Peroxidase OS=Betula platyphylla OX=78630 GN=POD10 PE=2 SV=1 |
| A0A0U2TQ78\|A0A0U2TQ78_BETPL | 73.89 | 3 | 3 | 34040 | Peroxidase OS=Betula platyphylla OX=78630 GN=POD11 PE=2 SV=1 |
| A0A0H4CNW3\|A0A0H4CNW3_BETPL | 219.1 | 30 | 25 | 33546 | Peroxidase OS=Betula platyphylla OX=78630 GN=POD3 PE=2 SV=1 |
| A0A0H4CRM9\|A0A0H4CRM9_BETPL | 147.3 | 9 | 7 | 33789 | Peroxidase OS=Betula platyphylla OX=78630 GN=POD7 PE=2 SV=1 |
| A0A0H4CNW8\|A0A0H4CNW8_BETPL | 156.7 | 11 | 9 | 29676 | Peroxidase OS=Betula platyphylla OX=78630 GN=POD8 PE=2 SV=1 |
| A0A1Q3D810\|A0A1Q3D810_CEPFO | 98.43 | 4 | 3 | 34752 | Peroxidase OS=Cephalotus follicularis OX=3775 GN=CFOL_v3_31875 PE=3 SV=1 |
| A0A3S5WGH6\|A0A3S5WGH6_9MAGN | 64.29 | 2 | 1 | 34694 | Peroxidase OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_00810400 PE=3 SV=1 |
| A0A443PN58\|A0A443PN58_9MAGN | 79.48 | 4 | 2 | 34689 | Peroxidase OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_02141600 PE=3 SV=1 |
| A0A068TUA6\|A0A068TUA6_COFCA | 60.07 | 2 | 1 | 29588 | Peroxidase OS=Coffea canephora OX=49390 GN=GSCOC_T00025901001 PE=3 SV=1 |
| I6XK01\|I6XK01_CORAV | 87.72 | 3 | 2 | 34625 | Peroxidase OS=Corylus avellana OX=13451 PE=2 SV=1 |
| A0A0A0KCF1\|A0A0A0KCF1_CUCSA | 62.32 | 2 | 1 | 33844 | Peroxidase OS=Cucumis sativus OX=3659 GN=Csa_6G216420 PE=3 SV=1 |
| A0A103YI01\|A0A103YI01_CYNCS | 81.39 | 3 | 2 | 34268 | Peroxidase OS=Cynara cardunculus var. scolymus OX=59895 GN=Ccrd_012179 PE=3 SV=1 |
| A0A2N9ISE3\|A0A2N9ISE3_FAGSY | 116.1 | 6 | 4 | 35508 | Peroxidase OS=Fagus sylvatica OX=28930 GN=FSB_LOCUS55157 PE=3 SV=1 |
| A0A2P5YUJ5\|A0A2P5YUJ5_GOSBA | 117.2 | 6 | 3 | 33168 | Peroxidase OS=Gossypium barbadense OX=3634 GN=GOBAR_AA01297 PE=3 SV=1 |
| A0A2I4HRC1\|A0A2I4HRC1_JUGRE | 67.3 | 2 | 1 | 37751 | Peroxidase OS=Juglans regia OX=51240 GN=LOC109020639 PE=3 SV=1 |
| A0A2I4HRI5\|A0A2I4HRI5_JUGRE | 65.45 | 2 | 1 | 35449 | Peroxidase OS=Juglans regia OX=51240 GN=LOC109020700 PE=3 SV=1 |
| A0A2J6K2F9\|A0A2J6K2F9_LACSA | 84.59 | 3 | 2 | 35572 | Peroxidase OS=Lactuca sativa OX=4236 GN=LSAT_1X68441 PE=3 SV=1 |
| I3S023\|I3S023_LOTJA | 105.3 | 7 | 4 | 36395 | Peroxidase OS=Lotus japonicus OX=34305 PE=2 SV=1 |

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|---|---|---|---|---|---|
| A0A2C9UBY7\|A0A2C9UBY7_MANES | 63.91 | 2 | 1 | 36094 | Peroxidase OS=Manihot esculenta OX=3983 GN=MANES_15G018400 PE=3 SV=1 |
| A0A371I0M4\|A0A371I0M4_MUCPR | 72.22 | 4 | 3 | 35550 | Peroxidase OS=Mucuna pruriens OX=157652 GN=HRPN PE=3 SV=1 |
| M0SEE4\|M0SEE4_MUSAM | 68.68 | 2 | 2 | 36193 | Peroxidase OS=Musa acuminata subsp. malaccensis OX=214687 GN=103978205 PE=3 SV=1 |
| M0TJQ8\|M0TJQ8_MUSAM | 63.35 | 2 | 1 | 30316 | Peroxidase OS=Musa acuminata subsp. malaccensis OX=214687 PE=3 SV=1 |
| A0A1U7VJ43\|A0A1U7VJ43_NICSY | 126.2 | 8 | 4 | 36143 | Peroxidase OS=Nicotiana sylvestris OX=4096 GN=LOC104214408 PE=3 SV=1 |
| A0A1J3GSY2\|A0A1J3GSY2_NOCCA | 65.67 | 2 | 1 | 36861 | Peroxidase OS=Noccaea caerulescens OX=107243 GN=LE_TR1968_c0_g1_i1_g.5216 PE=3 SV=1 |
| A0A2T7CFB3\|A0A2T7CFB3_9POAL | 78.62 | 3 | 2 | 34913 | Peroxidase OS=Panicum hallii var. hallii OX=1504633 GN=GQ55_9G551000 PE=3 SV=1 |
| V7BUP3\|V7BUP3_PHAVU | 60.42 | 1 | 1 | 34426 | Peroxidase OS=Phaseolus vulgaris OX=3885 GN=PHAVU_005G094200g PE=3 SV=1 |
| W8Q7S0\|W8Q7S0_POPTR | 76.66 | 2 | 1 | 35357 | Peroxidase OS=Populus trichocarpa OX=3694 GN=PRX24 PE=2 SV=1 |
| M5W1R7\|M5W1R7_PRUPE | 89.32 | 4 | 2 | 35831 | Peroxidase OS=Prunus persica OX=3760 GN=PRUPE_6G013800 PE=3 SV=1 |
| A0A2I0KYR8\|A0A2I0KYR8_PUNGR | 65.04 | 2 | 1 | 31429 | Peroxidase OS=Punica granatum OX=22663 GN=CRG98_005978 PE=3 SV=1 |
| A0A061DQB9\|A0A061DQB9_THECC | 84.71 | 3 | 2 | 39510 | Peroxidase OS=Theobroma cacao OX=3641 GN=TCM_004510 PE=3 SV=1 |
| A0A0S3RGH6\|A0A0S3RGH6_PHAAN | 63.6 | 2 | 1 | 35076 | Peroxidase OS=Vigna angularis var. angularis OX=157739 GN=Vigan.02G267500 PE=3 SV=1 |
| A0A0S3RLC2\|A0A0S3RLC2_PHAAN | 65.86 | 2 | 1 | 33773 | Peroxidase OS=Vigna angularis var. angularis OX=157739 GN=Vigan.03G110500 PE=3 SV=1 |
| A0A1S3UBP9\|A0A1S3UBP9_VIGRR | 90.16 | 4 | 2 | 34632 | Peroxidase OS=Vigna radiata var. radiata OX=3916 GN=LOC106763811 PE=3 SV=1 |
| ***Plant basic secretory protein (BSP) family protein (n=2)*** | | | | | |
| A0A061E155\|A0A061E155_THECC | 144 | 16 | 8 | 25548 | Plant basic secretory protein (BSP) family protein OS=Theobroma cacao OX=3641 GN=TCM_007153 PE=4 SV=1 |
| A0A061E214\|A0A061E214_THECC | 66.79 | 1 | 1 | 32190 | Plant basic secretory protein (BSP) family protein OS=Theobroma cacao OX=3641 GN=TCM_007157 PE=4 SV=1 |
| ***Plant basic secretory protein (BSP) family protein, putative (n=2)*** | | | | | |
| A0A061E211\|A0A061E211_THECC | 65.08 | 2 | 2 | 25479 | Plant basic secretory protein (BSP) family protein, putative OS=Theobroma cacao OX=3641 GN=TCM_007152 PE=4 SV=1 |

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|---|---|---|---|---|---|
| A0A061E7Y1\|A0A061E7Y1_THECC | 95.63 | 5 | 3 | 26267 | Plant basic secretory protein (BSP) family protein, putative OS=Theobroma cacao OX=3641 GN=TCM_007154 PE=4 SV=1 |
| ***Plasmodesmata callose-binding protein 3-like isoform (n=1)*** | | | | | |
| A0A1U8G5R4\|A0A1U8G5R4_CAPAN | 75.68 | 2 | 2 | 21225 | PLASMODESMATA CALLOSE-BINDING PROTEIN 3-like isoform X1 OS=Capsicum annuum OX=4072 GN=LOC107862333 PE=4 SV=1 |
| ***Plasmodesmata callose-binding protein 3-like (n=1)*** | | | | | |
| A0A1S3BDH0\|A0A1S3BDH0_CUCME | 68.22 | 2 | 2 | 20850 | PLASMODESMATA CALLOSE-BINDING PROTEIN 3-like OS=Cucumis melo OX=3656 GN=LOC103488471 PE=4 SV=1 |
| ***Polygalacturonase, putative isoform 2 (n=1)*** | | | | | |
| A0A061DSW1\|A0A061DSW1_THECC | 113.8 | 3 | 1 | 52341 | Polygalacturonase, putative isoform 2 OS=Theobroma cacao OX=3641 GN=TCM_005281 PE=3 SV=1 |
| ***probable alpha-glucosidase os06g0675700 (n=1)*** | | | | | |
| A0A2H3X455\|A0A2H3X455_PHODC | 81.22 | 2 | 1 | 103704 | probable alpha-glucosidase Os06g0675700 OS=Phoenix dactylifera OX=42345 GN=LOC103697882 PE=3 SV=1 |
| ***probable pectinesterase/pectinesterase inhibitor 51 (n=1)*** | | | | | |
| A0A2I4HSG5\|A0A2I4HSG5_JUGRE | 117.2 | 7 | 3 | 63654 | probable pectinesterase/pectinesterase inhibitor 51 OS=Juglans regia OX=51240 GN=LOC109021005 PE=3 SV=1 |
| ***probable polygalacturonase (n=1)*** | | | | | |
| A0A2I4GTY4\|A0A2I4GTY4_JUGRE | 77.34 | 3 | 2 | 52110 | probable polygalacturonase OS=Juglans regia OX=51240 GN=LOC109010872 PE=3 SV=1 |
| ***Putative acidic endochitinase (n=1)*** | | | | | |
| A0A251TW18\|A0A251TW18_HELAN | 63.3 | 2 | 1 | 32639 | Putative acidic endochitinase OS=Helianthus annuus OX=4232 GN=CHIT3 PE=3 SV=1 |
| ***Putative class I chitinase (n=1)*** | | | | | |
| Q9LEB2\|Q9LEB2_PHAVU | 69.32 | 2 | 1 | 37538 | Putative class I chitinase OS=Phaseolus vulgaris OX=3885 GN=chi2 PE=4 SV=1 |
| ***Putative eukaryotic aspartyl protease family protein (n=1)*** | | | | | |
| A0A251TTN9\|A0A251TTN9_HELAN | 78.12 | 3 | 2 | 50604 | Putative eukaryotic aspartyl protease family protein OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr09g0247561 PE=3 SV=1 |
| ***Putative galacturan 1,4-alpha-galacturonisdase (n=1)*** | | | | | |
| A0A2P6P6R8\|A0A2P6P6R8_ROSCH | 91.98 | 4 | 2 | 52259 | Putative galacturan 1,4-alpha-galacturonidase OS=Rosa chinensis OX=74649 GN=RchiOBHm_Chr7g0196791 PE=3 SV=1 |
| ***Putative germin-like protein 2-1 (n=1)*** | | | | | |
| A0A3S3MDZ1\|A0A3S3MDZ1_9MAGN | 63.47 | 1 | 1 | 94040 | Putative germin-like protein 2-1 OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_01057500 PE=3 SV=1 |
| ***Putative glucan endo-1,3-beta-D glucosidase (n=1)*** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| A0A2P6QCQ2\|A0A2P6QCQ2_ROSCH | 60.68 | 2 | 1 | 49917 | Putative glucan endo-1,3-beta-D-glucosidase OS=Rosa chinensis OX=74649 GN=RchiOBHm_Chr5g0041211 PE=3 SV=1 |
| ***Putative glycosidase (n=1)*** | | | | | |
| A0A2P6SNT2\|A0A2P6SNT2_ROSCH | 73.67 | 2 | 1 | 32398 | Putative glycosidase OS=Rosa chinensis OX=74649 GN=RchiOBHm_Chr1g0379721 PE=3 SV=1 |
| ***Putative peptidase M10A, Metallopeptidase, catalytic domain protein (n=2)*** | | | | | |
| A0A251SWK7\|A0A251SWK7_HELAN | 111.8 | 6 | 6 | 35405 | Putative peptidase M10A, Metallopeptidase, catalytic domain protein OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr13g0419141 PE=3 SV=1 |
| A0A251SY80\|A0A251SY80_HELAN | 61.34 | 1 | 1 | 20807 | Putative peptidase M10A, Metallopeptidase, catalytic domain protein OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr13g0423751 PE=3 SV=1 |
| ***Putative plant basic secretory protein (BSP) family protein (n=1)*** | | | | | |
| A0A251V7R2\|A0A251V7R2_HELAN | 116.5 | 10 | 5 | 25027 | Putative plant basic secretory protein (BSP) family protein OS=Helianthus annuus OX=4232 GN=HannXRQ_Chr03g0070441 PE=4 SV=1 |
| ***R 13 protein (n=1)*** | | | | | |
| Q84ZU9\|Q84ZU9_SOYBN | 60.68 | 1 | 1 | 72800 | R 13 protein OS=Glycine max OX=3847 GN=102666666 PE=4 SV=1 |
| ***Squamous cell carcinoma antigen recognized by T-cell 3-like (n=1)*** | | | | | |
| A0A2Z7CM07\|A0A2Z7CM07_9LAMI | 82.33 | 3 | 3 | 92195 | Squamous cell carcinoma antigen recognized by T-cell 3-like OS=Dorcoceras hygrometricum OX=472368 GN=F511_07799 PE=4 SV=1 |
| ***Subtilisin-like protease (n=2)*** | | | | | |
| A0A2R6RN74\|A0A2R6RN74_ACTCH | 69.08 | 2 | 1 | 81282 | Subtilisin-like protease OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc04770 PE=4 SV=1 |
| W9RMM1\|W9RMM1_9ROSA | 82.14 | 3 | 2 | 70707 | Subtilisin-like protease OS=Morus notabilis OX=981085 GN=L484_016023 PE=4 SV=1 |
| ***Subtilisin-like protease SBT1.7 (n=7)*** | | | | | |
| A0A199W419\|A0A199W419_ANACO | 72.38 | 3 | 2 | 79285 | Subtilisin-like protease SBT1.7 OS=Ananas comosus OX=4615 GN=ACMD2_04618 PE=4 SV=1 |
| A0A1S2YZ46\|A0A1S2YZ46_CICAR | 60.17 | 1 | 1 | 80755 | subtilisin-like protease SBT1.7 OS=Cicer arietinum OX=3827 GN=LOC101501767 PE=4 SV=1 |
| A0A1U8K442\|A0A1U8K442_GOSHI | 61.78 | 2 | 1 | 80743 | subtilisin-like protease SBT1.7 OS=Gossypium hirsutum OX=3635 GN=LOC107913264 PE=4 SV=1 |
| A0A2I4DH75\|A0A2I4DH75_JUGRE | 86.45 | 3 | 3 | 80882 | subtilisin-like protease SBT1.7 OS=Juglans regia OX=51240 GN=LOC108980118 PE=4 SV=1 |

| | | | | | |
|---|---|---|---|---|---|
| A0A1U8AYU8\|A0A1U8AYU8_NELNU | 75.45 | 2 | 1 | 79642 | subtilisin-like protease SBT1.7 OS=Nelumbo nucifera OX=4432 GN=LOC104607876 PE=4 SV=1 |
| A0A438ITL8\|A0A438ITL8_VITVI | 69.51 | 2 | 1 | 77991 | Subtilisin-like protease SBT1.7 OS=Vitis vinifera OX=29760 GN=SBT1.7_15 PE=4 SV=1 |
| A0A3S3Q3I8\|A0A3S3Q3I8_9MAGN | 61.54 | 2 | 1 | 81121 | Subtilisin-like protein protease SBT1.7 OS=Cinnamomum micranthum f. kanehirae OX=337451 GN=CKAN_00651000 PE=4 SV=1 |
| ***Subtilisin-like protein (n=1)*** | | | | | |
| Q94KL9\|Q94KL9_SOYBN | 101.5 | 5 | 3 | 81613 | Subtilisin-like protein OS=Glycine max OX=3847 PE=4 SV=1 |
| ***Subtilisin-like serine protease (n=1)*** | | | | | |
| A0A072V371\|A0A072V371_MEDTR | 65.59 | 2 | 1 | 80649 | Subtilisin-like serine protease OS=Medicago truncatula OX=3880 GN=25489941 PE=4 SV=1 |
| ***Tamarinin (n=1)*** | | | | | |
| E1AZ68\|E1AZ68_TAMIN | 88.99 | 3 | 3 | 31127 | Tamarinin (Fragment) OS=Tamarindus indica OX=58860 PE=2 SV=1 |
| ***Thaumatin (n=1)*** | | | | | |
| A0A2P5F7Y2\|A0A2P5F7Y2_TREOI | 67.38 | 2 | 1 | 24787 | Thaumatin OS=Trema orientale OX=63057 GN=TorRG33x02_102950 PE=4 SV=1 |
| ***Thaumatin-like protein Fragment (n=1)*** | | | | | |
| A0A2R6QJH9\|A0A2R6QJH9_ACTCH | 172.8 | 21 | 2 | 11988 | Thaumatin-like protein (Fragment) OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_Acc16579 PE=4 SV=1 |
| ***Thaumatin-like protein 1 (n=1)*** | | | | | |
| A0A314ZJG7\|A0A314ZJG7_PRUYE | 73.79 | 3 | 2 | 34203 | Thaumatin-like protein 1 OS=Prunus yedoensis var. nudiflora OX=2094558 GN=Pyn_14929 PE=4 SV=1 |
| A0A2P2NIH1\|A0A2P2NIH1_RHIMU | 61.37 | 2 | 2 | 26517 | Thaumatin-like protein 1 OS=Rhizophora mucronata OX=61149 PE=4 SV=1 |
| ***Thaumatin-like protein (n=6)*** | | | | | |
| L7TUK4\|L7TUK4_ACTDE | 60.9 | 1 | 1 | 24771 | Thaumatin-like protein OS=Actinidia deliciosa OX=3627 GN=TLP4 PE=2 SV=1 |
| A0A151RRI0\|A0A151RRI0_CAJCA | 131.9 | 9 | 6 | 23571 | Thaumatin-like protein OS=Cajanus cajan OX=3821 GN=KK1_033356 PE=4 SV=1 |
| A0A2H3XAJ0\|A0A2H3XAJ0_PHODC | 67.96 | 3 | 2 | 19584 | thaumatin-like protein OS=Phoenix dactylifera OX=42345 GN=LOC103700746 PE=4 SV=1 |
| A0A438JJ59\|A0A438JJ59_VITVI | 63.43 | 1 | 1 | 30463 | Thaumatin-like protein OS=Vitis vinifera OX=29760 GN=tlp_12 PE=4 SV=1 |
| A0A438JJ51\|A0A438JJ51_VITVI | 118.5 | 9 | 5 | 23964 | Thaumatin-like protein OS=Vitis vinifera OX=29760 GN=tlp_16 PE=4 SV=1 |

| | | | | | |
|---|---|---|---|---|---|
| A0A438JJ53\|A0A438JJ53_VITVI | 96.36 | 6 | 4 | 23908 | Thaumatin-like protein OS=Vitis vinifera OX=29760 GN=tlp_6 PE=4 SV=1 |
| ***Thaumatin-like protein/pathogenesis related protein-5*** | | | | | |
| A0A0D3QXF6\|A0A0D3QXF6_9ROSI | 116.3 | 8 | 4 | 24700 | Thaumatin-like protein/pathogenesis related protein-5 OS=Populus szechuanica OX=179740 GN=TLP2 PE=2 SV=1 |
| ***TMV resistance protein N-like (n=1)*** | | | | | |
| A0A2K3MVI5\|A0A2K3MVI5_TRIPR | 143.8 | 16 | 8 | 25306 | TMV resistance protein N-like OS=Trifolium pratense OX=57577 GN=L195_g018000 PE=4 SV=1 |
| ***Transmembrane 9 superfamily member (n=1)*** | | | | | |
| A0A444EPB9\|A0A444EPB9_ENSVE | 76.71 | 2 | 2 | 65200 | Transmembrane 9 superfamily member OS=Ensete ventricosum OX=4639 GN=GW17_00024150 PE=3 SV=1 |
| ***Trehalase (n=1)*** | | | | | |
| W9RWI4\|W9RWI4_9ROSA | 78.9 | 2 | 2 | 68016 | Trehalase OS=Morus notabilis OX=981085 GN=L484_007814 PE=3 SV=1 |
| ***Xylem cysteine peptidase 2B (n=1)*** | | | | | |
| A0A0E3VMV9\|A0A0E3VMV9_POPKI | 75.55 | 2 | 1 | 39502 | Xylem cysteine peptidase 2B OS=Populus kitakamiensis OX=34292 GN=PsgXCP2B PE=2 SV=1 |
| ***Xylem serine proteinase 1 (n=1)*** | | | | | |
| B9R726\|B9R726_RICCO | 67.8 | 2 | 1 | 80768 | Xylem serine proteinase 1, putative OS=Ricinus communis OX=3988 GN=RCOM_1588040 PE=4 SV=1 |
| ***Xyloglucan endotransglucosylase/hydrolase (n=1)*** | | | | | |
| A0A2K2BE83\|A0A2K2BE83_POPTR | 72.09 | 2 | 1 | 32595 | Xyloglucan endotransglucosylase/hydrolase OS=Populus trichocarpa OX=3694 GN=POPTR_002G060400 PE=3 SV=2 |

*proteins found by mass spec*
*Family: birch*

## References


1. Abbas, A. K., Lichtman, A. H. H. & Pillai, S. *Cellular and Molecular Immunology: with STUDENT CONSULT Online Access*. *Elsevier Health Sciences.* (2014).

2. Howell, M. & Shepherd, M. The immune system. *Anaesthesia and Intensive Care Medicine* (2018). doi:10.1016/j.mpaic.2018.08.014

3. Küppers, R. Overview of the immune system. in *The Lymphoid Neoplasms 3ed* (2010). doi:10.1201/b13424

4. Diagnostics, C. Innate and Adaptive Immunity. Available at: https://www.creative-diagnostics.com/innate-and-adaptive-immunity.htm.

5. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* (2006). doi:10.1016/j.cell.2006.02.015

6. Sabroe, I. Innate Immunity in Allergic Disease. in *Allergy and Allergic Diseases, Second Edition* (2009). doi:10.1002/9781444300918.ch9

7. Lacy, P. & Stow, J. L. Cytokine release from innate immune cells: Association with diverse membrane trafficking pathways. *Blood* (2011). doi:10.1182/blood-2010-08-265892

8. Küppers, R. Overview of the immune system. in *The Lymphoid Neoplasms 3ed* (2010). doi:10.1201/b13424

9. Valenta, R. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* **2**, 446–453 (2018).

10. Wahn, U. *et al.* Indoor allergen exposure is a risk factor for sensitization during the first three years of life. *J. Allergy Clin. Immunol.* (1997). doi:10.1016/S0091-6749(97)80009-7

11. Turner, H. & Kinet, J.-P. Signalling through the high-affinity IgE receptor FcεRI. *Nature* (2002). doi:10.1038/35037021

12. Niederberger, V., Niggemann, B., Kraft, D., Spitzauer, S. & Valenta, R. Evolution of IgM, IgE and IgG1-4 antibody responses in early childhood monitored with recombinant allergen components: Implications for class switch mechanisms. *Eur. J. Immunol.* (2002). doi:10.1002/1521-4141(200202)32:2<576::AID-IMMU576>3.0.CO;2-U

13. Geroldinger-Simic, M. *et al.* Birch pollen-related food allergy: Clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. *J. Allergy Clin. Immunol.* (2011). doi:10.1016/j.jaci.2010.10.027

14. Schenk, M. F. *et al.* Proteomic analysis of the major birch allergen Bet v 1 predicts allergenicity for 15 birch species. *J. Proteomics* (2011). doi:10.1016/j.jprot.2011.03.021

15. Breitenbach, M. *et al.* Biological and Immunological Importance of BET v 1 Isoforms. in (2011). doi:10.1007/978-1-4615-5855-2_16

16. Valenta, R., Seiberler, S., Kraft, D., Scheiner, O. & Lonsdale, D. Characterization of a birch pollen allergen, Bet v III, representing a novel class of Ca2+ binding proteins: specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca2+. *EMBO J.* (2018). doi:10.1002/j.1460-2075.1994.tb06654.x

17. Sekerková, A. & Poláčková, M. Detection of Bet v1, Bet v2 and Bet v4 specific IgE antibodies in the sera of children and adult patients allergic to birch pollen: Evaluation of different IgE reactivity profiles depending on age and local sensitization. *Int. Arch. Allergy Immunol.* (2011). doi:10.1159/000321819

18. Engel, E. *et al.* Immunological and biological properties of Bet v 4, a novel birch polen allergen with two EF-hand calcium-binding domains. *J. Biol. Chem.* (1997). doi:10.1074/jbc.272.45.28630

19. VIETHS, S., SCHEURER, S. & BALLMER-WEBER, B. Current Understanding of Cross-Reactivity of Food Allergens and Pollen. *Ann. N. Y. Acad. Sci.* (2010). doi:10.1111/j.1749-6632.2002.tb04132.x

20. Cadot, P. *et al.* Purification and characterization of an 18-kd allergen of birch (Betula verrucosa) pollen: identification as a cyclophilin. *J Allergy Clin Immunol* (2000).

21. Mahler, V. *et al.* cDNA cloning and characterization of a cross-reactive birch pollen allergen: Identification as a pectin esterase. in *International Archives of Allergy and Immunology* (2001). doi:10.1159/000053670

22. Asero, R. Is There a Role for Birch Pollen Immunotherapy on Concomitant Food Allergy? *Curr. Treat. Options Allergy* (2015). doi:10.1007/s40521-015-0045-1

23. Wang, X. *et al.* Mechanism of oral tolerance induction to therapeutic proteins. *Advanced Drug Delivery Reviews* (2013). doi:10.1016/j.addr.2012.10.013

24. Repa, A. *et al.* Susceptibility to nasal and oral tolerance induction to the major birch pollen allergen Bet v 1 is not dependent on the presence of the microflora. *Immunol. Lett.* (2008). doi:10.1016/j.imlet.2007.11.025

25. Nayak, P. A., Nayak, U. A. & Khandelwal, V. The effect of xylitol on dental caries and oral flora. *Clinical, Cosmetic and Investigational Dentistry* (2014). doi:10.2147/CCIDE.S55761

26. Ferreira, F. D. *et al.* Purification and characterization of recombinant Bet v I, the major birch pollen allergen. Immunological equivalence to natural Bet v I. *J. Biol. Chem.* (1993).

27. Scheiner, O. & Kraft, D. Basic and practical aspects of recombinant allergens. *Allergy* (1995). doi:10.1111/j.1398-9995.1995.tb01167.x

28. Ferreira, F. *et al.* Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* (1998). doi:10.1096/fasebj.12.2.231

29. Wingfield, P. T. Protein precipitation using ammonium sulfate. *Curr. Protoc. Protein Sci.* (2016). doi:10.1002/0471140864.psa03fs84

30. Burgess, R. R. Chapter 20 Protein Precipitation Techniques. in *Methods in Enzymology* (2009). doi:10.1016/S0076-6879(09)63020-2

31. Duong-Ly, K. C. & Gabelli, S. B. Chapter Seven - Salting out of Proteins Using Ammonium Sulfate Precipitation. in *Laboratory Methods in Enzymology: Protein Part C* (2014). doi:https://doi.org/10.1016/B978-0-12-420119-4.00007-0

32. Exalpha Biologicals Inc. *Ammonium Sulfate Precipitation Protocol*.

33. Sigma Aldrich. Fractional Precipitation of Affinity Chromatography Samples. Available at:

https://www.sigmaaldrich.com/technical-documents/protocols/biology/affinity-chromatography/fractional-precipitation.html.

34. Kendrick Labs Inc. Ethanol Precipitation of Protein: Protocol and % Recovery. Available at: https://kendricklabs.com/wp-content/uploads/2016/08/EtOHppt_2008.pdf.

35. Bactiridescence Cambridge. Ethanol Precipitation of Proteins. Available at: http://2011.igem.org/Team:Cambridge/Protocols/Ethanol_Precipitation_of_Proteins#/Protocols/Ethanol_Precipitation_of_Proteins.

36. JIANG Hongzhou, SAKAMOTO Yuichi, T. Y. and T. M. Proteins in The Exudation Sap from Birch Trees , Betula platyphyl / a Sukatchev var . japonica Hara and Betula verrucosa Her . *Eurasian J. For. Res.* **2**, 59–64 (2001).

37. Sanchez, L. TCA protein precipitation protocol. *October* (2001). doi:10.1145/2003653.2003657

38. Koontz, L. TCA precipitation. in *Methods in Enzymology* (2014). doi:10.1016/B978-0-12-420119-4.00001-X

39. CalTec. TCA_precipitation_protocol. *TCA_precipitation_protocol* Available at: http://www.its.caltech.edu/~bjorker/TCA_ppt_protocol.pdf.

40. Cutler, P. & Ó'Fágáin, C. Lyophilization of Proteins. in *Protein Purification Protocols* (2004). doi:10.1385/1-59259-655-x:309

41. Ó'Fágáin, C. & Colliton, K. Storage and lyophilization of pure proteins. in *Methods in Molecular Biology* (2017). doi:10.1007/978-1-4939-6412-3_9

42. Ratti, C. *Advances in Food Dehydration*. (2008).

43. ThermoFisherScientific. SpeedVac Vacuum Concentrators. Available at: https://www.thermofisher.com/at/en/home/life-science/lab-equipment/speedvac-vacuum-concentrators.html.

44. Woronowicz, K. *Use of the SpeedVac*.

45. Deutscher, M. P. Guide to protein purification: Preface. *Methods in Enzymology* (1990). doi:10.1016/0076-6879(90)82001-I

46. ThermoFisherScientific. Dialysis Methods for Protein Research. Available at: https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/dialysis-methods-protein-research.html.

47. Andrew, S. M., Titus, J. A. & Zumstein, L. Dialysis and concentration of protein solutions. *Curr. Protoc. Toxicol.* (2002). doi:10.1002/0471140856.txa03hs10

48. Dialysis. Available at: http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/ch02s04.html.

49. Citeq biologics. BCA and Bradford protein assays. Available at: https://www.citeqbiologics.com/difference-between-bca-bradford-protein-assays/.

50. He, F. BCA (Bicinchoninic Acid) Protein Assay. *BIO-PROTOCOL* (2016). doi:10.21769/bioprotoc.44

51. ThermoFisherScientific. *Pierce™ BCA Protein Assay Kit*.

52. Kendrick Labs Inc. How does 1D gel electrophoresis work? Available at: https://kendricklabs.com/1d-method/.

53. Garfin, D. E. Chapter 29 One-Dimensional Gel Electrophoresis1. in *Methods in Enzymology* (2009). doi:10.1016/S0076-6879(09)63029-9

54. E., H. & K., K. The mechanism of silver staining of proteins separated by SDS polyacrylamide gel electrophoresis. *Biotech Histochem.* (2017).

55. Liu, W. *et al.* Development of a simple and effective silver staining protocol for detection of DNA fragments. *Electrophoresis* (2017). doi:10.1002/elps.201700009

56. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* (1976). doi:10.1016/0003-2697(76)90527-3

57. National Diagnostic. Staining Proteins with Coomassie Blue.

58. Mahmood, T. & Yang, P. C. Western blot: Technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* (2012). doi:10.4103/1947-2714.100998

59. Ni, D., Xu, P. & Gallagher, S. Immunoblotting and immunodetection. *Curr. Protoc. Immunol.* (2016). doi:10.1002/cpim.10

60. Magi, B. & Liberatori, S. 23 Immunoblotting techniques. in *Methods in molecular biology (Clifton, N.J.)* (2005). doi:10.1016/0952-7915(89)90176-3

61. Bio, N. Western Blotting.

62. Expedeon. Dot Blot.

63. Uritani, M. & Hamada, A. A simple and inexpensive dot-blotter for immunoblotting. *Biochem. Educ.* (1999). doi:10.1016/S0307-4412(98)00292-1

64. Nature Education. Proteome Definition. (2014). Available at: https://www.nature.com/scitable/definition/proteome-297/.

65. Cox, J. & Mann, M. Quantitative, High-Resolution Proteomics for Data-Driven Systems Biology. *Annu. Rev. Biochem.* (2011). doi:10.1146/annurev-biochem-061308-093216

66. Feist, P. & Hummon, A. B. Proteomic challenges: Sample preparation techniques for Microgram-Quantity protein analysis from biological samples. *International Journal of Molecular Sciences* (2015). doi:10.3390/ijms16023537

67. Knol, E. F., Mul, F. P. J., Jansen, H., Calafat, J. & Roos, D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J. Allergy Clin. Immunol.* (1991). doi:10.1016/0091-6749(91)90094-5

68. McGowan, E. C. & Saini, S. Update on the performance and application of basophil activation tests. *Curr. Allergy Asthma Rep.* (2013). doi:10.1007/s11882-012-0324-x

69. Hubatsch, I., Ragnarsson, E. G. E. & Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* (2007). doi:10.1038/nprot.2007.303

70. Repetto, G., del Peso, A. & Zurita, J. L. Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. *Nat. Protoc.* (2008). doi:10.1038/nprot.2008.75

71. Srinivasan, B. *et al.* TEER Measurement Techniques for In Vitro Barrier Model Systems. *Journal of Laboratory Automation* (2015). doi:10.1177/2211068214561025

72. Fernández, D. A. *et al.* The kinetic, mechanistic and cytomorphological effects of palytoxin in human intestinal cells (Caco-2) explain its lower-than-parenteral oral toxicity. *FEBS J.* (2013). doi:10.1111/febs.12390

73. Maznah, I. The use of Caco-2 cells as an in vitro method to study bioavailability of iron. *Malays. J. Nutr.* (1999).

74. Pauli, G. *et al.* Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J. Allergy Clin. Immunol.* (2008). doi:10.1016/j.jaci.2008.09.017