

# **Destruction of Nasal Mucus Proteins by Pollen Proteases**

**Dissertation**

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## **2 Summary**

### **2.1 Introduction:**

The central topic of this dissertation is that besides allergic people also non-allergic people get allergy-like symptoms when they are exposed to pollen-enriched air. This means that pollen can harm humans not only in an allergic way. There must also be another way in which pollen affect the nasal mucosa. The purpose of this study was to investigate the reactions which occur when pollen of grasses, bushes and trees get in contact with nasal mucus proteins.

### **2.2 Material and methods**

1. The reactions of persons were observed when they got in contact with pollen-enriched surroundings.
2. Nasal mucus samples from these tested persons were investigated on protein content and by polyacrylamide-gel-electrophoresis (PAGE) the protein spectrum was obtained.
3. Pollen from grasses, bushes and trees were used in the experiments. They represent the most common pollen allergens. PAGE was performed to prove proteins in pollen dust. With zymographies of pollen extracts we detected that pollen expel proteases when they get in contact with liquid. These proteases usually have a physiological function in the process of floral replication.
4. In other PAGE experiments the mixtures of nasal mucus and pollen were analysed to explore the effect of pollen on nasal mucus.

## **2.3 Results:**

1. Allergic and non allergic patients suffer from symptoms like hyper secretion of nasal mucus and tear fluid of the anterior parts of the eye as well as an itchy nose when they get in contact with pollen.
2. With PAGE proteins of nasal mucus and pollen were characterized.
3. Polyacrylamide-gel-electrophoreses showed that pollen contain proteins which were identified as proteases in zymographies.
4. The examination of mixtures of nasal mucus and pollen showed that pollen proteases destroy nasal mucus proteins. This is how the protective layer on the mucosa becomes incomplete.

## **2.4 Discussion:**

The experiments show that the reactions that occur when non allergic persons are exposed to pollen-polluted surroundings depend on the destruction of nasal mucus proteins by pollen proteases. This degradation process of the nasal mucus can also trigger allergies because allergens first have to pass the nasal mucus to get to the inflammatory cells in the nasal mucosa.

# 3 Pollen

## 3.1 Genesis and function of pollen

Pollen grains contain the male part of the plant which are produced by meiosis in the inner end of the microsporangia. The outer part of the pollen is the exine, which consists of polysaccharide and sporopollenin. Inside the pollen are two cells that comprise the male gametophyte. The tube cell develops into the pollen tube. The germ cell divides by mitosis into two sperm cells [1, 2].

### 3.1.1 Pollination

The transfer of pollen from the male plant to a female one is called pollination. This is accomplished by wind, insects, birds, bats and humans. Some plants can pollinate themselves [3-7].

### 3.1.2 Gynoecium

The gynoecium consists of stigma, style and ovary containing one or more ovules. It is the female part for the fertilisation process.



Fig 1 Gynoecium



**Fig 2 Hibiscus gynoecium**

### **3.1.2.1 The stigma and style**

The stigma acts as a receptive surface on which pollen are attached and germinates its pollen tube. This is the part where pollen proteases have their physiological function. The tube needs them to make its way through the style to the ovary. The style serves to move the stigma some distance from the ovary. This distance is specific for each species. This is important for the pollen to find the right gynoecium to fertilize [8].

### **3.1.2.2 The ovary**

The ovary contains one or more ovules, which contain a female gametophyte. Only one ovule will develop into a seed.

### **3.1.2.3 The gametophytes**

The male gametophyte develops inside the pollen grain. The female gametophyte develops inside the ovule.

Male gametophytes have two haploid nuclei (the germ nucleus and the tube nucleus) located within the exine of the pollen grain.

Female gametophytes of flowering plants develop within the ovule located within an ovary at the base of the pistil of the flower.

### 3.1.3 Fertilization

After the pollen has reached the right stigma, the pollen tube grows with the help of proteases through the stigma and style toward the ovules in the ovary [8-12].



**Fig 3 Pollen with pollen-tube**

The germ cell in the pollen grain divides and releases two sperm cells which move down the pollen tube. Once the tip of the tube reaches the micropyle end of the embryo sac, the tube grows into the embryo sac to the egg. One sperm cell fuses with the egg, producing the zygote which will later develop into the next-generation sporophyte. The second sperm fuses with the two polar bodies located in the centre of the sac, producing the nutritive triploid tissue that will provide energy for the embryo's growth and development [13].



## 3.2 Structure

The casing which houses the male gametophyte has a very complex structure which is specific for each species. The exine is the outer layer of a living pollen grain. It is composed of sporopollenin with small quantities of polysaccharides. Sporopollenin is very chemically stable and it is resistant to almost all kinds of environmental damage. It is equipped with apertures. It is divided into two sub layers: the outermost sexine and the unsculptured underlying nexine. The sexine has surfaces that are sculptured in elaborate ways with reticularly arranged perforations. They are responsible for exine's amorphous or granular appearance. The inner layer of a living pollen grain is called the intine. It is composed of cellulose and is very similar in construction to ordinary plant cell walls. A layer called the endexine separates the sexine and intine. The endexine has a laminated appearance.

Pollen grains are generally classified according to their physical appearance. There are three criteria of classification:

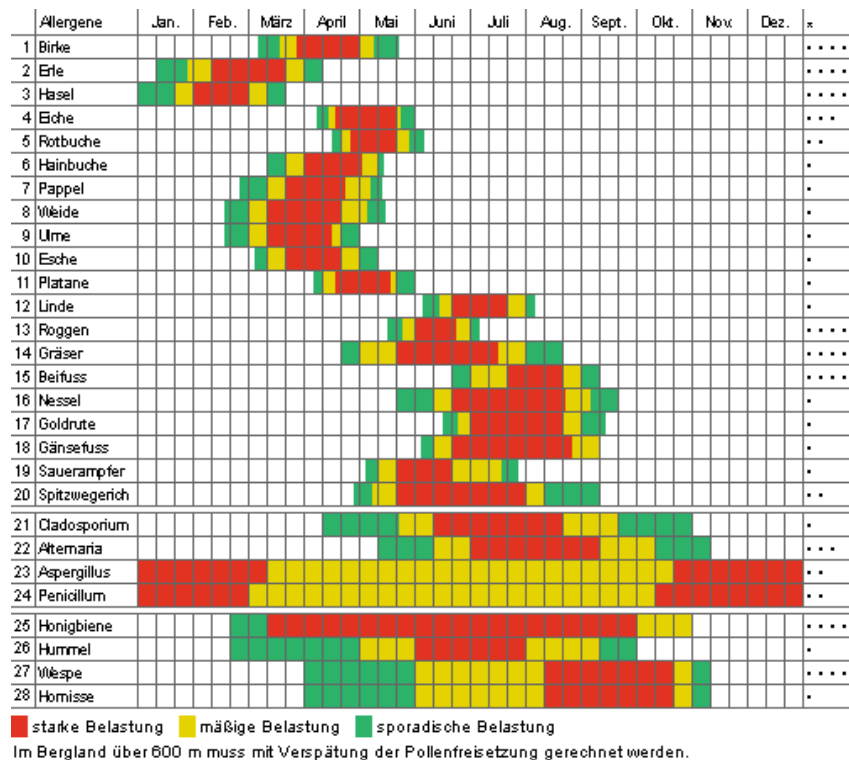
- 1.) The number and position of the apertures
- 2.) The shape of the pollen grain as a whole
- 3.) The fine elaborate structure on the sexine

In the exine are two types of apertures: Pori or pores are circular apertures, and colpi which are elliptic holes. Pollen with pori are called porate; those with colpi are called colpate; and those with both pori and colpi are called colporate. If their apertures are arranged around the equator of the pollen grains they get the prefix zono-; if they are spread all over the surface they get the prefix panto-. The number of apertures

is also indicated by prefixes: mono- for one aperture; di- for two apertures; tri- for three apertures.

### 3.3 Pollen calendar

This calendar shows the pollen-count of the air in Austria during the year. The green fields mean low, yellow fields mean medium and red fields mean high pollen-count of the air.



\* Klinische/ Therapeutische Relevanz

**Fig 6 Pollen calendar**

# 4 Allergology

## 4.1 Definition

An allergy is a hypersensitive response to an environmental airborne substance such as pollen, fungi, bacteria, dust mites, cockroaches and animal dander. It is an anaphylactic reaction by mast cells which bind IgE and degranulate mediators like histamine and leukotrienes which produce inflammatory reactions [14].

## 4.2 Statistics

More than 25% of the world population suffer from allergies and the number of affected patients is increasing since the last decade. The most common manifestation of allergies is atopic rhino conjunctivitis with a percentage of 15%. Other kinds of allergies are atopic (10%), allergic asthma (5%) and food allergies (2%). These numbers can not be generalized, because there are differences in age and the environment surrounding of the patients. Atopic dermatitis and asthma for example mainly affect children whereas young adults suffer from allergic rhinitis and allergic asthma. When growing up in a city it is more likely to get an allergy than living at the countryside. There are investigations running which should proof that this phenomenon is referred to traffic exhaustions. Passive smoking within the first three months of life is another factor which can increase the risk of getting an allergy. Further more a genetic disposition for allergies could be found in family studies [15].

# **5 Nasal mucus**

## **5.1 Functions**

### **5.1.1 Transport**

The nasal mucus is essential for the transport of the particles which are filtered from the inspiratory air. Though the active movement is performed by the cilia, particles could not be transported without the mucus trapping the particles. More information is available in the chapter of the mucociliar system [16].

### **5.1.2 Protection**

The nasal mucus is a chemical, immunological and bactericidal barrier which provides protection for the mucosa and the upper airway. Proteins like IgA are responsible for this. For more information see the next chapter about the contents of nasal mucus [16, 17].

### **5.1.3 Conditioning**

When the inspired air reaches the lung, the humidity is around 100% and the temperature is about 37° Celsius. Due to the relatively large surface of the nasal mucosa it does most of the warming and the humidisation. During this process the mucus becomes hyperosmolar and fluid is dragged out of blood vessels by transudation. The tracheal and bronchial mucosa only play a minor role in conditioning the air [16].

### 5.1.4 Olfaction

During inhalation of certain odors, nasal secretion is increased. This shows that nasal mucus helps the olfactory cells to detect various odors [16].

## 5.2 Nasal Secretions

### 5.2.1 Cellular Sources

Nasal mucus is a mixture of products made by secretory cells. Here is a summary of these:

<u>Protein</u>	<u>Source</u>
<i>Mucus glycoproteins</i>	Mucous cells, epithelium cells serous cells, submucosal glands
<i>Immunoglobulins</i>	Plasma cells
<i>Secretory component</i>	Epithelium, mucous glands
<i>Lactoferrin</i>	Serous cells
<i>Lysozyme</i>	Serous cells
<i>Kallikrein</i>	Epithelium
<i>Antiproteases</i>	Epithelium, submucosal glands

Table 1: Nasal mucus proteins [16].

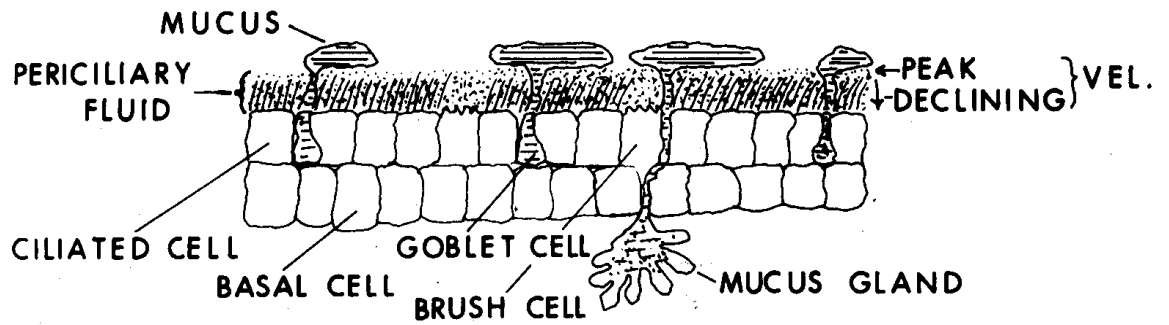


Fig 7 Nasal mucosa [16]

### 5.2.1.1 Mucous cells

The mucous cells, also called goblet cells contain granules which are mainly packed with acidic glycoproteins and also neutral glycoproteins. These granules extrude their content with exocytosis. In other words these cells perform apocrine secretion [16].

### 5.2.1.2 Serous cells

Serous cells contain smaller granules than mucous cells and they also have a compact core. They mainly contain neutral glycoproteins, lysozyme, lactoferrin and IgA. An interesting fact is that serous cells are more common in fetal airways than in adult ones [16][16]

### 5.2.1.3 Submucosal glands

These glands contain grouped acini which are connected with tubules and produce either serous or mucous secretion. Because of their great number of secretory cells they produce a major part of the nasal mucus. The following figures show the architecture of acini, secretory tubule, collecting duct and ciliated duct:

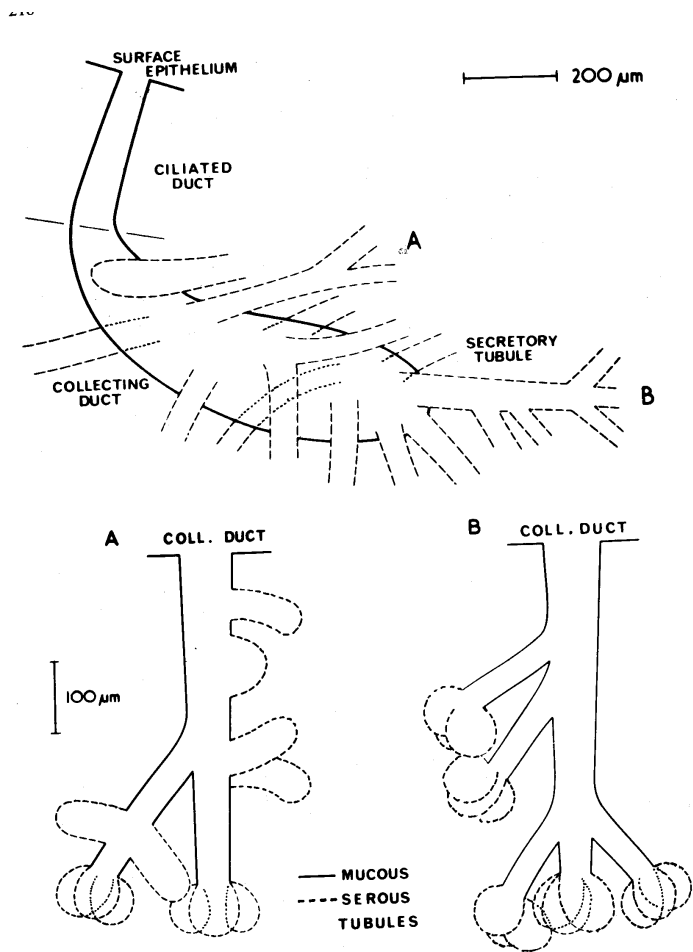


Fig 8 Structure of submucosal glands[Nose]218

The anterior nasal glands can be found in the vestibular region and produce serous neutral secretion and acidic mucus glycoproteins as well as IgA and other proteins. The secretion reaches the nasal cavity through long ducts. Inside these the water and ion-contents are changed.

Spread in the whole complex of the nasal mucosa mucosal glands can be found especially in the septum and the anterior areas. They produce a serous and seromucous secretion.

In the olfactory region Bowman-glands are located which are found in the submucosa. The composition of their secretion and their control is unknown.

In the paranasal sinuses submucosal and epithelial glands are found. Their secretions mix up with the mucus of the nasal cavity [16].

## **5.2.2 Control of secretion**

### **5.2.2.1 Parasympathicus**

Most parasympathetic fibres reach the nose via the vidian nerve. They stimulate the nasal glands to produce secretion mediated by acetylcholine. This mechanism can be blocked by atropine and enhanced by pilocarpine. There may also exist non cholinergic ways of stimulation, maybe through kallikrein [16].

### **5.2.2.2 Sympathicus**

Nasal secretion is still stimulated by the vidian nerve when the parasympathetic nerves are blocked with atropine. It is most likely that this results from a sympathetic innervation. This theory is reinforced by the fact that stimulation of the cervical sympathetic trunk causes nasal secretion. The receptors which cause these secretions are  $\alpha_1$ . An antagonist is for example phenoxybenzamine and an agonist is phenylephrine [16].

### **5.2.2.3 Reflex Control**

Intranasal allergens and other stimuli can lead to a secretion of nasal mucus. This reaction can be reduced by parasymphatholytical drugs such as atropine [16].

### **5.2.2.4 Mediators**

Inflammatory mediators such as histamine, 5-hydroxytryptamine, bradykinin, substance P and neuropeptides enhance the nasal secretion. They also have an effect on nasal vasculature and mucociliary transport [16, 18-21].

## **5.2.3 Biochemistry of airway secretions**

Nasal secretion is a mixture of several different fluids, the concentration always varies depending on physiological and pathological conditions.

The nasal mucus consists of a thin periciliary serous layer in which the cilia beat and a thick blanket of mucus in which particles are trapped [16, 22].

### **5.2.3.1 Mucus glycoproteins**

Glycoproteins make the major part of the macromolecules of nasal mucus. They represent 80% of the dry weight of nasal secrete.

Glycoproteins are a subdivision of glycoconjugates. They consist of carbohydrates which are covalently linked with proteins. There is a single central protein on which sugar side-chains are covalently

attached. The longer these side-chains are, the more viscous is the mucus [16, 22].

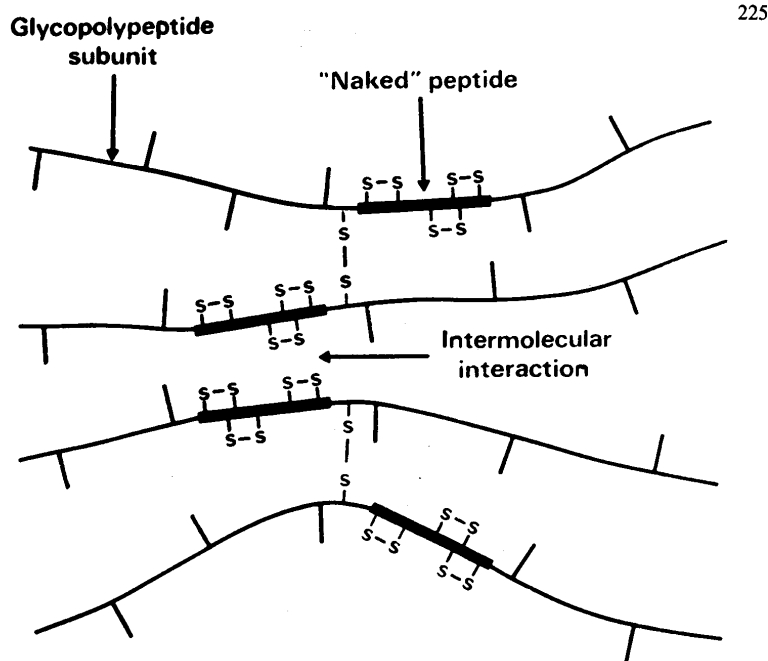


Fig. 2. Proposed structures involved in cross-linking glycopolyptide subunits. From Roberts [138].

**Fig 9 Glycopeptide [16].**

In nasal mucus two kinds of glycoproteins can be found. On one hand there are acid ones. As a terminal acid they contain sialic acids or sulphate. On the other hand there are neutral glycoproteins. They contain a large amount of fructose in the terminal positions.

Although glycoproteins can also be found in blood-plasma, they are not the same as in nasal mucus. The following table shows the differences:

<u>Contents</u>	<u>Mucus Glycoproteins</u>	<u>Plasma Glycoproteins</u>
<b>Amino acid content:</b>	High levels: serine, threonine, proline  Low levels: aromatic and sulphur-containing amino acids	Typical protein
<b>Carbohydrate content:</b>	More than 50%	Less than 25%
<b>Monosaccharides:</b> Fructose, galactose N-acetylglucosamine, N-acetylneuraminic acid	Present	Present
Mannose	Low levels or absent	Present
N-acetylgalactosamine	Present	Low levels or absent

*Table 2: Differences between mucus and plasma glycoproteins [16].*

### 5.2.3.2 Immunoglobulins

#### 5.2.3.2.1 IgA

IgA is specific for all human secretes and can of course also be found in nasal mucus.

There are two different kinds of IgA in the nasal mucus: A 7s monomer component and a 11s dimer. The 7s IgA is typical for plasma and makes only 20% of IgA in nasal mucus. The other 80% are 11s dimers. They are locally synthesized in the nasal submucosa by plasma cells and secreted by glands. The 7s monomers reach the nasal mucus through transudation from blood vessels.

The secretion of IgA is increased during inflammatory infections in the upper airways. The function of IgA is neutralizing viruses, without the

help of the complement system. The most common viruses in the nasal mucosa are parainfluenza type 1 and rhinovirus type 13. High titres of IgA provide a certain protection against these viruses.

Another function of IgA is the inhibition of allergy-induced histamine release from mast cells. This is the reason for an increased titre of IgA at patients with allergies against pollen, especially ragweed [16, 23-26].

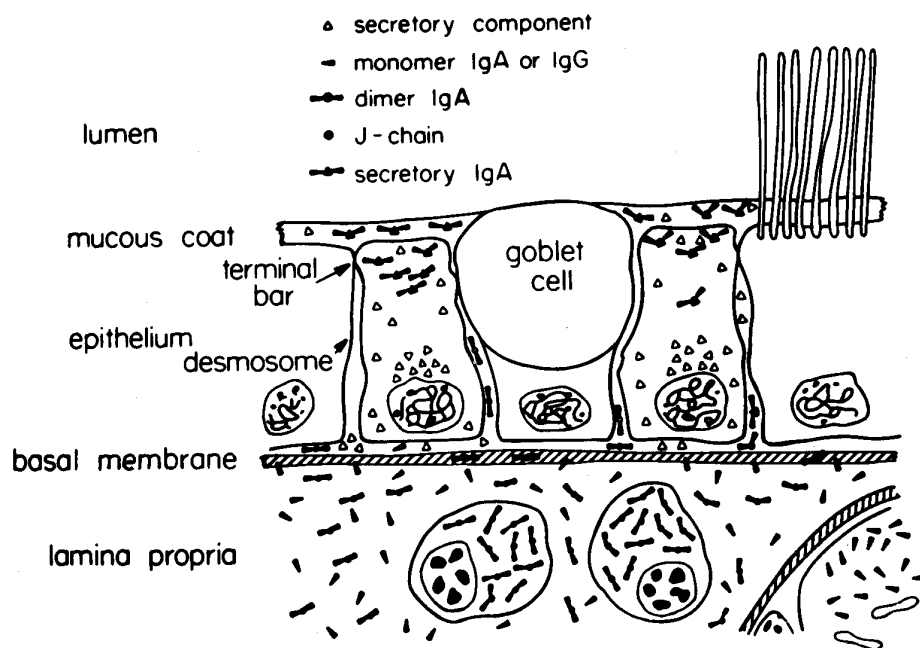


Fig 10 Mechanism of IgA-secretion [16].

### 5.2.3.2.2 IgE

The IgE titres in the nasal mucus can be used for a differential diagnosis between allergic rhinitis and non atopic conditions. These titres are much higher in allergic patients than in non-allergics.

10-80% of the IgE are produced locally depending on allergies. The rest is contributed by transudation from the blood [16].

#### **5.2.3.2.3 IgG**

The function of IgG is to react with antigens and to create antigen-antibody-complexes. These attract neutrophile granulocytes and the complement. By this way the antigens can be eliminated.

5 to 50% of the IgG are produced locally by plasma cells. The rest is provided like the other immunoglobulins by the blood through transudation [16].

#### **5.2.3.2.4 IgM**

Not all studies could detect IgM in nasal mucus. Because of its high molecular weight in plasma only a very low number of IgM can reach the nasal mucus through transudation. There were also some IgM producing plasma-cells detected in nasal mucosa, but the concentration is very low.

IgM helps at the process of phagocytosis with the agglutination of particles. It also influences the viscosity of nasal mucus at patients with cystic fibrosis [16].

#### **5.2.3.3 Albumin**

The concentration of albumin in nasal mucus varies widely. There are also discussions about where it comes from. It is proved that it migrates into nasal mucus via transudation. It could also be a sign of contamination of nasal mucus with blood when the measured levels are very high. Since the blood-vessels are very small and fragile in the nasal mucosa, little wounds are very common in this area. Usually the

concentration of albumin in tracheobronchial secretions is around 50 to 100 mg/100 ml. In the nose the percentage of albumin compared to other proteins is approximately 2 to 10%.

Albumin is a good indicator for the level of transudation since its concentration is rising proportionally with it. The transudation is increased for example during inflammatory processes. The main substances which increase the permeability of blood vessels are histamine, prostaglandins, bradykinin and lysozyme.

Another function of albumin lies in the viscoelasticity of the nasal mucus. It reacts with mucus glycoproteins and changes their rheological character [16].

#### **5.2.3.4 Lactoferrin**

Lactoferrin is an iron-binding protein similar to transferrin. Besides in nasal mucus it can be found in tears, bronchial secretion and other body secretions. It can bind two  $Fe^{+++}$  ions or other divalent or trivalent metal ions, but with less affinity.

The production site of lactoferrin lies in serous glands of the epithelium. The function of the unbound lysozyme is defence against microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* because they need  $Fe^{+++}$  [16].

#### **5.2.3.5 Lysozyme**

Lysozyme can be isolated in nasal mucus, tracheal secretion and sputum. Like lactoferrin it is produced by serous cells. Comparisons of

its concentration to the concentration of albumin show that it is actively secreted by serous glands.

The function of lysozyme is similar to lactoferrin: it has a bactericide function [16].

#### **5.2.3.6 Antiproteases**

Several antiproteases can be found in nasal mucus. There are  $\alpha$ -antitrypsin,  $\alpha$ 1-antichymotrypsin,  $\alpha$ 2-macroglobulin and various other antiproteases associated with leucocytes.

The fact that the titres of these antiproteases are increased during inflammatory processes such as bronchitides and pneumonias shows that they protect the tissue from destructive inflammatory proteases [16].

Antiproteases may also play a role in protection from pollen allergens. The pollen proteases can be inhibited by nasal mucus antiproteases [16, 27-33].

### **5.2.3.7 Other Macromolecules**

Here is a list of other macromolecules which can be found in nasal mucus:

Transferrin

Haptoglobin

$\alpha$ 1-Acid Glycoprotein

Ceruloplasmin

Hemopexin

$\beta$ -Glycoprotein

$\beta$ 1c-Globulin

$\beta$ 2a-Globulin

Kallikrein

Fibrinogen

DNA can be found in purulent sputum and is derived from damage to macrophages and epithelial cells. This DNA causes the changes of viscoelastic properties of purulent sputum [16].

### **5.2.3.8 Lipids**

Many kinds of lipids have been found in nasal mucus: Phospholipids, predominantly phosphatidylcholine are the main lipids in infected patients. In non infected patients neutral lipids such as glycerides are found.

The lipids make 25% of the dry weight of gel and 5% of the dry weight of nasal secrete.

The function of lipids is to interact with glycoproteins and to influence the physical characteristics such as the viscosity of secretes [16].

### 5.2.3.9 Ions and water

Because of evaporation, nasal mucus is hyperosmolar compared to plasma and there is also an active transport of ions in the epithelium which is directed to the lumen-side.

<b>Ion Concentrations</b>	<b><u>Na+</u></b>	<b><u>K+</u></b>	<b><u>Mg++</u></b>	<b><u>Ca++</u></b>	<b><u>Cl-</u></b>
<b>Plasma</b>	142	4	1	2	101
<b>Interstitial fluid</b>	145	4	1	1	114
<b>Intercellular fluid</b>	10	160	13	1	3
<b>Human nasal mucus</b>	128-150	17-41	5	4	139
<b>Dog nasal lateral serous gland</b>	14	30	-	-	24
<b>Human tracheobronchial mucus</b>	165	13	-	6	162
<b>Dog tracheobronchial mucus</b>	169	-	-	-	147
<b>Cat tracheobronchial mucus</b>	236	5	-	2	119

*Table 3: Ionic compositions of airway secretions [16].*

The pH of nasal mucus is usually lower than the pH of plasma, but during inflammation it can reach the one of plasma ( 7.4) [16].

### 5.3 Ciliar Transport of Nasal Mucus

The cilia located in the nasal epithelium are 5 to 6  $\mu\text{m}$  long and there are about 29 to 200 cilia on a ciliar cell. They can reach a beat-frequency up to 100 beats per minute. By this way the nasal mucus can be transported up to 25 mm per minute. The ciliar beat-frequency can be increased by various stimuli. For example rise of temperature increases the speed of ciliar transport, but also transmitters like serotonin, acetylcholine and  $\beta_2$  mimetic drugs have this effect [34, 35].

Another interesting thing is, although cilia already survived a very long time in evolution, they die immediately when they lose contact to water and dry.

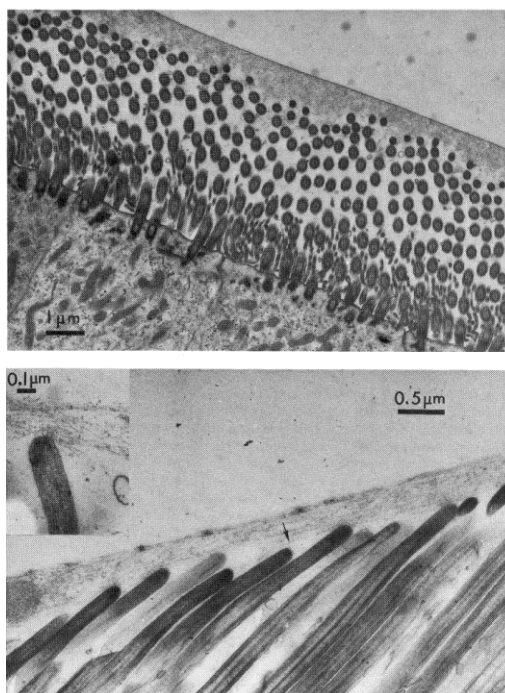
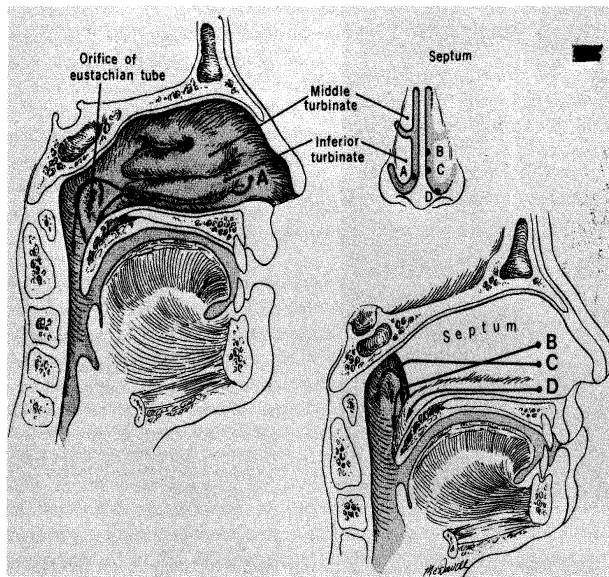
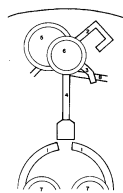
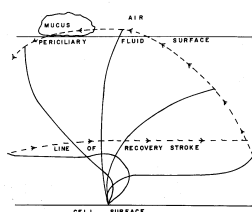


Fig 11 Cilia in the microscope [16]



**Fig 12 Ciliary clearance [16]**

Every single cilia beats individually in a constant direction which makes the transport of nasal mucus possible. First the cilia beats into the direction of the pharynx. During this movement it touches the mucus and propels it forwards. Then it performs a movement backwards in the opposite direction. During this backward movement the tip of the cilia is close to the cellular surface and does not have much contact to the mucus. This way the mucus can be transported in a constant direction to the pharynx, where it is swallowed [16, 36-40].



**Fig. 13** Above is shown the approximate motion of a cilium during one complete beat. In the middle of the periciliary fluid at its distal extremity. Below, diagram of a cilium in motion through a fluid showing the structure in one section. These are also each pair of microtubules plus the central pair in the cilium. Indicated are: 1 lateral projections of central microtubules; 2, 3 base and lower distal part of radial links; 4 and 5 lateral distal part of the base peripheral links; 7, central microtubules; 8, axoneme. Diagrams modified from Sligh [196].

**Fig 13 Ciliary movement [16].  
Fig 14 Ciliar structure [16].**

## **5.4 Proteases-Theory**

Nasal mucosa has a layer of mucus which has several functions. One of them is protection. Pollen on the other side can expel proteases when they get in contact with liquid. These proteases can destroy the proteins and consequently the structure of the mucus. This is an important fact, because the allergens of pollen first have to pass this protective layer before they can get in contact with inflammatory cells and cause a hypersensitive allergic reaction.

# 6 Methods

## 6.1 Collecting Pollen

One essential part for running my experiments were pollen. The Botanic institute of the Karl-Franzens-University Graz was so generous and provided a selection of grass- and tree- pollen.



**Fig 15 Hazelnut**

But because they were not fresh on one hand and for getting a comparison between fresh and old pollen I tried to get as many fresh pollen as possible. The only way of collecting them is to go outside to forests and meadows and to collect blossoms with pollen inside. At this part I have to thank the whole team of the laboratory with whose help we could collect an enormous amount of different kinds of pollen.

Here is a list of the pollen-species we collected:

- 1.) *Helianthus annuus* (Sunflower / Sonnenblume)
- 2.) *Corylus avellana* (Hazelnut / Haselnuss)
- 3.) *Betula pendula* (Birch / Birke)
- 4.) *Alnus glutinosa* (Alder / Erle)
- 5.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 6.) *Fraxinus excelsior* (Ash / Esche)
- 7.) *Fagus sylvatica* (Beech tree / Buche)
- 8.) *Salix caprea* (Willow / Weide)
- 9.) *Abies alba* (Fir tree / Tanne)
- 10.) *Quercus robur* (Oak / Eiche)
- 11.) *Dactylis glomerata* (Orchard grass / Knäuelgras)
- 12.) *Alopecurus pratensis* (Amaranth / Fuchsschwanz)
- 13.) *Phleum pratense* (Timothy grass / Lieschgras)
- 14.) *Bellis perennis* (Daisy / Gänseblümchen)
- 15.) *Brassica napus* (Rapeseed / Raps)
- 16.) *Anemone nemorosa* (Anemone / Buschwindröschen)

Collecting the blossoms with the right grade of development is not so easy. You have to be on the field exactly at the right time. When you are too early, the pollen are not yet fully developed and they are useless. When you are late, the wind has already blown the pollen away and the blossoms are empty.



**Fig 16 Pollen Probes**

Once the blossoms are collected, they must be dried to get the pollen out of them. The easiest way of doing this is to spread the blossoms on a tablet, leave them in a dry place for one or two days and then shake them. By shaking the pollen will fall on the ground of the tablet. The empty blossoms must be taken off the tablet and then the pollen could be collected. Since nature is not sterile, other particles than pollen such as dust and parts of blossoms are present on the ground of the tablet. It is almost impossible to get a 100% pure probe of pollen. Depending on the different kinds of trees and flowers one probe is not as pure as other ones. For example the hazelnut probes are almost pure whereas the willow probes are less clean.

For experiments which should be as realistic as possible the purity of the pollen probes does not play a major role because the inspired air which passes the nasal cavity is not sterile too. It is also polluted for example by fine dust and other particles.

## 6.2 Proteases extraction

When the pollen get in contact with water they seem to explode when watching this process with a microscope. They change their shape completely and emit substances such as proteases to the surrounding water immediately through their pores. For more information about this process go to the pollen-chapter.

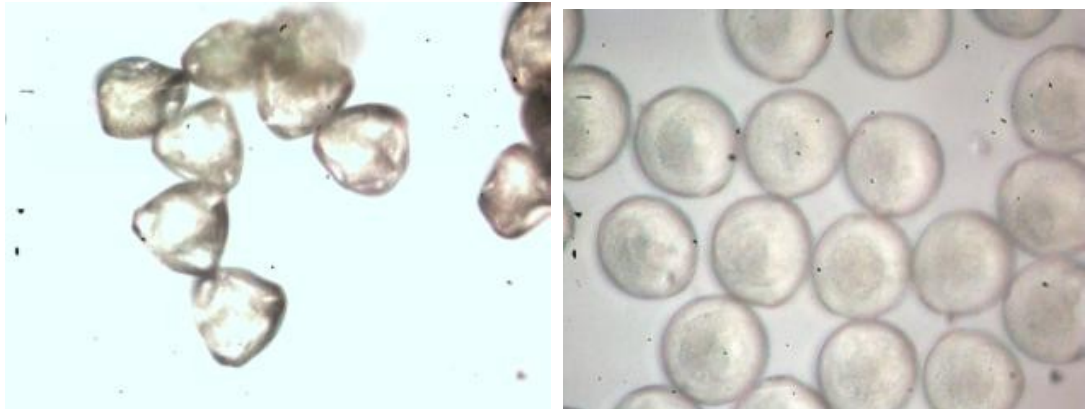


Fig 17 Dry birch-pollen  
Fig 18 Wet birch-pollen

As one can see the pollen change their triangular shape into a circular one. The little bobble-like structures on the edges of the triangles disappear too.

This process can also be used for the proteases extraction. I simply mixed 10  $\mu\text{g}$  of pollen with 100  $\mu\text{l}$  of physiological (0,9 %) NaCl solution. After centrifugation of this mixture a solution of salt solution and pollen proteins is obtained.

## **6.3 Protein determination (Bradford protein assay)**

### **6.3.1 Considerations for use**

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford assay is recommended especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

The method described below is for a 100  $\mu$ l sample volume using 1 ml colour reagent. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality. In assays using 1 ml colour reagent prepared in lab, the sensitive range is closer to 5 to 100  $\mu$ g protein. Scale down the volume for the "micro assay procedure," which uses 1 ml cuvettes.

### **6.3.2 Principle**

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

### 6.3.3 Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum.

### 6.3.4 Procedure

Bradford reagent: Dissolve 20 mg Coomassie Brilliant Blue G-250 in 25 ml 96 % ethanol, add 50 ml 85 % phosphoric acid. Dilute to 500 ml water when the dye has completely dissolved, and filter before use.

Warm up the spectrophotometer before use.

Dilute unknowns if necessary to obtain between 5 and 100 µg protein in at least one assay tube containing 100 µl samples.

If desired, add an equal volume of 1 M NaOH to each sample and vortex (see comments below). Add NaOH to standards as well if this option is used.

Prepare calibration standards containing a range of 0,5 – 0.005 mg bovine serum albumin (Merck Ch. Nr. 1.12018.0025) in 1 ml volume.

#### 6.3.4.1 Calibration curve

<b>Protein mg/ml</b>	0,5	0,25	0,1	0,05	0,025	0,01
<b>µl calibration solution</b>	100	250	100	50	25	10
<b>µl water/puffer</b>	0	250	400	450	475	490

Add 1 ml dye reagent and incubate 5 min.

Measure the absorbance at 595 nm.

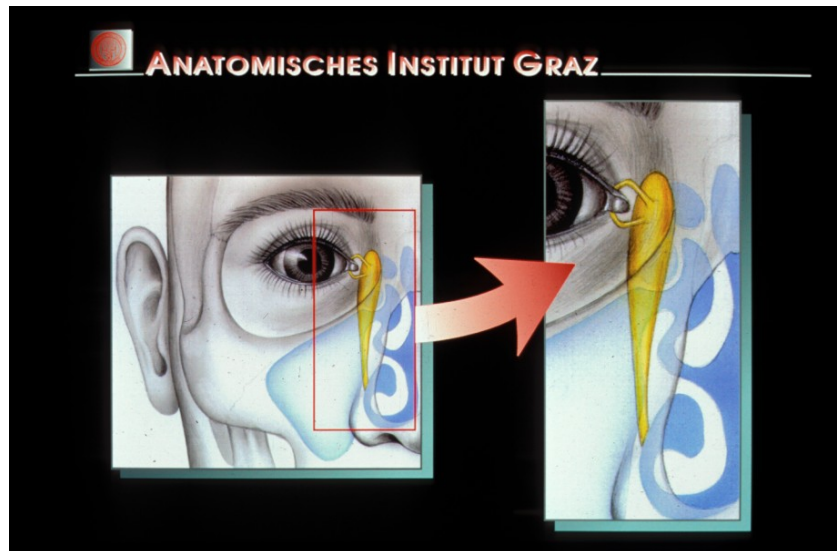
### **6.3.5 Analysis**

The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan and phenylalanine residues. Obviously, the assay is less accurate for basic or acidic proteins. The Bradford assay is rather sensitive to bovine serum albumin, more so than "average" proteins, by about a factor of two. Immunoglobulin G (IgG - gamma globulin) is the preferred protein standard. The addition of 1 M NaOH was suggested by Stoscheck (1990) to allow the solubilization of membrane proteins and reduce the protein-to-protein variation in colour yield.

## **6.4 Three different kinds of nasal mucus**

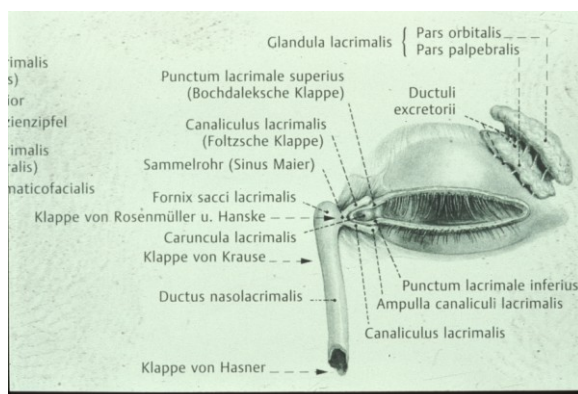
For my experiments I decided to examine the nasal mucus of three different groups of patients. The first group consists of male healthy adults with no allergies. The second group are male adults with allergies. The first two groups were chosen to investigate differences in the non allergic reactions between allergics and non allergics. The third group consists of patients with dry eyes which have punctum-plugs implanted. From this group I wanted to find out, if the tear-liquid plays a role in the composition of nasal mucus proteins.

Tear fluid is mixed up with nasal mucus. because of the anatomic relation between the eye and the nose, which are connected to the nasolacrimal duct,



**Fig 19 Nasolacrimal duct**

For the determination, which kind of proteins are produced in the nose and which get into the nose through the nasolacrimal duct, the nasal mucus of persons with and without punctum plugs were compared. Punctum Plugs are little silicone-plugs which obdurate the puncta lacrimalia, the entrance to the nasolacrimal duct from the eye. From these persons probes pure nasal mucus without tear liquid can be extracted.



**Fig 20 Punctum lacrimale**

**Fig 21 Punctum plug**

## 6.5 Polyacrylamide-gel-electrophoresis



Fig 22 Electrophoresis-computer and power supply

Fig 23 Polyacrylamide-gel

The reason for performing electrophoreses was to proof that the structure of nasal mucus proteins are modified by pollen proteases. Therefore, electrophoresis is an easy and effective method, because in the gel the proteins are sorted by their molecular size and charge.

Comparing electrophoresis of pure nasal mucus with a mixture of nasal mucus and a pollen-extract one can see that large proteins disappear and smaller proteins newly appear in the mixture of nasal mucus and pollen.

First of all I had to find out basic things such as the amount of pollen and nasal mucus I had to use and how long the suspension of those two has to be incubated to get nice results in the electrophoreses.

After a lot of attempts I found out that 10 mg of our pollen-probes have the right amount of proteins and proteases to get nice results.

Nasal mucus is more difficult to investigate. You always need the same amount of mucus and its components. This is impossible, because the mixture of components always varies. For example when the donor of the nasal mucus has a cold or suffers from an allergic attack the mucus

is more fluid than usually. I decided to take the probes with 2 Q-tips from each proband. This procedure brought very constant results concerning the amount of proteins in the probe.

Next the right time which the pollen proteases need to destruct the nasal mucus proteins had to be found. Several incubation times: 5 min, 10 min, 30 min, 60 min, and 24 hours have been tested. The best results were received with 1 hour of incubation.

So in the end I used 2 Q-tips of nasal mucus-probes, 10 mg of pollen and 140  $\mu$ l of physiological salt solution to get the right mixture for the electrophoreses.

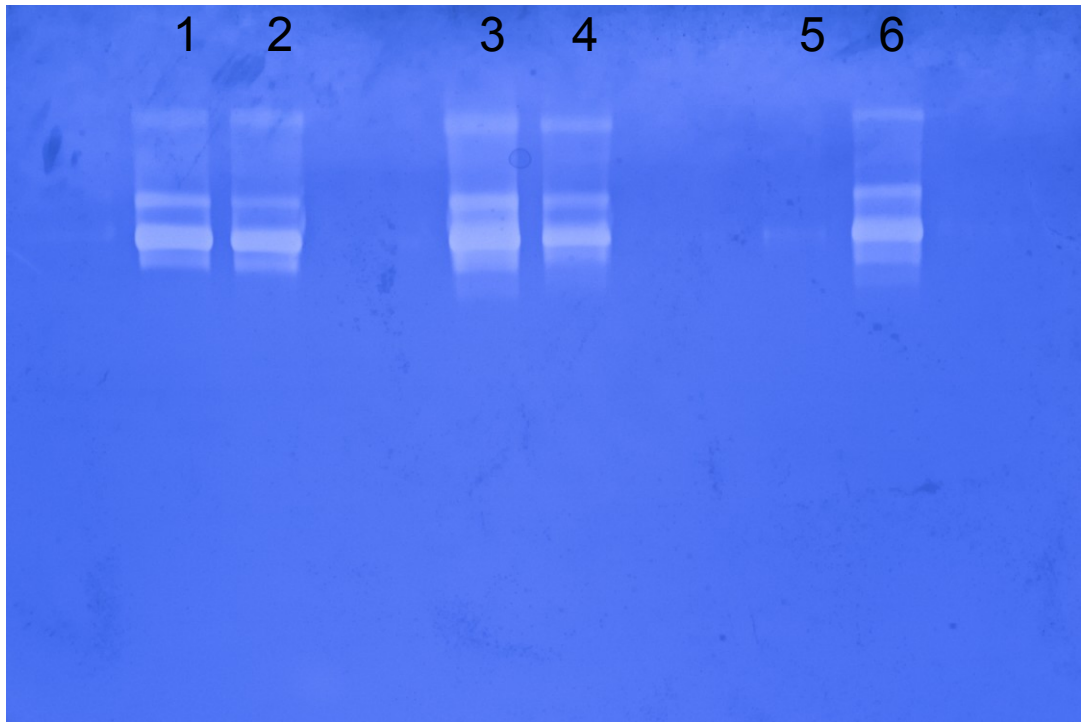
The gel-electrophoresis-chamber ran with 200 Volts at 0.78mA for 35 minutes. These settings appeared to be the best for my experiments.

## **6.6 Zymography**

Zymography is a special kind of gel-electrophoresis which indicates proteases. The procedure is similar to the polyacrylamide-gel-electrophoresis: 30  $\mu$ l of a solution which contain proteins (especially proteases) in this case pollen and nasal mucus is mixed with 30  $\mu$ l of running buffer. 13  $\mu$ l of this mixture are deposited on a gelatine gel.

This gel is electrified in the electrophoresis chamber. After that the proteins in the gel become renaturised and the gel gets into the incubator for 24 hours at 37° Celsius. During this time the proteases have time to destroy the gelatine proteins on a certain place on the lane. The proteins migrate depending on their size and electronic charge. After the

incubation the gelatine on the gel is coloured, except for the spots where the gelatine is destroyed by the proteases.



**Fig 24 Zymography-gel**

In the end the gel looks like a negative of a polyacrylamide-electrophoresis-gel. The white spots in the lanes are the proteases.

# 7 Results

## 7.1 Laboratory

Proteins do exist in the pollen-water-solution and they are expelled immediately from pollen when they get in contact with water. Protein-determinations showed what quantity and kind of proteases were present. Finally with the zymography it could be proofed that pollen contain proteases.

Further more I could proof that nasal mucus proteins get destructed when they get in contact with pollen. Therefore, nasal mucus was mixed with several different kinds of pollen. Then a polyacrylamide-gel-electrophoresis was done as well as with natural nasal mucus and natural pollen as a control. When you compare the results you can exactly see which nasal proteins were destructed by pollen proteases, which smaller protein-chains newly developed from the bigger proteins and which proteins did not change at all.

Because of using pollen from different species, a different grade of aggressivity the various plants' pollen could be shown in the polyacrylamide-gel-electrophoreses.

The table in chapter 7.3 shows the exact differences of aggressivity of different pollen types

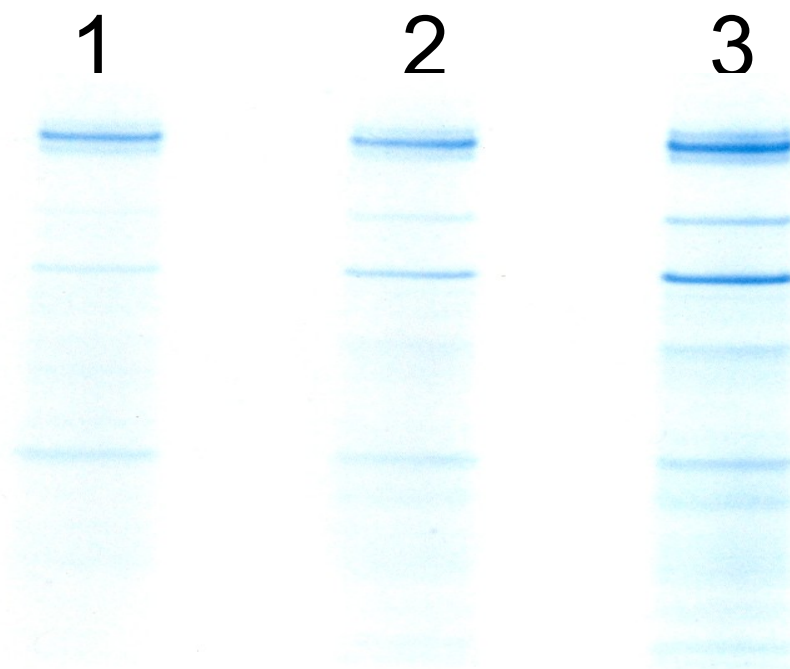
## 7.2 Experiments

As mentioned before, different Experiments had to be done to secure the theory that the nasal mucus gets destructed by the pollen proteases. Here is a chronologic list of these:

### 7.2.1 Experiment no.1, 26.02.2007

In the first experiment I tried to find out, how long it takes until the proteases have dissolved in fluid.

10mg of pollen were mixed with 50  $\mu$ l of 0,9% NaCl and incubated for 5, 30 and 60 minutes.



1.3.2007

Fig 1 three different incubation times

Here can be seen that there is no significant difference between the first lane (5 min incubation) and the second and third lane (30 and 60 min. incubation). This experiment showed, that pollen proteases immediately dissolve in liquid when they get in contact with it.

This is a very important fact, because if it took longer for the pollen to start destroying nasal mucus proteins, it would not be relevant for affected persons. Since the cilia move nasal mucus 2,5 cm per minute It does not take long until the pollen are swallowed in the larynx.

### **7.2.2 Experiment no.2, 27.02.2007**

In This experiment Electrophoreses of following kinds of pollen were performed to get a clue of the molecular sizes and weight of pollen proteins.

- 1.) *Helianthus annuus* (Sunflower / Sonnenblume)
- 2.) *Corylus avellana* (Hazelnut Haselnuss)
- 3.) *Betula pendula* (Birch / Birke)
- 4.) *Alnus glutinosa* (Alder / Erle)
- 5.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 6.) *Fraxinus excelsior* (Ash / Esche)
- 7.) *Fagus sylvatica* (Beech tree / Buche)

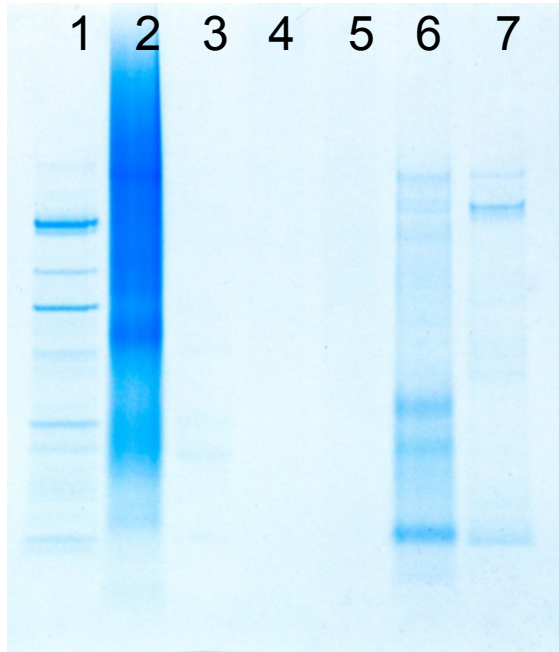


Fig 25 27.2.

10 mg of each pollen probe were incubated with 50  $\mu$ l of 0,9 NaCl solution and then incubated for one hour After that 10  $\mu$ l each were dispensed in the electrophoresis-chamber.

### 7.2.3 Experiment no.3, 13.03.2007

10 mg of pollen probes were mixed with 100  $\mu$ l of nasal mucus suspension and then incubated for one hour and after that 10  $\mu$ l each were dispensed in the electrophoresis-chamber.

Pollen Probes:

1.) Nasal Mucus

2.) *Helianthus annuus* (Sunflower / Sonnenblume)

- 3.) *Corylus avellana* (Hazelnut / Haselnuss)
- 4.) *Betula pendula* (Birch / Birke)
- 5.) *Alnus glutinosa* (Alder / Erle)
- 6.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 7.) *Fraxinus excelsior* (Ash / Esche)
- 8.) *Fagus sylvatica* (Beech tree / Buche)
- 9.) Nasal mucus

Two chambers ran with pure nasal mucus suspension.

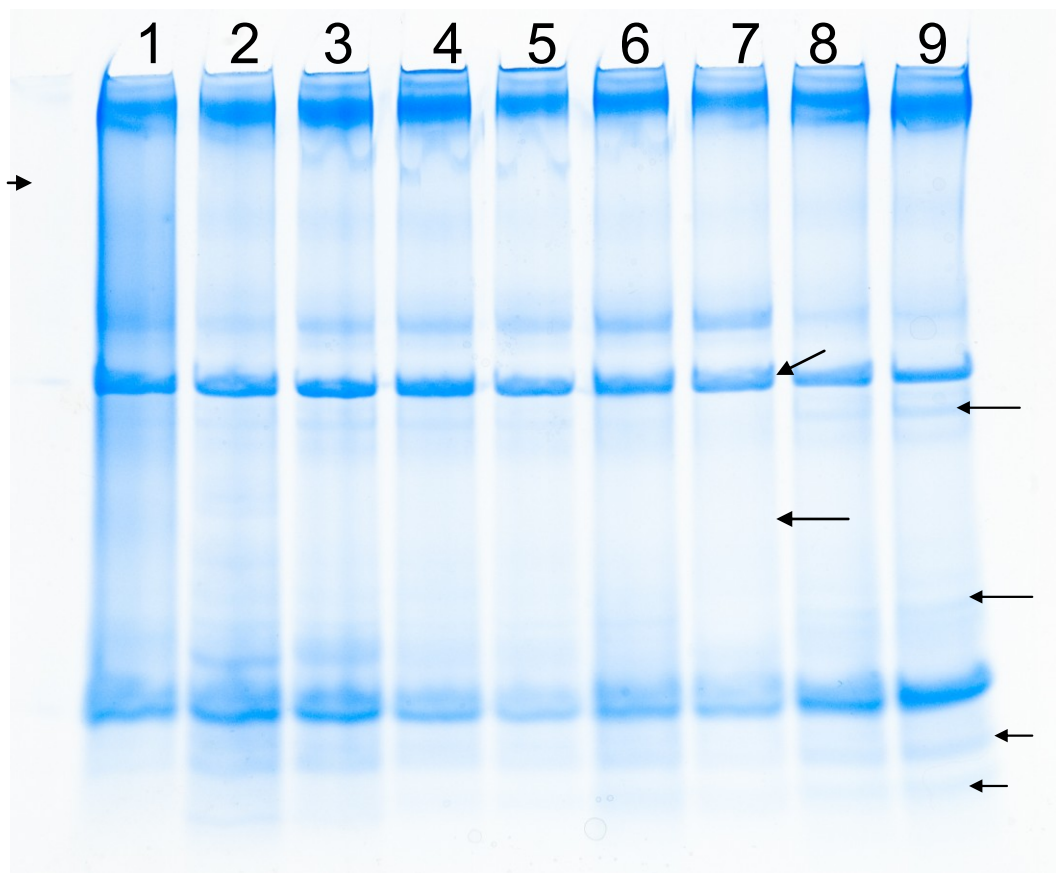


Fig 26 13.3.

This electrophoresis was made to see how nasal mucus proteins disappear when probes get mixed up with pollen. The disappearing bands are marked with arrows

#### 7.2.4 Experiment no.4, 08.05.2007

This experiment is the same as no.2 but with pollen of other tree species:

1.) *Betula pendula* (Birch / Birke)

2.) *Caprinus betulus* (Hornbeam / Hainbuche)

3.) *Corylus avellana* (Hazelnut Haselnuss)

4.) *Salix caprea* (Willow / Weide)

5.) *Abies alba* (Fir tree / Tanne)

6.) *Quercus robur* (Oak / Eiche)

The gel shows that some bands of nasal mucus proteins disappear.

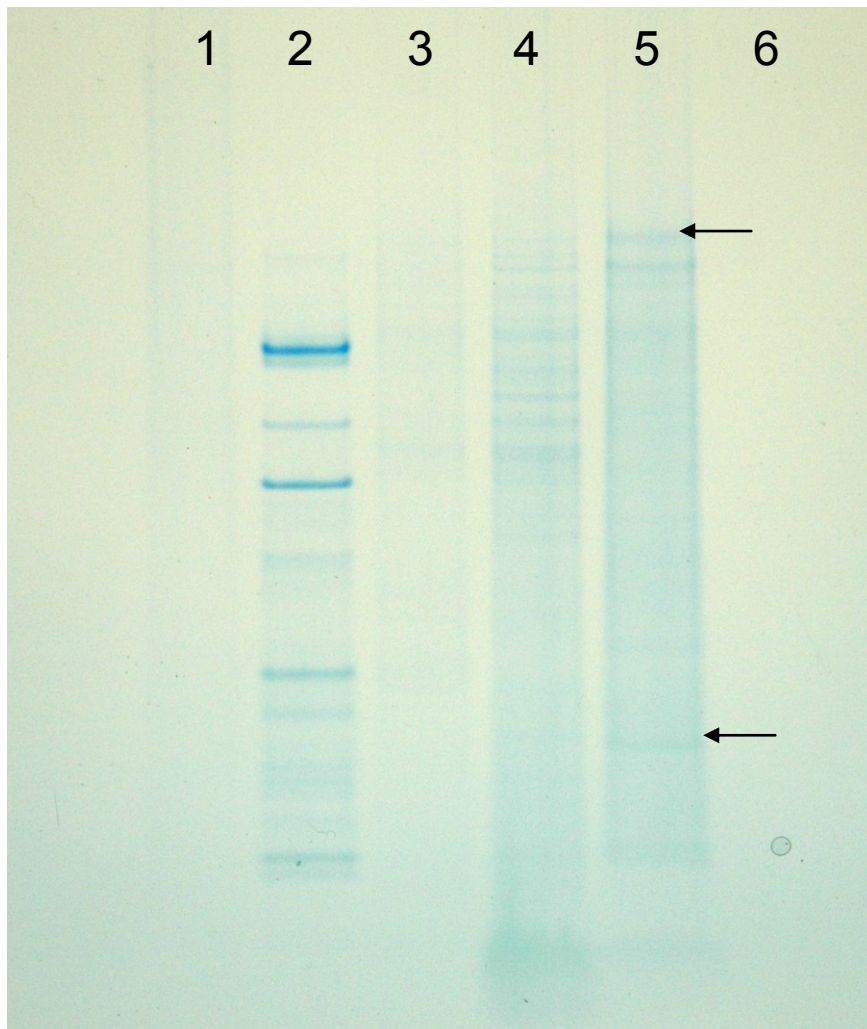


Fig 27 8.5.

### **7.2.5 Experiment no.5, 13.05.2007**

A zymography was made to proof that pollen contain proteases.

10 mg of pollen were mixed with 500  $\mu$ l of 0,9% NaCl and then incubated for one hour. 20  $\mu$ l each were disposed in the chamber and then it ran for 55 minutes.

These pollen were used:

- 1.) *Betula pendula* (Birch / Birke)
- 2.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 3.) *Corylus avellena* (Hazelnut Haselnuss)
- 4.) *Salix caprea* (Willow / Weide)
- 5.) *Abies alba* (Fir tree / Tanne)
- 6.) *Quercus robur* (Oak / Eiche)<sup>7</sup>

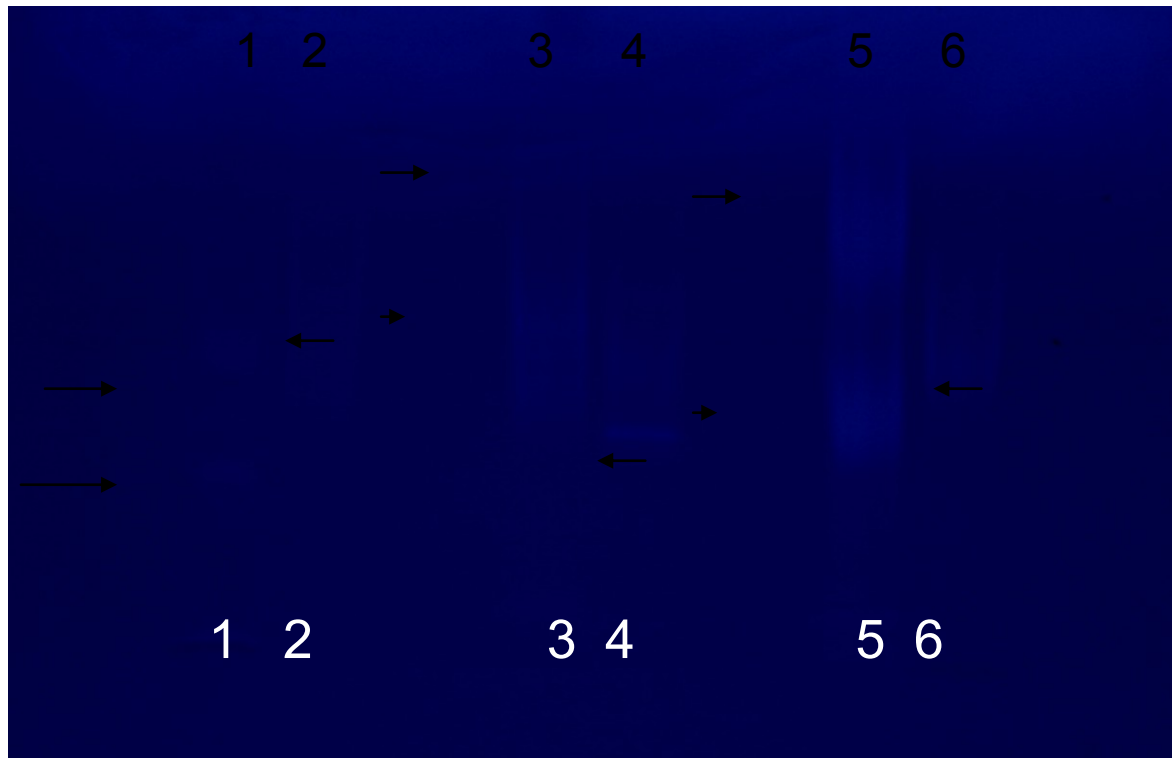


Fig 28 13.5.

### 7.2.6 Experiment no.6, 15.05.2007

120  $\mu$ l of a suspension of nasal mucus of a non-allergic patient was mixed with 10mg of each kind of pollen:

- 1.) *Betula pendula* (Birch / Birke)
- 2.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 3.) *Corylus avellena* (Hazelnut Haselnuss)

4.) *Salix caprea* (Willow / Weide)

5.) *Abies alba* (Fir tree / Tanne)

6.) *Quercus robur* (Oak / Eiche)

7.) Nasal Mucus

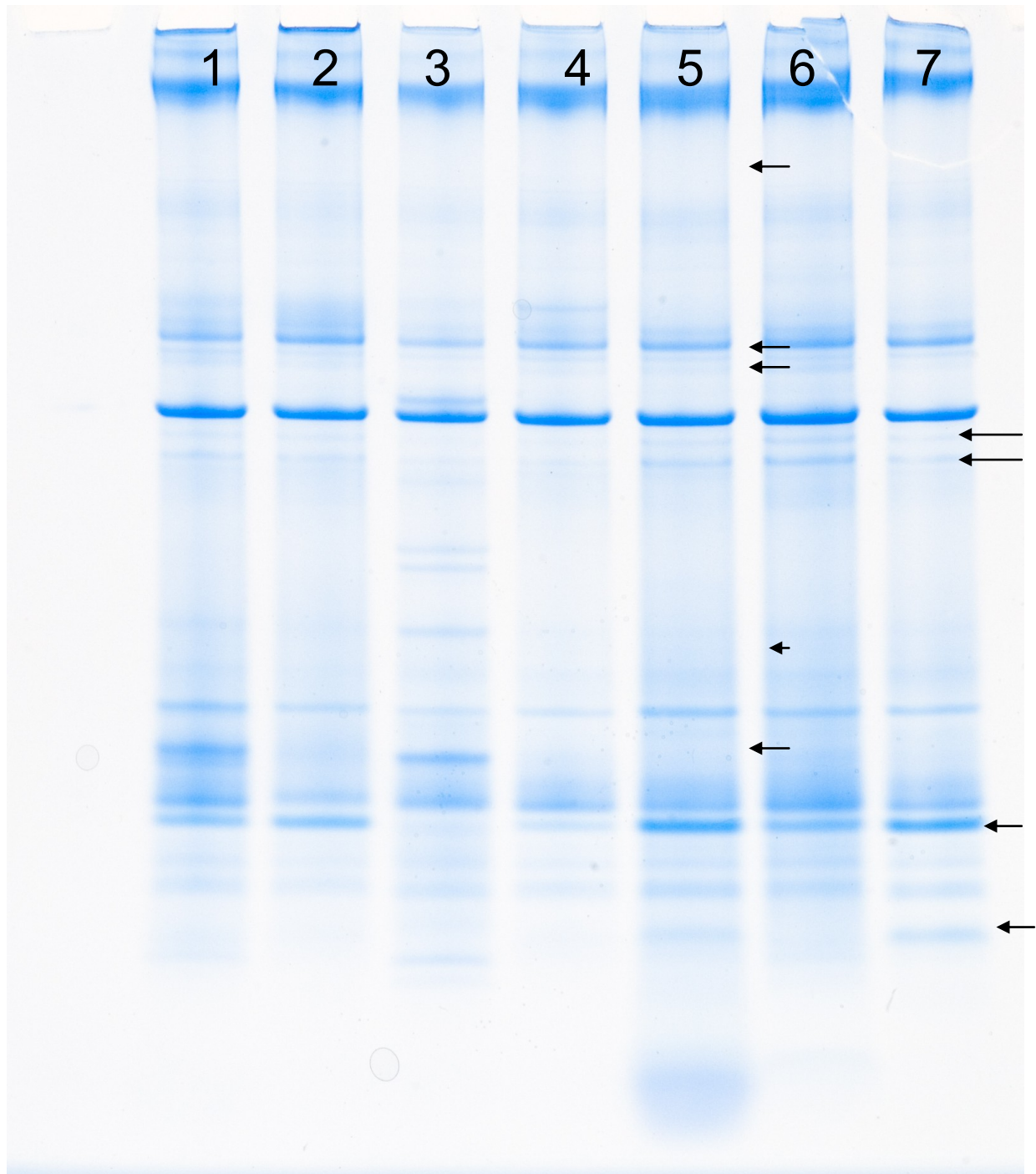


Fig 29 15.5.

After one hour of incubation and centrifugation 10  $\mu$ l of the mixture was disposed in the electrophoresis chamber.

The disappearing of protein-bands shows that these proteins were destroyed by pollen proteases. These bands are marked with arrows

### 7.2.7 Experiment no.7, 21.05.2007

A Zymography of probes of nasal mucus of six different patients (nr. 1,2 and 3 are allergic and 4,5 and 6 arenon allergic) was done. 20  $\mu$ l of each probe was put into the electrophoresis chamber and the runtime was one hour.

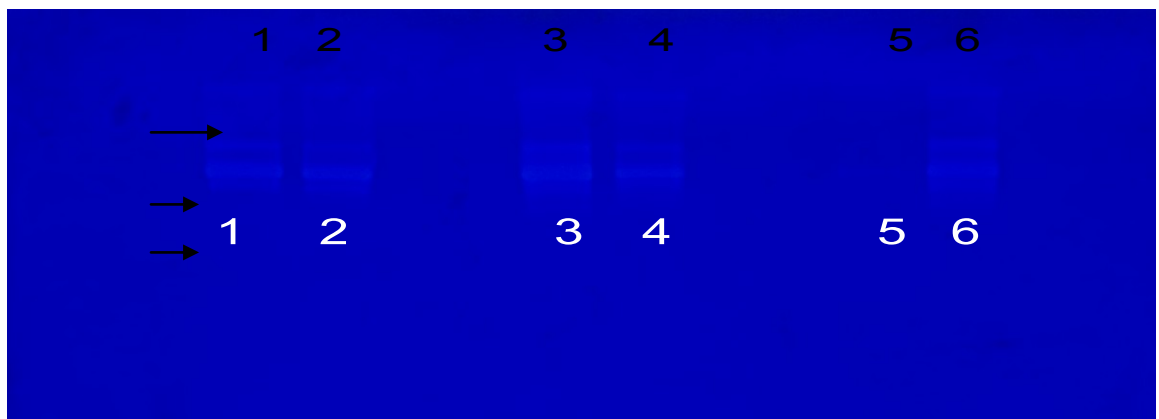


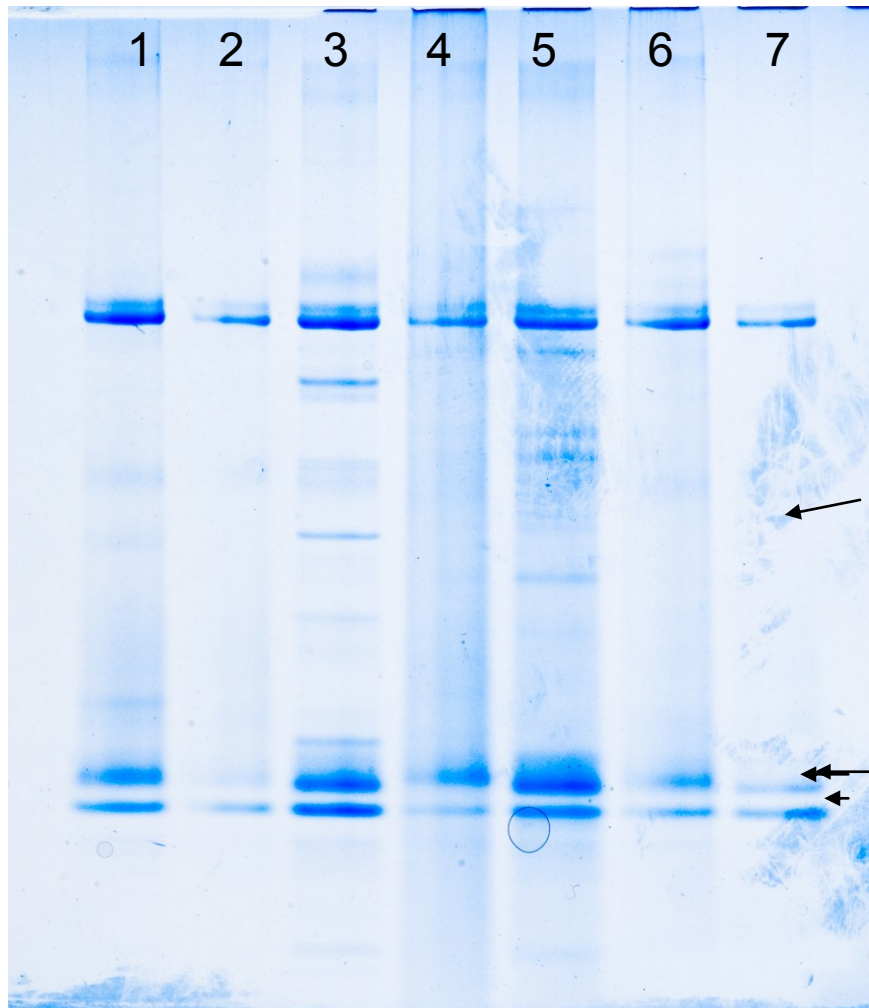
Fig 30 Experiment no. 7 21.5.

As the gel shows, there is no relevant difference between the composition of nasal mucus proteases of allergic and non allergic patients.

### **7.2.8 Experiment no.8, 22.05.2007**

120 µl of a suspension of nasal mucus of an allergic patient was mixed with 10mg of each kind of pollen:

- 1.) *Betula pendula* (Birch / Birke)
- 2.) *Caprinus betulus* (Hornbeam / Hainbuche)
- 3.) *Corylus avellena* (Hazelnut Haselnuss)
- 4.) *Salix caprea* (Willow / Weide)
- 5.) *Abies alba* (Fir tree / Tanne)
- 6.) *Quercus robur* (Oak / Eiche)
- 7.) Nasal mucus



**Fig 31 Experiment no. 8 22.5.**

After one hour of incubation and centrifugation 10  $\mu$ l of the mixture was disposed in the electrophoresis chamber.

The disappearing of protein-bands shows that these proteins were destroyed by pollen proteases. And There is also no difference in the reaction between non-allergic (experiment no. 6) and allergic probes. This fact proves that also non-allergic people can get problems when they get in contact with pollen.

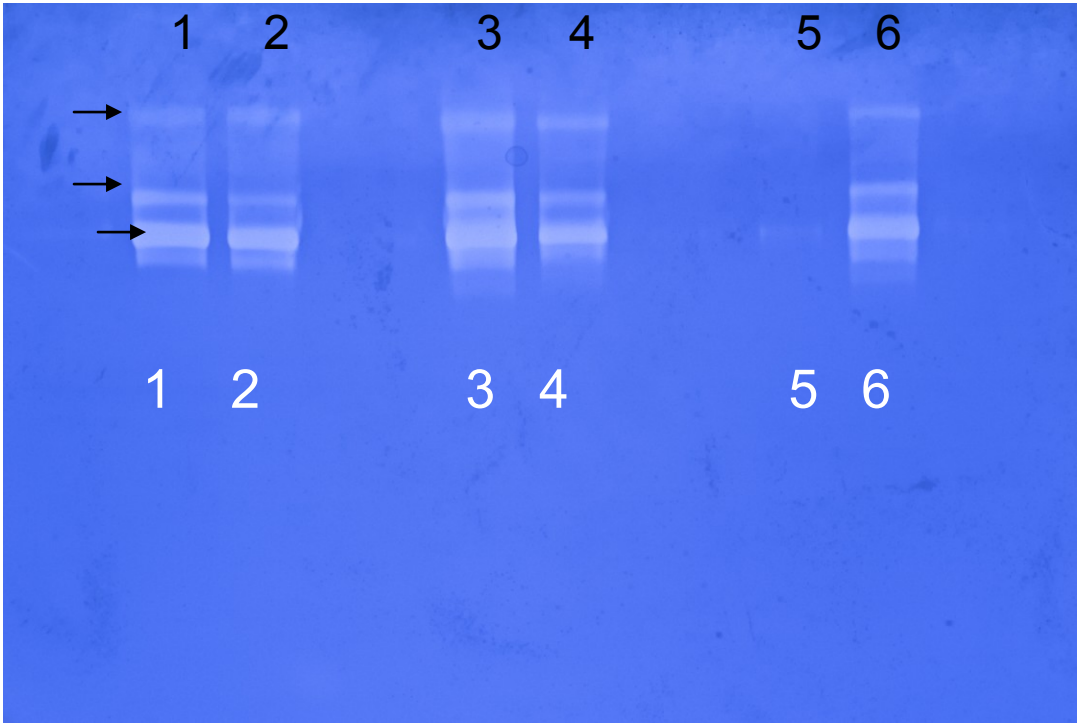
### 7.2.9 Experiment no.9, 23.05.2007

A Zymography of a mixture of 140µl of a nasal mucus suspension and 10 mg pollen of different kinds of grasses and composite plants:

- 1.) *Dactylis glomerata* (Knäuelgras)
- 2.) *Alopecurus pratensis* (Fuchsschwanz)
- 3.) *Phleum pratense* (Lieschgras)
- 4.) *Bellis perennis* (Gänseblümchen)
- 5.) *Brassica napus* (Raps)
- 6.) *Anemone nemorosa* (Buschwindröschen)

The mixture was incubated for one hour and then 13 µl were disposed into the electrophoresis-chamber which ran for one hour.

This experiment showed that there are proteases in pollen of grasses



## **Experiment no.10, 24.05.2007**

An electrophoresis of 140 µl of a suspension of nasal mucus of an allergic patient was mixed with each 10mg of pollen probes from grasses:

- 1.) *Dactylis glomerata* (Knäuelgras)
- 2.) *Alopecurus pratensis* (Fuchsschwanz)
- 3.) *Phleum pratense* (Lieschgras)
- 4.) *Bellis perennis* (Gänseblümchen)
- 5.) *Brassica napus* (Raps)
- 6.) *Anemone nemorosa* (Buschwindröschen)

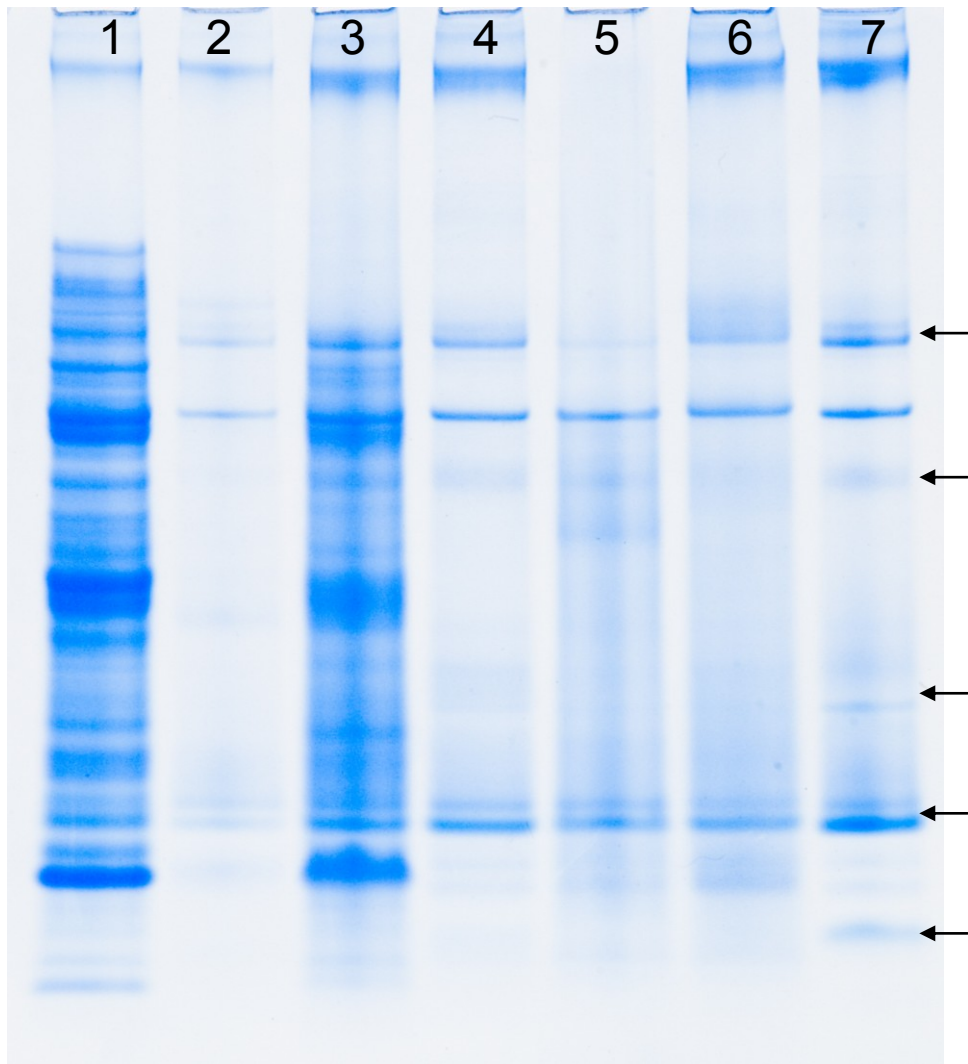


Fig 32 24.5.

The mixture was incubated for one hour and then 11,5  $\mu$ l were disposed into the electrophoresis-chamber which ran for 35 minutes.

The disappearing of protein-bands shows that these proteins were destroyed by pollen proteases.

### **7.2.10 Experiment no.11, 29.05.2007**

An electrophoresis of 10 mg of pollen probes mixed with 100 µl of 0,9% NaCl

- 1.) *Dactylis glomerata* (Knäuelgras)
- 2.) *Alopecurus pratensis* (Fuchsschwanz)
- 3.) *Phleum pratense* (Lieschgras)
- 4.) *Bellis perennis* (Gänseblümchen)
- 5.) *Brassica napus* (Raps)
- 6.) *Anemone nemorosa* (Buschwindröschen)

The mixture was incubated for one hour and then 9 µl were disposed into the electrophoresis-chamber which ran for 35 minutes.

This experiment was performed to compare pure pollen proteins to those from mixtures of pollen with nasal mucus.

### **7.2.11 Experiment no. 12, 15.06.2007**

In This experiment an electrophoresis of tear secrete, usual nasal mucus and the mucus of a patient with a punctum plug was performed.

This was made to find out, what role the mixing of tear secrete with nasal mucus in the nose.

The result is that there is no relevant difference between nasal mucus of patients with punctum plugs and normal nasal mucus.

### **7.2.12 Experiment no. 13, 09.08.2007**

This experiment shows a direct comparison of pure nasal mucus probes, nasal mucus mixed with pollen probes and pure pollen probes.

These pollen were tested:

- 1.) *Pinus sylvestris* (Föhre)
- 2.) *Corylus avellana* (Hasel)
- 3.) *Abies alba* (Tanne)
- 4.) *Carpinus betulus* (Hainbuche)

In this gel the difference between the protein bands of nasal mucus and those of the mixtures can be seen very well. Some bigger were destroyed by the pollen proteases.

## 7.3 Summary

In this table the results of the experiments of all three groups are summarized:

<u>Pollen sample</u>	<u>Protein determination</u>	<u>PAGE</u>	<u>Zymography</u>	<u>Reactions with nasal mucus</u>
<i>Corylus avellana</i> (Hazelnut / Haselnuss)	Proteins detected	Protein bands detected	Proteases detected	Nasal mucus proteins were destroyed
<i>Betula pendula</i> (Birch / Birke)	Proteins detected	Protein bands detected	No proteases detected	No reactions were found
<i>Alnus glutinosa</i> (Alder / Erle)	Proteins detected	Protein bands detected	Proteases detected	Nasal mucus proteins were destroyed
<i>Caprinus betulus</i> (Hornbeam / Hainbuche)	Proteins detected	Protein bands detected	No proteases were detected	No reactions were found
<i>Fraxinus excelsior</i> (Ash / Esche)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Fagus sylvatica</i> (Beech tree / Buche)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Salix Caprea</i> (Willow / Weide)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Abies alba</i> (Fir tree / Tanne)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Quercus robur</i> (Oak / Eiche)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Dactylis glomerata</i> (Orchard grass / Knäulgras)	Proteins detected	Protein bands detected	Proteases were detected	Nasal mucus proteins were destroyed
<i>Alopecurus pratensis</i> (Amaranth / Fuchsschwanz)	Proteins detected	Protein bands detected	A light proteases reaction was detected	Nasal mucus proteins were destroyed
<i>Phleum pratensae</i> (timothy grass / Lieschgras)	Proteins detected	Protein bands detected	Proteases were detected	Nasal mucus proteins were destroyed

Table 3: Summary of the results

# 8 Discussion

## 8.1 Pollen

When inspecting the pollen in the microscope big differences in the structure of pollen can be seen. All different plant species produce pollen in different shapes and sizes. When water is added to the pollen, in a split of a second they blow up and change their shape. During this process the proteases which usually have a function in fertilisation are freed from the body of the pollen. This is a very important fact, because it proves that when pollen get into the nose, the nasal mucus gets attacked immediately by the pollen-proteases. If the process of liberating the proteases from pollen took longer, there would not be enough time for the proteases to act because of the ciliar flow of the nasal mucus.

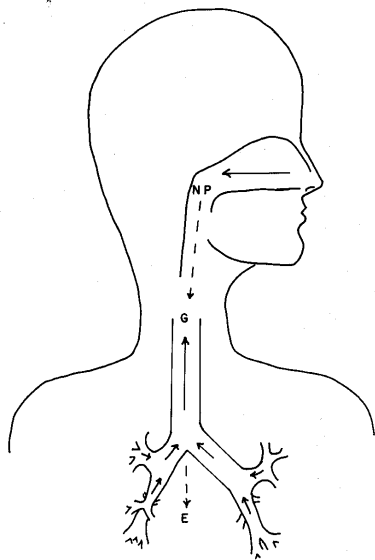


Fig 33 Ciliar flow of nasal mucus

All the nasal mucus is transported to the larynx, where it is swallowed together with the particles, such as pollen, which are filtered out of the inspiratory air.

Pollen which were stored for six years at room temperature and such which came freshly from blossoms were examined. Both of them showed the same effects in all the experiments.

## **8.2 Pollen proteases**

The experiments showed that pollen proteases do destroy nasal mucus proteins and they do cause allergy like symptoms when subjects get in contact with them.

## **8.3 Differences between different pollen species**

With the help of electrophoreses and zymographies differences between different pollen species could be proofed. The results can be seen in a table at 6.7.1.1.14

On one hand different kinds of proteins could be detected in all the probes which were investigated. And on the other hand proteases activities could be found as well. There were differences in the impact of proteases on the nasal mucus. Some pollen proteases destroyed more nasal mucus proteins than others. This means that some pollen species are more aggressive than others.

## **8.4 Nasal Mucus**

The nasal mucus of three patient groups were examined (Subjects with pollen allergies, without pollen allergies and subjects with punctum plugs). Our experiments showed that they have the same characteristics when they get in contact with pollen proteases. This shows that it does not matter whether one is allergic to pollen or not, the nasal mucus proteins will get destructed by pollen-proteases. The group with punctum plugs did not show a difference in the reactions as well. This explains that also non-allergics can get nasal problems when they get in contact with pollen when the air is polluted with them.

## **8.5 Effects of this study for the future**

Tests have shown that antiproteases can stop the destruction of nasal mucus proteins by pollen proteases. Doxycyclin, an antibiotic drug which is an antiprotease was mixed with pollen and nasal mucus. The procedure was the same as with pollen and nasal mucus. Before incubation three  $\mu\text{g}$  of doxycyclin were added. The electrophoreses and the zymographies have shown that the pollen proteases were inactivated and that there was no change in the nasal mucus proteins.

This could have an effect on the medication for persons who have pollen-allergies

## 9 References

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