AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD TECHNOLOGY

MicroMol GmbH in KARLSRUHE

POSTGRADUATE PROGRAM

Characterization of antibodies for the detection of celery in food

By

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A THESIS

Submitted to the Department of Food Technology at the Agricultural University of Athens

in partial fulfillment of the requirements for

the degree of Master of Science in

Food Science and Technology and Human Nutrition

Athens, Greece
May, 2012

Agricultural University of Athens
Department of Food Technology

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Acknowledgements

I would like to express my thanks to the company MicroMol GmbH in Karlsruhe for making this work possible. Especially Mrs. Dr Andrea Dreusch and Mr. Dr. Andreas Dreusch for giving me the thesis and made this study happen. I thank Mr. Dr. Wolfgang Rudy for his instructions, advice, guidance and attention and all my colleges in the laboratory for their help. At MicroMol GmbH it was nice to work with smiling people.

Also my supervisor Mr. Dr. Panagioti Skandami at the Agricultural University of Athens I would like to give many thanks for his willingness to support me abroad and helping me at my postgraduate thesis.

My gratitude also goes to the whole advisory committee for its assistance.

Especially I would like to thank my friend Mrs. Dr. Daniela Wittek for making corrections at this work and giving me advices.

Special thanks go to my mother Renate Politis and to my husband Christos Conomis for making it possible to have the time to work on this thesis in supporting me with the children.

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Abbreviations

APS	Ammoniumpersulfate
AP	Alkaline phosphatase
BCIP	5-Brom-4-Chlor-3-Indolylphosphat
BSA	Bovine serum albumin
DBPCFS	double blind placebo controlled food challenge
DMSO	Dimethylsulfoxid
DTT	Dithiothreitol
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
LTP	Lipid transfer protein
MW	Molecular weight
NBT	Nitrobluetetrazolium salt
OAS	Oral Allergy Syndrome
OD	Optical density
O/N	Over night
PCR	Polymerase chain reaction
RAST	Radioallergosorbent test
RT	Room Temperature
SDS	Sodium dodecylsulfat
SIT	Specific Immunotherapy
TEMED	Tetramethylethylendiamin
TMB	Tetramethyl benzidinedihydrochloride
WAO	World Allergy Organization

Summary in Greek

Τα αυξανόμενα κρούσματα αλλεργίας από κατανάλωση τροφίμων οδήγησαν την Ευρωπαϊκή Αρχή Ασφάλειας Τροφίμων (EFSA) στη θέσπιση του κανονισμού 2007/68/EC που κάνει υποχρεωτική τη σήμανση αλλεργιογόνου συστατικού στην ετικέτα του τροφίμου. Μεταξύ άλλων στη λίστα των συστατικών που προκαλούν αλλεργιογόνες αντιδράσεις συγκαταλέγεται και το σέλινο. Ως εκ τούτου η βιομηχανία τροφίμων αντιμετωπίζει το πρόβλημα σχεδιασμού πολύπλοκων και ακριβών συστημάτων διαχείρισης των αλλεργιογόνων και ειδικότερα του σέλινου εφόσον δεν υπάρχουν εύκολες και φτηνές ανοσολογικές μέθοδοι ανίχνευσης και ποσοτικού προσδιορισμού του. Ο μέχρι τώρα τρόπος ανίχνευσης του σέλινου είναι η PCR, μέθοδος που απαιτεί εκπαιδευμένο (εξειδικευμένο) προσωπικό και ακριβό εργαστηριακό εξοπλισμό, ενώ η ανάλυση περιορίζεται αυστηρά στον εργαστηριακό χώρο.

Στόχος της εργασίας ήταν η ανάπτυξη μιας ανοσοενζυμικής μεθόδου ανίχνευσης σέλινου. Είναι γνωστό από τη βιβλιογραφία ότι στο σέλινο υπάρχουν διάφορα αλλεργιογόνα μόρια, ένα εκ των οποίων αποτελεί η πρωτεΐνη Api g 1, η οποία βρίσκεται σε ποσοστό 20 % στο σύνολο των πρωτεϊνών στο σέλινο και υπάρχει σε δύο ισομορφές, την Api g 1.01 και την Api g 1.02. Η πρωτεΐνη αυτή παράχθηκε βακτηριακά από εισαγωγή πλασμιδίου στο γονιδίωμα του *E.coli* με σκοπό να χρησιμοποιηθεί στα πειράματα.

Σε ένα πρώτο σχήμα ανοσοποιήθηκαν δώδεκα κουνέλια. Χρησιμοποιήθηκαν σέλινο, πρωτεϊνικό εκχύλισμα από σέλινο, ανασυνδυασμένη πρωτεΐνη Api g 1 και ένα μίγμα πέντε πεπτιδίων (όλα τμήματα από την πρωτεϊνική ακολουθία της Api g 1.01) για την ανοσοποίηση των ζώων. Σε ένα άλλο δεύτερο σχήμα ανοσοποίησης, τέσσερα άλλα κουνέλια εμβολιάστηκαν το καθένα με ένα διαφορετικό πεπτίδιο. Τα δύο πεπτίδια αποτελούσαν τμήμα από την πρωτεϊνική ακολουθία της Api g 1.01 και τα άλλα δύο από την ισομορφή Api g 1.02. Σε αυτά τα τέσσερα ζώα, τα μόρια για την ανοσοποίηση ήταν μικρά αλλά επιλεγμένα έτσι ώστε η πρωτεϊνική ακολουθία να μην παρουσιάζει ομολογίες με πρωτεΐνες άλλων συγγενικών προς το σέλινο φυτών.

Εργαστηριακά χρησιμοποιήθηκε μια στήλη καθαρισμού ανοσοσυγγένειας πάνω στην οποία ήταν ακινητοποιημένη η ανασυνδυασμένη πρωτεΐνη Api g 1. Σκοπός ήταν η απομόνωση αντισωμάτων ικανών να ενώνονται ειδικά και συγκεκριμένα με την αλλεργιογόνο πρωτεΐνη. Όταν εξετάστηκαν σε τεστ Indirect-ELISA και κατόπιν σε Western Blot, για να προσδιοριστεί η ικανότητα τους να ενωθούν με το πρωτεϊνικό εκχύλισμα της σελινόριζας και με τη ανασυνδυασμένη πρωτεΐνη Api g 1 αντίστοιχα, έδειξαν επιτυχή ένωση με την αλλεργιογόνα πρωτεΐνη. Τα αντισώματα έδειξαν υψηλό βαθμό καθαρότητας. Από τη βιβλιογραφία είναι γνωστό ότι η πρωτεΐνη Api g 1 στην ηλεκτροφόρηση παρουσιάζεται στο δείκτη μοριακού βάρους των 15 kDa. Στα Western Blot τεστ παρατηρήθηκε αυτή η πρωτεΐνη στο χαρακτηριστικό ύψος της πηχτής, γεγονός που μας επιτρέπει να συμπεράνουμε ότι η ικανότητα των αντισωμάτων να ενώνονται με τη σελινόριζα οφείλεται σε συγκεκριμένη ένωση με την αλλεργιογόνα πρωτεΐνη Api g 1. Τα αντισώματα τα οποία έδειξαν τη μεγαλύτερη τάση ένωσης επιλέχθηκαν για να χρησιμοποιηθούν σε μετέπειτα πειράματα Sandwich-ELISA ως επισημασμένο πρώτο αντίσωμα.

Ως δεύτερο επισημασμένο αντίσωμα στο Sandwich-ELISA επιλέχθηκε ένα αντίσωμα (antiApi1-2Biotin) το οποίο αποκτήθηκε όταν ορός από το ζώο το οποίο είχε ανοσοποιηθεί με πεπτίδιο(aa121-140), τμήμα από την Api g 1.01 πέρασε μέσα από τη στήλη ανοσοσυγγένειας και στη συνέχεια συζεύτηκε με βιοτίνη έτσι ώστε να είναι εύκολη η ανίχνευσή του.

Πριν αναπτυχθεί το σύστημα Sandwich-ELISA έγιναν πειράματα ώστε να εξακριβωθεί αν το συγκεκριμένο αντίσωμα (antiApi1-2Biotin) δείχνει προτίμηση ένωσης με το σέλινο ή αν ενώνεται και με πρωτεΐνες άλλων φυτών με παρόμοια πρωτεϊνική ακολουθία. Όταν ίσες ποσότητες σελινόριζας, καρότου και μαϊντανού χρησιμοποιήθηκαν για να εξακριβωθεί η συμπεριφορά του συγκεκριμένου αντισώματος, αυτό επέδειξε ικανότητα να ενώνεται ειδικά με τη σελινόριζα και όχι με το καρότο και το μαϊντανό.

Στο Sandwich-ELISA τεστ που ακολούθησε, το παραπάνω αντίσωμα έδειξε να ενώνεται πολύ καλά με τη ανασυνδυασμένη πρωτεΐνη Api g 1, δεν κατάφερε όμως να εφαρμοστεί στη περίπτωση του Api g 1 στη φυσική του μορφή ως συστατικού της σελινόριζας, γεγονός το οποίο μπορεί να οφείλεται στην αναδίπλωση της πρωτεΐνης μέσα στη σελινόριζα.

Εφόσον κάτω από αυτές τις συνθήκες ένα τέτοιο σύστημα δεν ήταν εφικτό, έγινε προσπάθεια να εξακριβωθεί η εφαρμογή του αντισώματος antiApi1-2Biotin σε Indirect-ELISA τεστ. Σε μια προσπάθεια προσέγγισης της πραγματικότητας όπου το σέλινο βρίσκεται μονάχα σε ίχνη μέσα στο τρόφιμο ενώ το καρότο σε μεγαλύτερες συγκεντρώσεις, χρησιμοποιήθηκαν σε δοκιμή Indirect-ELISA δεκαπλάσιες συγκεντρώσεις καρότου και μαϊντανού. Τα αποτελέσματα έδειξαν ότι δεν ήταν εφικτή η διάκριση, οπότε η χρήση του αντισώματος είναι περιορισμένη. Μπορεί να χρησιμοποιηθεί σε τρόφιμα που ως γνωστό δεν περιέχουν καρότο, όπως για παράδειγμα μαρινάδες για κρέας. Επίσης μια άλλη πιθανή εφαρμογή του, θα ήταν για τον έλεγχο καθαρισμού σε μονάδες τροφίμων που επεξεργάζονται σέλινο. Μετά από ένα σωστό καθαρισμό, στα νερά απορροής δεν θα πρέπει να ανιχνεύονται ίχνη ούτε από σέλινο ούτε από καρότο.

Σε μια προσπάθεια παραλαβής άλλων αντισωμάτων ικανών να χρησιμοποιηθούν για την ανάπτυξη ενός συστήματος βασιζόμενο στο Sandwich-ELISA πρότυπο, οροί των ζώων που είχαν ανοσοποιηθεί κατά του σέλινου, πέρασαν μέσα από στήλη καθαρισμού ενεργοποιημένης σεφαρόζης ενωμένης με πρωτεϊνικό εκχύλισμα σέλινου. Σκοπός ήταν η απομόνωση αντισωμάτων με ευαισθησία ένωσης ειδικά με το σέλινο. Τα αντισώματα αυτά σε τεστ Indirect-ELISA και Western Blot έδειξαν να ενώνονται χωρίς ιδιαίτερη επιλεκτικότητα, με πολλές πρωτεΐνες της σελινόριζας αλλά και του καρότου.

Στο μέλλον θα πρέπει να εξεταστεί πως θα καταστρωθεί ένα ELISA σύστημα για να συμπληρωθεί το ήδη υπάρχον σύστημα. Ενδεχομένως πρέπει γίνουν επιπρόσθετες ανοσοποιήσεις. Πρέπει να ευρεθούν μικρά πεπτίδια για την ανοσοποίηση, τα οποία δεν εφαρμόζουν ομολογίες με άλλες πρωτεΐνες φυτών και τα οποία είναι σταθερά στη θερμοκρασία έτσι ώστε να μπορούν να χρησιμοποιηθούν και σε θερμοκρασιακά επεξεργασμένα τρόφιμα. Παράλληλα πρέπει να μελετηθεί η ανοσοποίηση άλλων

ζώων, όχι κουνελιών, τα οποία αντιδρούν στη σελινόριζα με μεγαλύτερο αριθμό αντισωμάτων.

Λέξεις κλειδιά
Api g 1 ,celery, food allergy, directive 2007/68/EC, food labeling, celery allergy, detection of allergy, carrot, immunoblot, immunodetection, Politis Anna γεωπονική σχολή Αθηνών, Τμήμα τροφίμων, αλλεργιογόνα, σέλινο, ανοσολογική μέθοδος , ανίχνευση σέλινου, Πολίτου Άννα.

1. Introduction

1.1.Definition of allergy

Allergy is a specific reaction of the body´s immune system to a substance that would normally be thought of as harmless. It is not a failure of the immune system, but rather an over activity. The substance that is responsible for the allergy is called allergen. The response of an allergic person to an allergen can cause a wide range of symptoms. Many people suffer because of such reactions. The incidence of allergies is worldwide increasing, especially in the developed countries. Climate change, exposure to air pollutant and migration from one country to another which has the consequence of contact with new allergens, new diet, new housing conditions, are reasons of the increasing allergy incidence.[1] Genetic, environment and lifestyle factors interact with each other and influence the way allergies are geographical spread.[16] Estimated 20-50% of the population is affected.[23] Very problematic is the increase in children, who bear the largest burden of the rising trend which has occurred over the last twenty years.[11]

1.1.1 Immunological reasons for allergies

White blood cells or leucocytes are cells of the immune system involved in defending the body against foreign materials and infectious disease, yet there are several different types of leucocytes. All have many things in common but they are distinct in form and function. Figure 1.1 shows how the white blood cells can be categorized.

Lymphocyte blood cells work with body´s chemicals to destroy specific antigens (foreign particles). The blood has three types of lymphocytes:

- B-cells which are responsible for the production of antibodies that bind to pathogens and destruct them. Upon an antigen binding to a B-Cell's receptor they proliferate and secrete a free form of those receptors (antibodies).
- Natural killer cells (NK) which kill cells infected by a virus or cells that have become cancerous.
- T-Cells which are divided into two subclasses:
    1. T-Helper-Cells (T_H). When they get activated they divide rapidly and secret proteins (cytokines) that activate the immune system. There are five different T_H groups. They secrete different cytokines, so they can aid in different kinds of immune responses.
    2. Cytotoxic T-Cells. They are also known as CD8+cytotoxic T-Cells which bind antigens of virus- infected or cells and kill them.
    3. Suppressor T-Cells. Their role is to shut down T-Cell-mediated immunity at the end of an immune reaction and to suppress auto-reactive T-Cells, that escaped the procedure of negative selection in the thymus.

It is known that allergies result from an imbalance in the T-Cell compartment of the immune-system. Precisely, allergies are accompanied by an increased activity of the T-Helper2 (T_H2) cells relative to T-Helper1 (T_H1) cells, giving rise to an increased production of IgE (Immunoglobulin). IgE specific for an allergen is not normally detected in the blood but in the case when a person becomes sensitized to a substance.

Substances causing an allergy (allergen) produce a specific IgE that is unique and will only react with it. The reaction is like lock and key. The combination of IgEs with their corresponding allergen is responsible for a release of chemicals such as histamines which cause the allergic symptoms. A person can have a specific IgE to more than one substance and therefore be allergic to more than one substance.

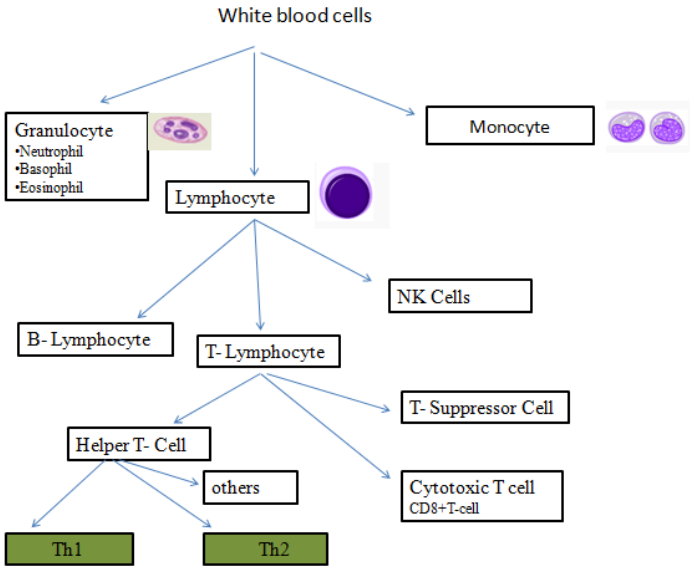


Fig.1.1: Schematic representation of the classification of blood cells

The allergic reaction can be divided in different stages. During the primary contact phase, immature B-Cells are transformed into plasma cells through a complex series of processes. At the same time antibodies, IgE are produced. They attach themselves onto mast cells as Fig.1.2 shows. Mast cells are tissue based cells that have an affinity for blood vessels. When the antigen enters the body at a second time the “antibody armed” mast cells recognize the specific antigen, their receptor is cross linked by the antigen and they get activated causing allergic reactions.

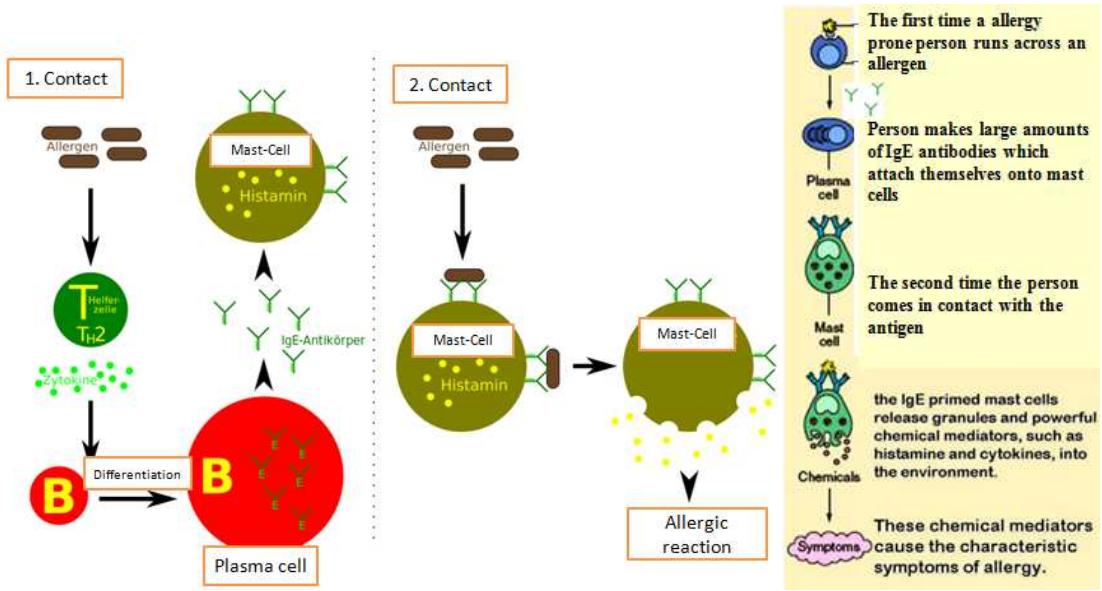


Fig. 1.2: The procedure of allergic reaction

B-Cells are transformed into memory cells having the ability to respond quickly when the antigen enters the body for the second time. The antibody production can be either primary, occurring at the first exposure to an antigen and is called sensitization, or secondary, after exposure to the same or similar antigen at a later date.

1.2 Food allergenicity

Some foods are specifically linked to allergic reactions. According to WAO (World Allergy Organization) in 2011 globally 220 – 520 million people suffered from food allergy. In the European population 11-26 million people are affected. [1] Children continue to be the most affected part of the population. The highest incidence occurs during their first year of life. Children are sensitive to minute amounts of allergen so that attention must be paid to food contaminations. Many infants hopefully stop the clinical reaction to food as they become toddlers. It is reported that 90% of infants allergic to cow´s milk and 50% of infants allergic to egg could tolerate these at the end of their third year. On the other hand 80 % of patients allergic to peanuts or codfish never got rid of the allergy. So it is necessary at periodical intervals to re-evaluate the avoidance strategies one might have structured.

Nevertheless a food allergy has an important socio-economic impact. It influences the quality of people's life, reduces self-esteem and inhibits family activities. Children cannot eat from school canteens, vacation overseas is mostly rejected. Economical, the medical treatment of the patients costs millions of Euros. Hidden costs occur through product recalls and because of expensive allergen management systems needed at the food manufactory.

For all these reasons to prevent and treat allergic reactions to food is a major challenge. Although worldwide there is an increase of symptoms of asthma, allergic rhinconjuctivitis and eczema in childhood, there is no information about the prevalence of food allergy over the time so as to estimate how the tendency will be. This is due to the lack of reports. [11]

1.2.1 Factors that influence the distribution of food allergies

Some parameters influence the distribution of food allergenicity.

- Kinds of food

Food differs in its allergenic potential. Some food has larger intrinsic capacity to induce allergic sensitization than others. High allergenic foods are peanuts, shrimps and sesame seeds. On the contrary potatoes even in high consumption levels don`t induce allergy.

- Consumption factor

This factor has two levels:

- The eating habits.
- The protein allergen level of the particular food.

In the region where a certain food is very often consumed, there is a high possibility of allergy. Fish allergy is more common to Norway. The

relationship dose-response for the development of allergy for a specific food is unknown. It is sure that a certain amount of consumption is necessary for the development of the allergy. If the dose-response curve is linear, exponential or whether it levels out after a particular level of exposure is unknown. Some proteins are in large quantities in food. Statistically these proteins have a greater chance of becoming allergens, than proteins that are present in small quantities.

- Food processing

Processing may alter the allergenicity. According to local traditions and cultural factors some foods are differently cooked or processed which leads to different levels of allergens because of degradation or creation. This is particularly true for peanuts which are either dried or roasted in the western hemisphere ahead of consumption, in China on the other hand, they are mostly fried and then contain less allergens.

- Cofactors

o People with infections or people with pollen exposure are more sensitive and may have a higher allergen uptake.
o Thunderstorms during pollen season may hydrate the pollen grains and their fragmentation. Atmospheric biological aerosols are built which carry allergens and can easily proceed into the patient's body.[1]
o Physical exercise, alcohol, and non-steroid antiinglammatory drugs advance allergenicity.
o Very important is the "matrix effect". It has been shown that the fat content of the food has a basic role in the allergenicity. The material surrounding the allergen in foods may influence whether or not an allergic response will be developed, how strong it will be and how fast it will develop. Consequently, the dish eaten and food habits may influence to some extent that an allergen will trigger a reaction. A Norwegian National Reporting System found out that food allergy is more common in women and the female to male ratio is 3:2 at food allergies. The explanation is possibly the physiological differences and health seeking behavior [9],[11].

1.2.2 Food Allergens

Food allergen refers to both, the whole food as a complex and to the chemically defined compounds which are responsible for allergenicity. Mostly these compounds are proteins. They are natural substances with various biological properties. They can be storage- proteins, metabolic, protective, transport proteins, regulatory proteins or enzymatically active (enzyme inhibitors, carbohydrases, proteases). They mostly have a globular compact structure, stabilised by hydrogen and disulphide bonds.

Often, after post translational modification, they get glycosylated. The 3-D structure and glycosylation are factors which contribute to the stability of the protein, related sometimes with allergenicity. Nevertheless also labile proteins, with loose structure, sensitive to degradation through protease, may be allergens. Most of the protein allergens are stable to processing and resistant to digestive enzymes. Allergenicity is never due to one single protein rather many different proteins in the food contribute to the symptom. Of course not all of them are recognized by all patients allergic to this

food. These which are recognised by more than 50% of the population are the major allergens. This characterisation relates only to the frequency of recognition by the IgE and doesn`t relate to the severity of the clinical manifestations of an allergic reaction. Triggered by major or minor allergens the clinical reactions may be similar.

In every single protein there are many molecular immunoreactive structures, called epitopes onto which the IgEs bind. The epitopes are correlated with the allergenicity and are widely spread within the protein molecule. Epitope mapping is the characterization of all the epitopes into the protein structure. Depending on their structure, sequential and conformational epitopes are present in food allergens. Conformational epitopes are associated to the secondary and tertiary structure of the protein allergen. They are mostly affecting B-Cells. Such epitopes once the protein gets denatured they become modified or even destroyed. The sequential epitopes are linear. They are formed on the peptide chain of the allergic protein by a sequence of amino acid residue. [11] They are mostly recognized by T-Cells. It has been shown that there is a particular significance of some epitopes which depend on their structure and location within the protein. Short linear epitopes which may be located in hydrophobic parts of the protein can be used as markers of persistent food allergy. [7] This may provide information on persistence and severity of clinical reactions.

Sometimes cross reactions between allergens is possible. Food and pollen or food with other food can cross react. An example is birch pollen and hazelnut. This makes the situation very complex. Such cross reactions are due to proteins that have similar sequence homology and/or structural features or common epitopes. These sequences are mostly highly conserved and are translated to important biological activities and functions, vital for the plant. [9]

1.2.3 Symptoms

Allergic reactions can occur after ingestion, inhalation or contact with foods. A two-step process is required for an allergic reaction to take place:

1) First, the capacity must be established to respond with an allergic reaction when exposed to the particular allergen. This requires an immune response to take place, at which the immune system answers with IgE antibody production against the allergen. This is the so called induction phase, or sensitization.
2) Once an individual has become sensitized to a particular allergen, the individual can develop a symptomatic allergic reaction when again exposed to the allergen in question. This is called the provocation or triggering phase. [9]

A reaction to food can cause clinical symptoms as shown in Fig.1.3 [9].They can be categorized in:
a) immune-mediated reactions (IgE med) to foods mediated either by IgE antibodies or other immunological pathways reactions. The symptoms occur immediately or within 1-2 hours after ingestion then the situation is described as food allergy.
b) Non-immune mediated responses (non-IgE med) that are dependent on enzyme deficiencies, pharmacological reactions, or unknown mechanisms. These reactions occur later. They are described as food intolerance [11][19].

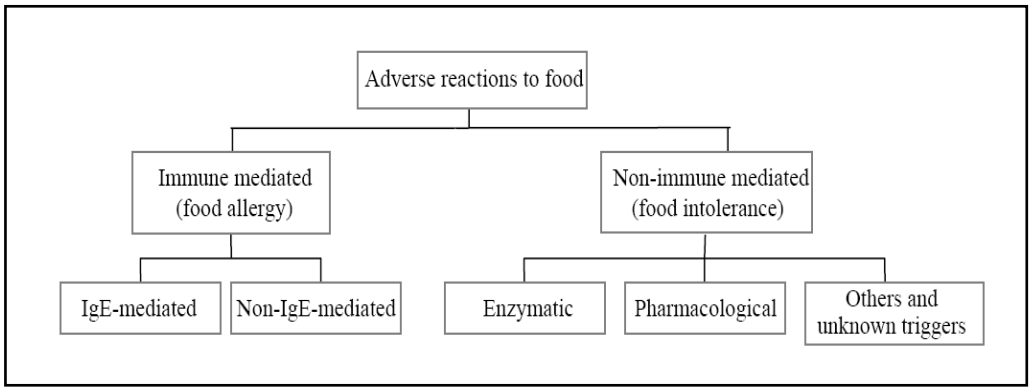


Fig: 1.3: Categorization of food reaction symptoms [9]

The associated symptoms are listed in Table 1.1

Table 1.1- Symptoms related with food allergy [11]

IgE-Mediated	Mixed IgE and non-IgE	Non- IgE mediated
Angiodema	Eosinophilic gastroentropathies	Protein-losing enteropathy
Nausea and vomiting	Gastroesophageal reflux	Dietary protein proctocolitis
Rhinoconjunctivitis	Colic	Heiner syndrome
Laryngeal edema	Pulmonary hemosiderosis	
Systemic Anaphylaxis	Urticaria	
Oral allergy syndrome	Oral itching and abdominal pain	
	Diarrhea	
	Wheeze, Asthma	
	Atopic dermatitis	

People with the Oral Allergy Syndrome (OAS) complain about symptoms of itching, tingling swelling of the tongue, mouth or throat, occurring after eating a food. This syndrome is associated with pollinosis. 23-47% of these patients are suffering from allergy to some kind of pollen. Due to similar chemical structures there is a relationship of the pollen to the food [5] [4].

The most severe manifestation of food allergy is anaphylaxis. In the USA the estimation is that every year there are 30.000 anaphylaxis episodes, 2.000 have to stay at hospital and 200 of these occasions dye. For children the mortality is 0.6-5% of such episodes. Asthma or rhinitis caused by food is rather unusual. [11]

1.2.4 Diagnosis of food allergy

The family's history is a very useful tool at the procedure of food allergy diagnosis. If one parent is atopic the possibility that the child develops this type of condition is 20-

40%. When two parents are atopic the possibility increases at 50-80%. The tests that are commonly used for the diagnosis of food allergies are:

Skin prick test. When there is suspicion about IgE mediated reactions to food according to a diet diary, then skin prick test are performed. A small amount of the allergen is placed onto the skin and then introduced into the epidermis by pricking the skin. After 15 min the reaction is observed. A positive reaction is shown by the fast development of localized reddening and swelling. Negative reactions have an accuracy of 95% while positive reactions only 50-60%. [9] In order to get comparable results the allergen extracts that are being used at these tests must be standardized. [11]

RAST. The radioallergosorbent test and other derived immunochemical tests are used to determine food-specific IgE antibodies. Used are microwell plastic strips, which have been coated with the allergen proteins. Serum is added to the microwell and the bound IgE antibody is detected using an anti-human IgE antibody enzyme labeled.

DBPCFS (double blind placebo controlled food challenge). At a hypo allergic basic nutrition, foods are introduced at one step procedure and then the reaction of the allergic person is observed. By this way the food that causes the allergy can be identified. Also by this way patients can find out many foods that are harmless for them. This procedure is expensive and takes time. Also the DBPCFC is sometimes impractical, as there is a risk of severe reactions. [9]

1.2.5 Treatment

The best treatment is to avoid the food identified as allergenic for the patient. Ingestion, inhalation and skin contact can cause severe reactions, so every three of the above must be avoided. Also there must be concern about potential exposures to relevant food allergens. [11] For some food, heat treatment can alter the allergen so that an allergic person can consume the cooked food even if he is allergic to the raw material. [17]

In the past few years some desensitization protocols have been studied so as to create an active induction of tolerance. Small portions of the food are introduced into the allergic person´s diet. The dose is getting larger until the person is taking the normal portion and is not any more nutritional restricted. The diet has to be followed strictly.
Hyposensibilisation (specific Immunotherapy SIT) is based on the same principle. The patient is vaccinated in this case with increasingly larger doses of an allergen. The aim is to induce immunologic tolerance. After the discovery of the T_H1/T_H2 subsets, it became clear that specific immunotherapy is able to restore the relative imbalance and to correct the T_H2 biased response. Subcutaneous injection immunotherapy SCIT is a modified form of SIT. The patient gets the allergen subcutaneous. It is a highly cost-effective treatment strategy which results in an improvement in patient´s life. Due to a rare serious side-effect of anaphylaxis, its use is possible only at specialist centers.

Epidemiological data highlights the involvement of micro flora of the intestinal in the development of allergic diseases. Strategies have been designed so as to involve “success factors” rather than to exclude risk factors. The effect of pro-biotics and pre-biotic supplementation on the development of allergy is being studied. [11]

1.2.6 Legislation

In order to protect themselves, consumers have the right to get information about what they eat. So as to choose a product without the risk of adverse reactions, they must be able to identify the allergen. For food allergic consumers labelling is a helpful tool. Large areas in the world lack legislation on food labeling at all. In the USA Food manufacturers have to list in lain and in common language, if any of the eight major allergens are present. These are: milk, egg, peanut, tree nut, fish, shellfish, wheat and soy.

According to the EU-Legislation manufacturers are required to declare all ingredients present in pre-packaged foods sold in the EU. Allergenic ingredients that are by law required to appear on food labels are listed at Annex IIIa of the Directive 2007/68/EC which is attached as Annex 1 in this work. At 26 November 2007 Directive 2005/26/EC was replaced. Products placed on the market before 31 May 2009 in compliance with the alt Directive can be marketed until their stocks are exhausted. In the Directive the phrase “Products thereof” include all derived products, originating from an ingredient included in Annex IIIa, obtained after one, or several processing stages. Allergenic ingredients, as part of carry-over processing aids, additives and solvents are considered ingredients and must be clearly indicated on the label with a clear reference to the name of the ingredient from which they originate. The legislation does not apply to no-intentional cross contamination of foods with allergenic ingredients. [8]

Responsible for the appearance of this list is the European Food Safety Authority (EFSA).The legislation is going to be altered when new data will appear. So foods may be added or deleted from the list.

This legislation makes manufacturers develop strategies to be able to detect which batch might contain allergens and to label it properly. Also traceability is a useful tool so as to recall suspicious batches. The obligation to label every item on the list of the Directive arises concern that too many foods containing only small amounts of these allergenic foods are over labeled. Many manufacturers, so as to avoid expensive allergen management systems, label their products with the indication “may contain”. [11] By this way allergic consumers are restricted from the consumption of potentially safe food.

Most of the foods listed at the Annex IIIa of the Directive 2007/68/EC are basic products with high nutritional value at a healthy diet, but cannot be eaten by allergic people. [20] Some of the food allergies are very widely distributed all throughout Europe, while others are more geographically restricted. Nevertheless how spread the allergy is, Directive 2007/68/EC obliges food manufacturer to label all ingredients or their derivatives that can cause allergic reaction or intolerances to sensitive consumers. On this list celery is included. Consequently, products with celery as ingredient, must clearly list it on the label [9].

1.3 Celery- Hazard Identification

Apium graveolens is a plant species in the family *Apiaceae* commonly known as celery or, depending on whether the stalks or roots are eaten: celery refers to the former and celeriac to the latter. The plant grows wild in the Mediterranean area and at the Himalayas in Asia. It is also widely cultivated. Celery is not only consumed raw in salads, but is often found in prepacked food. It is a constituent of sauces and soups and as spice, dried and powdered, it is used in many processed foods. Because of its aromatic flavour it is widely used in the food industry.

1.3.1 Allergenicity of celery

The fact that ingestion of celery may lead to intolerance reactions in humans, has been known for almost 70 years. The first case of an allergic reaction to celery root was observed by Jadassohn and Zaruski in 1926 in Zurich. Nowadays such reactions are common especially in Switzerland, Germany and France. They occur predominantly to raw celery and less frequently to cooked celery. Celery powder can also cause reactions comparable to these of raw celery. The frequency of allergy to celery tuber is higher than to celery stick.

In France about 30% of allergic reactions to food are assumed to be caused by celery. In Switzerland approximately 40% of patients with food allergy are sensitized to celery root. Not every individual who suffers from a celery allergy react to the same components of that foodstuff. [9]

1.3.2 Clinical Symptoms

Some food allergies induce only mild symptoms. But allergic reactions to celery are frequently more severe. Hopefully people with sensitization to celery and carrot have symptoms after digestion of these particular foods only in 21% of the cases. (Chi-squared Test, p =0,01) [9].While in people with sensitization to apple symptoms occur in 90% of the cases. The clinical syndromes of the allergy as being analysed at different studies are listed in Table 1.2. [29]

Table 1.2: Frequency of reactions after digestion of celery

Symptoms / Ref.	(1)	(2)	(3)	(4)	(5)	(6)
Anaphylaxis	19%	15%	2.9%	20%	%	
Shock fragments	10%		2.9%			
Cutaneous						
Angio-oedema	29%		31%			14%
Contact urticaria			17%			
Urticaria	10%		5.7%		17%	
Urticaria / Angio-oedema		85%		90%		
Urticaria / flush / pruritus						23%
Oral allergy syndrome					87%	55%
Gastrointestinal					20%	14%
Diarrhea	6.5%		2.9%			
Respiratory		40%		35%		
Asthma	6.5%		8.6%		10%	
Dyspnoe	10%		20%			
Dyspnoe / cough						14%
Rhinitis / conjunctivitis	10%		8.6%			14%
No. of patients	31	20	35	20	30	22

(1) celery allergic patients (clinical history)
(2) patients with celery allergy and concomitant pollen allergy (mugwort, birch) (clinical history)
(3) celery allergic patients (clinical history)
(4) patients (14-49 years of age) with celery allergy (clinical history)
(5) adults with celery and pollen allergy (clinical history)
(6) after DBPCFC in celery allergic patients (13-55 years of age)

(1) Wüthrich & Hofer 1984
(2) Pauli et al. 1985
(3) Wüthrich & Dietschi 1985
(4) Pauli et al. 1988
(5) Jankiewicz et al. 1998
(6) Ballmer-Weber et al. 2000

Anaphylaxis is the most severe manifestation of celery allergy. It typically presents itself along with many different symptoms within an average onset of 2 hours.

1.3.3 Dose

A very important point is to determine the minimum tolerable celery dose that sensitized patients. This varies from parts of gram up to gram levels. There is no sufficient data to determine a safe threshold dose. At sensitized individuals there is a high variability in sensitivity about the amount of allergens required to trigger an adverse reaction. [9]

At one study from Ballmer-Weber *et al.* (2000) patients with the history of an allergic reaction to celery were examined to raw celery, cooked celery and celery spice by DBPCFC. There were no patients complaining of allergy to cooked celery without symptoms to the raw. This indicates that new allergens are not created by the heating process, and that residual activity of the native celery allergens is responsible for the allergenic activity of the cooked vegetable. The results are shown at Table 1.3. [3]

Table 1.3: Symptoms of patients examined by DBPCFC to raw, cooked and spice celery.

Patient number*	Celery raw			Celery cooked		Celery spice	
	Symptom history	Symptoms DBPCFC	Dose	Symptoms DBPCFC	Dose	Symptoms DBPCFC	Dose
1	OAS	OAS	0.7	OAS	0.9	OAS	0.16
2	U,AE,R,C,D	–	–	OAS	1.8	–	–
3	OAS	OAS	0.7	OAS	0.9	–	–
4	OAS	OAS	0.7	OAS	1.8	OAS,R,F	0.16
5	OAS	–	–	OAS	0.9	–	–
6	R,C,D,GIT	OAS,D,R,C	0.7	F,P,C,D	34.5	–	–
7	OAS,U	F,V	28.5	neg	–	–	–
8	F,GIT	AE	28.5	neg	–	F,AE,GIT	5.85
9	OAS	OAS	0.7	neg	–	–	–
10	OAS	OAS	0.7	neg	–	OAS,R,C,AE	0.32
11	U,GIT	OAS	0.7	neg	–	–	–
12	OAS	AE	28.5	–	–	OAS	0.16

OAS: oral allergy syndrome; U: urticaria; AE: angiedema; R: rhinitis; C: conjunctivitis; D: dyspnea; GIT: gastrointestinal symptoms; F: flush; V: vertigo, P: pruritus; neg: no symptoms

1.3.4 Identified celery allergens

The identified allergens in celery are listed at Table 1.4 [30] . Each of them belong to different protein families

Table 1.4: Allergens in celery

Species	Allergen	Biochemical name	MW(SDS-PAGE)	Food Allergen	Entry Date	Modified Date
Apium graveolens (Celery)						
	Api g 1	Pathogenesis-related protein, PR-10	15	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
	Api g 2	Non-specific lipid-transfer protein, type 1	9 kDa	Yes	2010-04-29 16:57:55	2011-04-13 08:21:05
	Api g 3	Chlorophyll a-b binding protein, chloroplast		Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
	Api g 4	Profilin	14	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
	Api g 5	FAD-containing oxidase	58	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
	Api g 6	Non-specific lipid transfer protein type 2	7 kDa	Yes	2011-04-13 08:17:49	2011-04-13 08:17:49

1.3.4.1 Api g 1

The major allergen in celery is the Api g 1. Another name of it is Api g 1.01. Its molecular weight is 16 kDa and it represents about 20% of total soluble proteins in celery tuber [10] .It is recognized by 59–80% of celery allergic patients. This protein has an isoform, the Api g 1.02. It is heat labile and associated with pollinosis (birch pollen). It belongs to the BetVI family. It is a pathogenesis related protein. Such proteins are induced in several plant species upon environmental stress, when infected by viruses, viroids, fungi or bacteria [14]. They form a barrier against pathogens by gathering at infection sites and act to decrease susceptibility of plants. They may have

anti-fungal or anti-bacterial activity. Api g 1.01 on immunoblot exhibits a stronger IgE-binding capacity compared to Api g 1.02. [15] One important known IgE binding epitope is the P-loop region, which is at the amino acid area between 46-52 in the Api g 1 protein structure. [10] The Table 1.5 shows its code at the Databanks GenBank Nucleotide and UniProt. Its secondary protein structure is shown at Fig.1.4 and precisely at Annex 3. Also its protein sequence can be found in Annex 2.

Table 1.5: Identification code of Api g 1 and its iso-form in the databanks

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 1.0101	Z48967	P49372
Api g 1.0201	Z75662	P92918

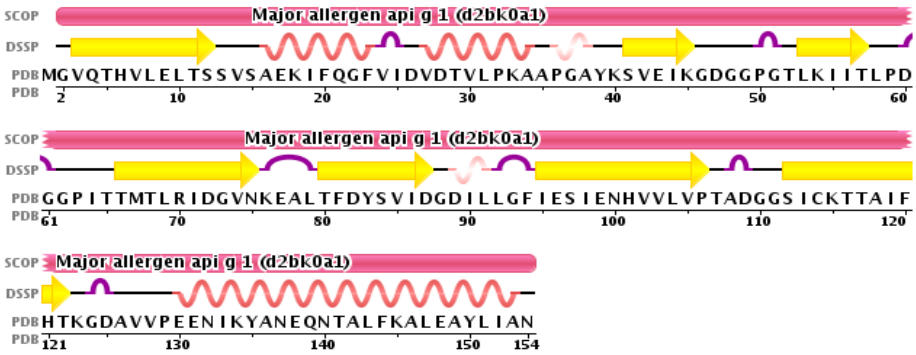


Fig.1.4: Secondary protein structure of Api g 1 (helices, beta sheets) [22]

1.3.4.2 Api g 2

Api g 2 represents a relevant celery stalk allergen in the lipid transfer protein family. It belongs to the group of monomeric proteins (around 7-9 kDa). They are frequent and potentially severe allergens [12]. They have mostly a compact 3D structure, they get glycosylated, factors that contribute to the protein`s stability which is related to allergenicity. [9]

Lipid transfer proteins (LTPs) are named after their ability to transfer phospholipids between vesicles and membranes. [14] These protein-allergens, among them the Api g 2, are often found in herbs and spice mixes [31] Thermal denaturation does not affect the binding capacity of Api g 2 to IgE. Also, Api g 2 shows highly resistance to simulated gastrointestinal digestion [12]. In the following Table 1.6 its code at the GenBank Nucleotide is shown. In Annex 4 its protein sequence is illustrated.

Table 1.6: Identification code of Api g 2 in the GenBank Nucleotide Databank

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 2.0101	FJ643539	

1.3.4.3 Api g 3

Another allergenic protein is the Api g 3. It is a chlorophyll-binding protein but its relevance is still unclear. Its molecular weight is 28, 1 kDa. [10] Its code in the databanks is shown at Table 1.7 and its protein sequence at Annex 4.

Table 1.7: Identification code of Api g 3 in the databanks

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 3.0101	Z75663	P92919

1.3.4.4 Api g 4

Api g 4 is a minor allergen and temperature stable. Its molecular weight is 15 kDa [9]. Api g 4 is a Profilin that belongs to a ubiquitous family of actin phosphatidylinositol 4, 5-bisphosphate-binding proteins. They play a key role in cell motility form the outer cell membrane to the inner cell through the regulation of actin microfilament polymerization dynamics. In plant cells they are also involved in processes like growth of pollen tubes and root hairs. Because of their involvement in essential cellular processes, profilins can be found in all organisms and that is why they are considered as pan-allergens. They are responsible for a lot of cross reactions between inhalant and nutritive allergen sources. [14]

Because of its cross reactivity it is important for patients with a birch-mugwort-celery sensitization.[9] Table 1.8 shows its code at the GenBank-Nucleotide and UniPort databank and Annex 4 its protein sequence.

Table 1.8: Identification code of Api g 4 at the databanks

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 4.0101	AF129423	Q9XF37

1.3.4.5 Api g 5

The glycoprotein Api g 5, with two isoforms, (53 and 57 kDa large), shows homology to FAD (flavin adenine dinucleotide) containing oxidases. The allergenicity of Api g 5 depends on N-glycans containing xylose and fucose residues, (the glycosylation level) and does not depend on the protein sequence. [27] The allergen Api g 5 is cross reactive to birch pollen and mugwort pollen. It is involved in stress response mechanisms. This protein is stable to temperature. In patient-studies, Api g 5 showed IgE-reactivity in 55% of the cases. [10] Table 1.9 shows its code in UniProt databank and Annex 4 its protein sequence.

Table 1.9: Identification code of Api g 5 at the databanks

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 5.0101		P81943

1.3.4.6 Api g 6

About Api g 6 very few is known. Its code at the UniProt databank is shown above at Table 1.10.

Table 1.10: Identification code of Api g 6 at the databanks

Isoallergen and variants	GenBank Nucleotide	UniProt
Api g 6.0101		P86809 (to be released upon publication)

1.3.5 Cross reactivity

The allergens of celery belong to protein families with homology in many fruits and vegetables well known to cross-reactivity. [10] That is why some of the patients allergic to celery, also exhibit identical reactions after ingestion of other plant-foods belonging to the taxonomic families *Rosaceae* (apple, pear, cherry, peach), *Apiaceae* (carrot) and *Solanaceae* (tomato, potato). [9]

The allergens of celery also show similarity to some pollen allergens. Allergy to celery root is highly associated with birch and mugwort pollen sensitization. That is why terms like “birch-mugwort-celery syndrome” or “celery-carrot-mugwort-spice syndrome” are common. There is evidence that birch pollen and celery allergy are highly related in Central Europe, while celery allergy related to carrot, spices and to mugwort pollen is more frequently in Southern Europe and is known as “celery-carrot-mugwort-spice syndrome”. [24]

The serological IgE cross-reactivity with celery is due to at least three classes of allergenic proteins:

- Api g 1, homologous to the major birch pollen allergen (Bet v 1) and to fruits and vegetables (Apple and carrot). These proteins are known to be strongly up-regulated in plants by pathogens.
- Api g 4, the celery 15 kDa profilin is associated with birch mugwort cross reactivity.
- Api g 5 the 53/57 kDa glycoprotein cross reacts with birch pollen and mugwort pollen [28]

New studies has proven that IgE antibodies specific for Api g 2 also cross-react with peach and mugwort pollen. [12]

1.3.5.1 Api g 1.01 similarities with other food allergens

The similarities of Api g 1.01 with other food allergen and the protein sequence homology of the most common of them is shown at Annex 5.

1.3.5.2 Api g 1.02 similarities with other food allergens

The similarities of Api g 1.02 isoform with other food allergen and the protein sequence homology of the most common of them is shown at Annex 6.

1.3.5.3 Api g 3 similarities with other food allergens

The similarities of Api g 3 with other food allergen and the protein sequence homology of the most common of them, is shown at Annex 7

1.3.5.4 Api g 4 similarities with other food allergens

The similarities of Api g 4 with other food allergen and the protein sequence homology of the most common of them, is shown at Annex 8.

1.3.5.5 Api g 5 similarities with other food allergens

About the similarities of Api g 5 with other food allergen, only few is known and it is shown at Annex 9.

1.4 Detection of allergens in food

The food industry is aware of the fact that celery consumption can cause many problems to allergic people and is trying to offer celery-free products. This attempt is not easy once it is common in the food manufactory to use the same pipes for more than one product. Celery free products and products that include celery have very often been processed by the same equipment. This situation cannot guarantee celery absence. There is the need for a test which analyses samples and can distinguish whether or not small concentrations of celery are present. Many testing-systems are in use and commercially available to help food manufacturer screen their raw ware and the production lines for unforeseen ingredients´ cross-over. Basic problems that remain by the detection of allergens are their minute amount in food, the difficulty in extracting them, cross-reactivity, matrix effects and the fact that food- processing may denature protein allergens so that a test that has been developed for native proteins may not work for processed food. A good analytical method must be based on defined reference material and a reliable standard curve. It must fulfill the criteria of sensitivity, specificity, reproducibility, precision and accuracy. The methods used for detection are listed at Table 1.11. [9]

Table 1.11[9]: Methods to detect allergens.

Physicochemical methods	**Immunological methods**
Kjeldahl nitrogen	Immunodiffusion
Nephelometry, high performance liquid chromatography (HPLC)	Counterelectrophoresis
	Radioimmunoassay
Mass spectrometry	Enzyme-linked immunosorbent assay (ELISA)
Capillary electrophoresis	Radioallergosorbent inhibition
PCR for allergen-specific DNA	Immunoblotting

PCR = polymerase chain reaction

The most frequently used detection technique is ELISA. LFA and Dipstick Tests are modified versions of ELISA. They satisfy the need of cheap, portable, reliable and user friendly tests. Using strip test readers, quantification is possible. These tests are immunochromatographic tests, with a mobile phase that allows movement of the

allergen-antibody complex and/or the sample along a test strip. For LFAs using a Sandwich-ELISA an allergen-specific detection antibody, deposited on the membrane, is solubilised upon introduction of a liquid sample and moves along with the sample until it reaches where the capture antibody has been immobilized. At that point the allergen-antibody complex is trapped. When the allergen is present, color development is taking place. The result can be obtained in 3-15 min. Dipstick tests on the other hand don´t have a mobile phase. Here an incubation time is needed. Most of these tests are based on Sandwich-ELISA. The result can be obtained into 10 min till 3 hours. [25]

Sometimes DNA-based methods are used. Also ELISA and PCR can been combined to detect food allergens with high sensitivity. DNA-based methods for detecting allergens have emerged as an alternative when effective protein-based methods are not available. They have high accuracy and sensitivity and can be used when the proceeding conditions are harsh. The disadvantage is that in some food the DNA is degraded, which leads to false positive signals. Also the matrix of the food may reduce the efficiency of DNA amplification. In comparison to immunological protein detection systems, the DNA based method is more complicated and requires more equipment. For routine detection they are therefore impractical. Three DNA-based approaches are available to detect food allergens.

- PCR with gel-Electrophoresis

At this method the DNA must be extracted from the food, and then with help of primers, specific DNA fragments get amplified. The amplified product is loaded to run agarose gel electrophoresis. This method is good for qualitative detection.

- PCR combined with ELISA

The DNA that encodes for the allergen is amplified by help of specific primers. Then the amplified PCR product is immobilized onto a micro plate, the double strand is being denaturized, hybridized and finally detection is possible by an antibody that is enzyme conjugated.

- Real time PCR

This method makes both detection and quantification possible. The amplified DNA is detected as the reaction progresses. The detection of products is possible by help of non specific fluorescent dyes that intercalate with any double-stranded DNA or by sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter. This reporter permits detection. [25] [21]

1.4.1 Detection of celery in food

For the detection of celery in food a number of DNA-based methods have been published and commercialized. As mentioned before, these methods are for laboratory use, expensive equipment is needed and also educated staff. Therefore immunochemical methods for the quantification of celery allergens in foods would be much easier but not available at the moment [9] [10]. Many efforts to that direction have been made in the past. At the latest published attempts [10] [26] 2010 and 2011

respectively the developed Sandwich-ELISA cross reacted with carrot and potato. Therefore, it was the purpose of this study to develop a stable system that would detect traces of celery in foods and help food manufacturer at their attempt to offer celery free products to people who suffer from celery allergy.

To the current and sensitive topic of food allergy many additional improvements must be done. The importance of the problem is not well recognized even in developed countries. Other than an easy detecting method, screening and diagnosis of food allergy must be implemented. Clinicians must get more education so as to recognize possible celery allergy symptoms. Epidemiological data is required so as to understand better the food allergy. The medical community very often does not treat adequately patients with food anaphylaxis. Pediatrics should know the special nutritional needs of children with food allergy and advice them individually. [11]

2. Objectives of the study

The objective of this study was to characterize antibodies gained from the immunization of rabbits regarding their ability to bind to celery and the major allergen protein in celery, the Api g 1. The application of these antibodies in the development of a stable, sensitive, specific prototype system based on an ELISA test, suitable to detect traces of celery in food and its possible commercialisation was the final goal of this project.

For this reason, various preparations were given to sixteen rabbits in order to elicit respond of their immune system. Celeriac crude, celeriac protein extract, recombinant protein Api g 1.01, a mixture of five peptides of the protein sequence of Api g 1, as well as four different peptides of the Api g 1 protein skeleton, were used to immunize the rabbits. The crude sera-antibodies and secondly the antibodies received after immunoaffinity purification, were examined in Indirect-ELISA tests regarding their ability to bind with celery protein and recombinant protein Api g 1. The ones that showed high binding ability were selected and further examined at Sandwich-ELISA tests as this kind of system is more stable and robust for the development of commercialized kits. The cross reactivity of these antibodies with other allergen proteins, similar to celery plants, like carrot and parsley was tested in order to examine the binding specifity of the antibodies.

3. Materials and methods

For this work the following materials and methods were used:

3.1Buffers

Nr.1 Protein-Extraction-Buffer

Tris 0,174 M
SDS 0,0173 M
DTT 0,129 M
Addition of 150 ml Glycerin 87% then fill up to 1L with ddH_20.

Nr.2 ELISA Buffers

Nr.2a: PBS-(coating Buffer)
Potasiumchlorid 2,7mM
Di-Sodiumhydrogenphosphat 10,1 mM
Sodiumchlorid 137mM
Potasiumhydrogenphosphat 1,76 mM
pH 7.4

Nr.2b: ELISA-Incubation Buffer
PBS,
1% BSA
0,05%, Tween20

Nr.2c: ELISA-Blocking Buffer
PBS
3%BSA
0,05%Tween20

Nr.2d: PBST, ELISA-Washing-Buffer
PBS
0,05% Tween20

Nr.2e: ELISA-Substrate Buffer
0,2 M $Na_2HPO_42H_2O$
0,1 M citric acid-H_20
25,7 ml from the first dilution, 24,3 ml of the second dilution are mixed with 50 ml dest. water. In 11 ml of this mixture 2 µl H_2O_2 are added and one pill of Tetramethylbenzidinedihydrochloride (SigmaAldrich/USA)

Nr.2f: ELISA Stop-Buffer
1 M of H_2SO_4

Nr.3 SDS-PAGE Buffers

Nr.3a:Electrophoresis Buffer
Glycin 99.8% 0,192 M
Tris Ultrapure 0,017 M
SDS Ultrapure 0,1%

Nr.3b: Seperating GelBuffer for 1 L
Tris Ultrapure 181,71 g pH 8,8
SDS Ultrapure 4 g

Nr.3c: Stacking GelBuffer for 1L
Tris Ultrapure 60, 57 g pH 6, 8
SDS Ultrapure 4 g

Nr. 3d: Coomasie discolor mixture
Acetic acid 10%
Ethanol 20%

Nr. 3e: Laemmli loading dye 3 x stock
1 M Tris pH 6,8 2,4ml
20% SDS 3ml
Glycerol 100% 3ml
B-Mercaptoethanol 1,6 ml
Bromophenol blue 0,006g
DTT 300 µl
Total 10,3 ml

Nr. 4 Western-Blot Buffers

Nr.4a:Western-Blot Transfer Buffer
50 mM Tris ultrapure
380 mM Glycine
0,01%SDS
20% Methanol

Nr. 4b:Western-Blot TBS
0,003M KCl
0,136 M NaCl
0,02 M Tris Ultrapure

Nr. 4c:Incubation Buffer for Western Blot
TBST
5% powdered milk

Nr.4d:Western-Blot Washing Buffer (TBST)
TBS+0,5%Tween20

Nr. 4e:Western-Blot Blocking Buffer
TBST+ 10% powdered milk

Nr.4f: Buffer for colorimetric detection of Western Blot

- A: 0,5 g NBT in 10 ml DMSO
- B: 0,5 g BCIP in 10 ml DMSO
- Incubation-buffer:
  100 mM NaCl
  5 mM MgCl2
  100 mM Tris pH 9,5

66 µl of Reagent A+ 33 µl of Reagent B in 10 ml Incubation buffer

Nr.5 Buffers for the purification of the antibodies by immunoaffinity

Nr. 5a:Linkage Buffer:
0,1 M $NaHCO_3$,
0,5 M NaCl/ pH 8,3

Nr. 5b:Blocking Buffer:
100 mM Tris/ pH 7,5

Nr. 5c:Regeneration Buffer I:
0,1 M Tris,
0,5 M NaCl /pH 8,5

Nr. 5d:Regeneration Buffer II:
0,1 M Na-Acetat,
0,5 M NaCl/pH 4,5

Nr. 5e:Elution Buffer :
100 mM Glycin /pH 2, 5

Nr. 5f:Neutralization Buffer:
Tris 1 M /pH 8

3.2 Media

MaxiSorp microtiterplate (Roth/Germany)
Immunoaffinity column Hydra (Charles River/USA)
PVDF Membrane (Amersham/USA)
Filtepapers (Whatman/Germany)
Cell strainer (BD Biosciences/USA)

3.3 Reagents and KITS

Tetramethylbenzidinedihydrochloride -ELISA Substrate- (SIGMA Aldrich /Germany)
BCIP/NBT (Carl Roth /Germany)
NHS-Biotin (Pierce /USA)
Protein Marker MarkItblue (Annex 11) (MicroMol /Germany)
Antibody goat antiRabbitHRP (MicroMol /Germany)

Antibody goat antiRabbitAP (MicroMol/ Germany)

3.4 Instrumentation

Athos microplate reader (Biochrom/UK)
Hybridization oven 6/12 (Störktronik/Germany)
Electrophoresis power supply (Amersham Biosciences/USA)
UV/Visible Spectrophotometer Ultrospec 2000 (Pharmacia Biotech/Germany)
Centrifuge Biofuge fresco (Heraues instruments/Germany)
Sonification instrument (UP 50H, Hielscher/Germany

3.5 Bio-molecular methods

3.5.1 Protein extraction from celeriac, carrot, parsley

To extract the protein-allergens from celeriac, carrot and parsley the following procedure was performed. Two grams of the vegetable were ground in a pestle with liquid nitrogen. 5 ml Protein-Extraction-Buffer (Nr.1) were added, the mixture was homogenized and filtrated through a cell strainer. After addition of 20 ml Ethanol, the sample was kept at - 20°C for 1 hour. Then it was centrifuged for 15 min at 5000 rcf, the supernatant was removed, 20 ml cold Ethanol were added and through sonification, lysis of the plant cells was achieved. The sample was centrifugated again for 15 min at 5000 rcf and the supernatant removed. Finally 1 ml PBS (Nr 2a) was added to the pellet, the total protein content was determined by measurements with a UV/Visible Spectrophotometer at 280nm and the protein extract was kept at - 20°C for later use. [13] [3]

3.5.2 Indirect-ELISA method

Principle: The Indirect-ELISA requires the immobilization of antigens on a polystyrene based modified surface. After that, a primary antibody is added, which binds specifically to the antigen that is coating the well. A labeled secondary antibody is used for detection. This antibody binds specifically to the primary antibody. Due to the labeling a spectrometer can be employed to quantify the color strength.

Procedure: The upper row of wells on a micro titer plate - was coated in duplicates or triplicates with 75 µl PBS (Nr.2a) into which either 5 µg/ml celeriac or carrot or parsley protein extract were added or in another case 1 µg/ml recombinant protein Api g 1. In the rest rows of wells 50 µl PBS were added. By taking out 25 µl PBS of the mixture of the first raw of wells and adding them into the second raw of wells dilutions of 1:3 were made. By the same procedure 5 dilutions were prepared. The immobilization of the protein onto the plate took place overnight at 4°C. In order to minimize the amount of unbound protein four washing steps with 200 µl washing buffer (Nr.2d) followed. To reduce unspecific binding, the coated plate got blocked by 300 µl blocking buffer (Nr.2c) for 1 hour at RT. 50 µl purified antibodies or sera-antibodies in concentrations of 1 µg/ml and 1:1000 respectively were loaded onto the plate and incubated for 1 h at RT. The above concentrations were achieved by dilution with incubation buffer (Nr.2b). After 4 washing steps with 200 µl washing buffer (Nr. 2d), the goat antibody anti-RabbitHRP (1:20.000/ 50 µl) was added or in another case the antiApi1-2Biotin (1µg/ml/50 µl). Incubation took place for 1 h at room

temperature (RT). Four washing steps with 200 µl washing buffer (Nr. 2d) followed. By the addition of 50 µl chromogenic substrate TMB (Nr.2e) and incubation for 10 min the reaction was visualized and finally stopped with 50 µl 1 M H_2SO_4. In the case when antiApi1-2Biotin was used as detector antibody one more step was enclosed in the procedure; Streptavidin (50 µl of a 0,5µg/ml solution) was added and incubated for 1 hour at RT. Then 4 washing steps followed and the chromogenic substrate was added as in the case before. Using the ELISA plate reader the absorbance at 450 nm with reduction at 630 nm was measured.

3.5.3 Immunoblotting experiments

In order to estimate the specific binding affinity of antibodies to the antigen (i.e. the Api g 1-protein or celeriac, carrot, parsley proteins respectively) immunoblots were performed. Firstly an SDS-PAGE gel had to be run.

Principle of electrophoresis: Onto a gel matrix formed of polyacrylamide, protein samples that have been denatured can move in the gel on the basis of their molecular weights only and get separated.
The gel matrix consists of chains of polyacrylamide that are crosslinked by N,N-methylene bis-acrylamide co-monomers. Polymerization is initiated by ammonium persulfate and catalysed by TEMED. The concentration of an acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. Ahead of analyzing the proteins on the gel, they need to be denatured. Sodium dodecyl sulfate, which is an anionic detergent, is used for denaturation. One SDS molecule binds to 2 amino acids. Mercaptoethanol assists the protein denaturation by reducing disulfide bonds. Proteins become linearized and can be separated on the gel.

Procedure of Electrophoresis: Gel plates were set up. Then the components for the gels were mixed. Two different layers of acrylamide were made and poured between the glass plates. The lower layer (separating, or resolving, gel) was responsible for actually separating polypeptides by size. The upper layer (stacking gel) was designed to compress the proteins into micrometer thin layers when they reached the separating gel. The gels were made according to the recipe in the following Table 3.1

Table 3.1: Recipe of stackgel and separating gel.

Components	Separating gel 12%	Stacking gel
H_2O	3,4 ml	3 ml
4X Sep.Gel-Buffer (Nr.3b)	2,5 ml	-
30% Acrylamide	4 ml	0,66 ml
TEMED	10 µl	5 µl
10%APS	50 µl	25 µl
4X Stack.Gel.Buffer(Nr.3c)	-	1,25

A comb was placed into the upper stacking gel before the polymerization. After the gels had been polymerized the comb was removed. The slots were washed out to remove unpolymerised acrylamide. Meanwhile the protein samples were prepared. They were mixed with 2 x sample loading buffer (Nr. 3d) at 1:1 dilution. The samples got heated at 95°C for 3 minutes and finally they were be loaded onto the slots of the

gel. 15 µl of the protein sample, were added at each slot. After the gel had run approximately 1 hour at 25 mA at RT, the electrophoretically separated proteins were transferred to a PVDF membrane where they were detected using antibodies specific for the target protein. To prepare the membrane, it was soaked in ethanol for a few minutes and rinsed with distilled water. Six pieces of Whatman paper (3mm) were cut. Then the membrane, pads, filter papers and gel were stacked in the following order: black frame (negative electrode) >> pad >> 3 pieces of filter papers >> SDS-PAGE gel >> membrane >> 3 pieces of filter papers >> pad >> red frame (positive electrode). The blotting cell was filled with Western blot transfer buffer (Nr 4a), and blotting was performed at 400 mA for 2 hours.

After the blotting procedure, the membrane was taken out of the equipment and placed in a blocking solution (Nr.4e) for 1 hour. Meanwhile the primary antibody solution was being prepared with incubation buffer which contained 5% powdered milk. Depending on the antibody`s reactivity, concentrations of 1 µl/ml for purified antibodies or 1:1000 dilutions for sera-antibodies were used. The membrane was incubated with 10 ml primary antibody-solution for 1 hour at RT. After 4 washing steps with TBST (Nr.4d) the membrane was incubated with 10 ml of the second antibody, which was a goat anti-Rabbit conjugated with alkaline phosphatase (1 hour RT). The second antibody was diluted 1:1000 in incubation Buffer (Nr. 4c). Finally after four further washing steps with washing buffer (Nr. 4d), the detection of the characteristic bands was possible by adding BCIP/NBT buffer onto the membrane for colorimetric detection (Nr. 4f).
An alternative method that had been followed in some experiments for the identification of proteins was Coomassie blue staining of the gel to detect proteins in the banding pattern. To stain gels they were placed in Coomassie solution and shaken for approximately 1h at RT. Then the staining solution had to be poured off and in order to destain the gel it was put into a discolor mixture (3d) for 30 min.

3.5.4 Purification of sera-antibody on an antigen-affinity-column (Api g 1-Hydra affinity column)

The gained sera-antibodies were further purified via an Api g 1-HYDRA column onto which recombinant protein Api g 1 was immobilized. After washing the column with PBS (Nr.2a), 10 ml of sera were given onto it. Sera and solid phase were incubated at 4°C overnight, rotating. After washing three times with 4 ml PBS (Nr.2a), 10 ml elution-buffer (Nr.5e) was poured onto the matrix. Ten fractions of 1 ml each were collected. To each fraction 100 µl neutralization buffer (Nr. 5f) was added. In order to test which fractions included the antibody, a SDS-PAGE electrophoresis was performed using a 12% gel. The fractions which contained the antibody were identified, pooled and the concentration of the antibody-sample was measured at 280 nm with an UV/Visible Spectrophotometer

3.5.4.1 Determination of protein concentrations

Principle: Quantification of proteins in a solution is possible by simple spectrophotometry. The absorption of proteins at 280 nm depends on their content of Tyr and Trp therefore one has to keep in mind that it varies greatly between different proteins and is just an approximation. For the determination of a given protein

concentration the sample was diluted with PBS accordingly. The concentration was then calculated using the Lambert-Beer formula.

3.5.4.2 Ammoniumsulfat precipitation

In order to obtain a higher antibody concentration, an ammonium precipitation was carried out. For this purpose an 5,75M ammoniumsulfat solution pH 7,4 was prepared and 10 ml of this solution was poured carefully onto 10 ml antibody solution. At 4°C the mixture was stirred overnight. After a centrifugation step of 30 min at 400 rpm the pellet was disolved in 1 ml PBS and dialyzed against 1 L of PBS.

3.5.4.3 Dialysis through a Semi-Permeable Membrane

Principle: Dialysis is the movement of molecules through a semi-permeable membrane, from high concentration to low concentration. Only molecules that are small enough to fit through the membrane pores are able to move and reach equilibrium with the entire volume of solution in the system. There is a stop of movement when molecules are moving through the pores into and out of the dialysis unit at the same rate. Large molecules that cannot pass through the membrane pores will remain at this side of the membrane as they were when dialysis was initiated. By changing the buffer, additional unwanted substance can be removed. After the precipitation of the antiApi1-2, unwanted salts which would disturb the protein function (e.g. binding to an antigen) had to be removed. In order to substitute these negative substances, dialysis against PBS was performed.

Procedure: The antibody sample was placed in a dialysis bag and put into a container with 1 L PBS. Everything was stirred overnight (O/N) at 4°C for 1 hour. Three PBS changes in intervals of one hour were required in order to obtain a salt minimization in the sample.

3.5.5 Biotinylation of antiApi1-2

Principle: A method to make an antibody detectable is to attach biotin covalently to it. Biotinylation is rapid, specific and is unlikely to alter the natural function of the molecule due to its small size. Biotin binds to streptavidin with an extremely high affinity and specificity. It is resistant to extreme heat, pH and proteolysis. Multiple biotin molecules can conjugate to an antibody of interest, which means also multiple streptavidin binding-sites, and thereby increasing sensitivity of detection.

Procedure: According to the instructions of Thermo Fisher a 10mM Biotin solution in DMSO was prepared. In order to estimate how much Biotin was necessary for the conjugation of 1 ml antibody with the concentration of 1,55 mg/ml, the following equation was used.

$$\frac{1\ ml\ IgE\ x\ 1{,}55\ IgE\ x\ 1mmol\ IgG\ x\ 20mmol\ Biotin}{1\ ml\ IgG\ x\ 150.000\ mg\ IgG\ x\ 1mmol\ IgG} =$$

$$= \frac{0{,}20\ 10^{-3} mmol\ Biotin\ x\ 1.000.0000\ \mu l\ x\ 1L}{L\ x\ 10mmol} = 20\ \mu l\ Biotin$$

20 µl Biotin solution with 1 ml antibody was incubated for 1 hour at RT.

3.5.6 Sandwich-ELISA

Principle: This type of assay is called Sandwich because the analyte to be measured is bound between two antibodies. One of these antibodies is the capture antibody that binds onto the micro plate and the other one is the detection antibody. Usually the detection antibody is coupled with an enzyme. When the substrate of the enzyme is added a color development is taking place. The higher the concentration of the antigen the stronger the color changes. A spectrophotometer can be used to give quantitative values for color strength. The Sandwich-ELISA method is more sensitive and specific than other ELISA methods. The prerequisite is that the two antibodies used, don't bind to the same epitope.

Procedure: Microtiter plates were coated in duplicates with 50 µl/well PBS containing Api g 1-specific antibodies or celery-specific antibodies at concentrations of 2 µg /ml The immobilization onto the ELISA plate took place O/N at 4°C. After 4 washing steps with 200 µl ELISA-washing buffer (Nr.2d) to remove unbound antibody, the samples were blocked at RT with 300 µl blocking buffer (Nr.2c) for 1 hour. At the upper raw of wells on the micro titer plate 75 µl celery protein extract or recombinant Api g 1 protein were added with a starting concentration of 5 µg /ml and 1 µg /ml respectively. In the rest rows of wells 50 µl PBS was added. By taking out 25 µl PBS of the mixture of the first row of wells and adding them into the second raw of wells dilutions of 1:3 were made. By the same procedure 5 dilutions were proceed. The incubation took place at RT for 1 hour. Four washing steps with 200 µl ELISA-washing buffer followed. Then 50 µl of detection antibody antiApi1-2-Biotin were applied at a concentration of 1 µg /ml. After 1 h incubation at RT and four washing steps with 200 µl washing buffer, StreptavidinHRP (50 µl of a 0,5 µg /ml solution) were added onto the plate. The StreptavidinHRP complex was incubated for 1h at RT. Four washing steps with 200 µl PBS followed and finally 50 µl/well TMB substrate (Nr.2e) were given onto the plate. After 10 min the reaction was stopped by 50 µl 1 M H_2SO_4. The absorbance was measured at 450 nm using an ELISA plate reader. In order to test the binding affinity of the capture antibody to the detection antibody a negative control was performed by adding every reactant except the antigen.

3.5.7 Purification of sera-Antibodies on NHS-activated-sepharose coupled with celeriac protein

Principle: To gain antibodies that are specific for celery and efficient in a Sandwich-ELISA setting, an affinity matrix was prepared.

Procedure: In order to swell 1 g NHS-activated sepharose, the sepharose was treated with 10 ml 1 mM HCl for 30 min. After washing firstly with 30 ml PBS (Nr.2a) and secondly with 12 ml linkage Buffer (Nr.5a), 8 ml of celeriac protein extract with a concentration of 3,92 mg/ml was poured onto the matrix and incubated at 4°C overnight. In this way the celeriac extract proteins were immobilized onto the matrix. The following day unbound ligand was washed out with 40 ml PBS (Nr.2a). Subsequently the matrix was blocked for 2 hours with 4 ml blocking buffer (Nr.5b). 10 ml of sera-antibodies were given onto the column and incubated for 4 hours at 4°C. The flow-through was discarded and washing steps with washing buffer followed (2x40 ml). Finally the purified antibodies were eluted with 10 ml of elution buffer. (Nr.5e). Ten fractions of 1 ml each were selected. These fractions were neutralized by

100 μl neutralization buffer (Nr.5f). In order to select the fractions that contained the purified antibodies, 12% SDS-PAGE electrophoresis was performed. The fractions that included the antibodies were pooled, dialyzed against PBS (Nr 2a) and stored at -20°C for further experiments. The concentration of the antibodies was measured at 280 nm with a UV/Visible Spectrophotometer.

4. Results

In this chapter the results of the experimental part of the thesis are presented.

4.1Previous work

The following steps were not carried out during the experimental work of this thesis yet they are briefly described below.

4.1.1Expression of recombinant protein Api g 1

For the experiments the recombinant Api g 1 was used. For this reason the DNA sequence of the protein Api g 1 was cloned into the pQE-30 expression vector and subsequently the recombinant plasmid was transformed into *E.coli* cells. The expressed protein Api g 1 was then used for the immunization and for further experiments as a binding agent.

4.1.2 Immunization schema

Two groups of rabbits were immunized.
In the first group, twelve animals got different preparations in order to built antibodies. The antigen solution they got, as described in Table 4.1, was crude celeriac (unheated and heated at 95°C), celeriac protein extract (unheated and heated at 95 °C), recombinant protein Api g 1.01, and a mixture of five peptides of the protein sequence of Api g 1.01 respectively. The immune system of these rabbits became fortified against these immunogens.
In the second group, four rabbits got immunized. The agents used to activate their immune system were in this case, small molecules on the contrary to the first group where large agents were used. Each of these animals got a preparation of only one small peptide in order to elicit response. Two isoforms of the protein exist, and therefore two peptides of the Api g 1.01 isoform were used as well as two peptides derived from the second isoform, the Api g 1.02 protein sequence. In this way polyclonal antibodies were gained

Table 4.1: Agent used for the immunization

	Imunisation ID	Immunisation through:
Immunization 1	2400/10 Animal 1,2	celeriac-extract
	2400/10 Animal 3,4	celeriac-extract at 95°C
	2400/10 Animal 5,6	protein-extract from celeriac
	2400/10 Animal 7,8	protein-extract from celeriac at 95°C
	2400/10 Animal 9,10	Recombinant Api g1 Protein incl. His-tag
	2400/19 Animal 11,12	PepMix(5 Peptides) from the Api g1 Api30(aa 39-52) KSVEIKGDGGPGT; Api55(aa 55-65) IITLPDGGPIT; Api68(aa 68-79) TLRIDGVNKEAL Api105(aa 105-113) VPTADGGSI Api120(aa 120-140) FHTKGDAVVPEENIKYANEQ
Immunization 2	1959/08 Animal 1	Pep-Api1-1(aa 1-12) MGVQTHVLELTS
	1959/08 Animal 2	Pep-Api1-2(aa 121-140) HTKGDAVVPEENIKYANEQN
	1959/08 Animal 3	Pep-Api2-1(aa 11-31) PSTVSAEKMYQGFLLDMTVF
	1959/08 Animal 4	Pep-Api22(aa 121-138) NTKGDAVLPEDKIKEATE

The gel matrix of immunoaffinity column HYDRA was coupled with recombinant protein Api g 1 in order to purify sera of some animals and to gain antigen specific antibodies. Table 4.2 shows which sera had been selected for purification.

Table 4.2: Sera of animals which were purified in previous experiments

Imm.1	Ser.1	Ser.4	Ser.5	Ser.6	Ser.7	Ser.8	Ser.9	Ser.10	Ser.11	Ser.12
Purif.	-	-	-	-	+	+	+	+	+	+
Imm. 2	Ser.1	Ser. 2	Ser. 3	Ser. 4						
Purif.	-	-	-	-	-	-	-	-	-	-

Imm.-Immunization, Purif.: purification, Ser.-Serum
- a purification was conducted, + no purification step was conducted

4.2 Experimental work of this thesis

4.2.1 Examination of the binding capacity of sera and purified-antibodies with celeriac-protein/recombinant protein Api g 1 by the Indirect-ELISA method

The reactivity of crude immune sera and their purified antibodies (Api g 1 specific antibodies) with recombinant protein Api g 1 and celeriac protein extract were tested by Indirect-ELISA test. The method was followed according to 3.5.2 in Materials and Methods.

Purified antibodies of the first immunization: 7p, 9p, 10p, 11p, 12p, and sera-antibodies of animals 1, 4, 5, 6, 7, 8, 9, 10, 11, 12 as well as the sera-antibodies of the second immunization were tested by this way. All assays were carried out in duplicate. The negative control samples included all the reactants except the antigen. The results are shown in Fig.:4.1a-Fig.:4.6b

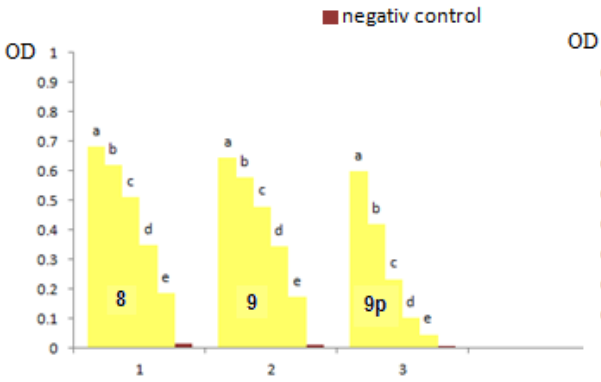


Fig.4.1.a: Indirect-ELISA: Reaction of **celeriac**

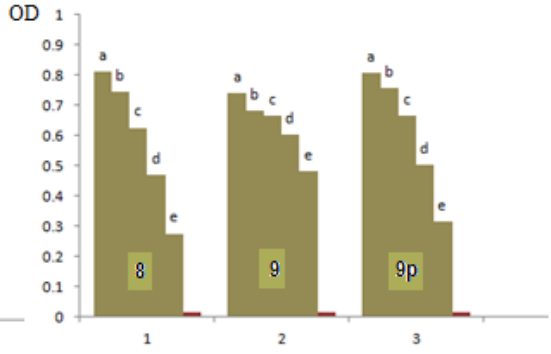


Fig.4.1.b: Indirect-ELISA: Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333** µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

With antibodies from the first group of Immunization
1: Serum /Animal 8 (1:1000)
2: Serum /Animal 9 (1:1000)
3: purified antibody from Animal 9 (1 µg/ml, well: 0, 05 µg)

Detection Antibody: goat aRabbitHRP 1:20.000

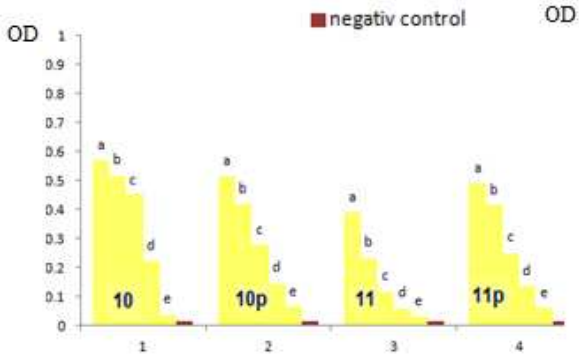


Fig.4.2.a: Indirect-ELISA: Reaction of **celeriac**

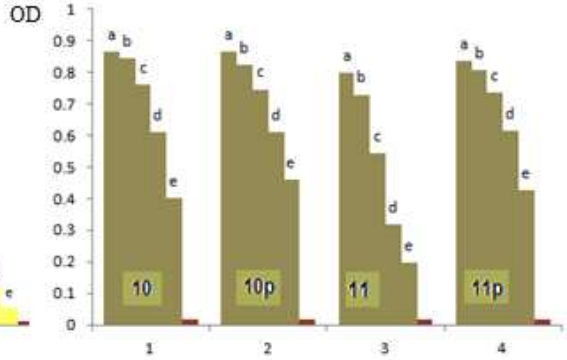


Fig.4.2.b: Indirect-ELISA: Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

With antibodies from the first group of immunization
1: Serum /Animal 10(1:1000)
2: purified antibody from Animal 10 (1 µg/ml, well: 0,05 µg)
3: Serum /Animal 11(1:1000)
4: purified antibody from Animal 11 (1 µg/ml, well: 0,05 µg)

Detection Antibody: goat aRabbitHRP 1:20.000

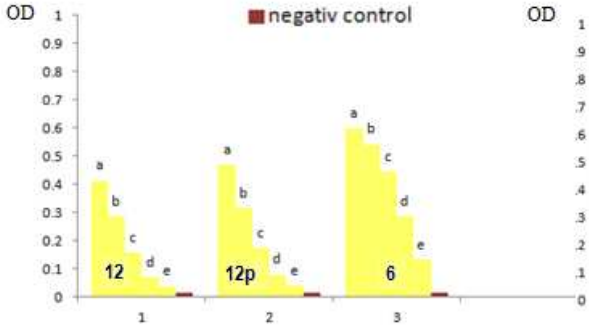


Fig.4.3.a: Indirect-ELISA: Reaction of **celeriac**

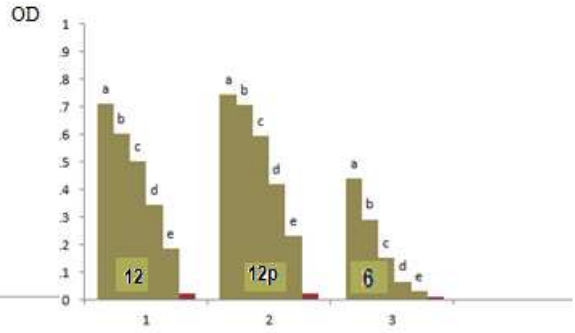


Fig.4.3.b: Indirect-ELISA: Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

With antibodies from the first group of immunization
1: Serum /Animal 12(1:1000)
2: purified antibody from Animal 12 (1 µg/ml, well: 0.05 µg)
3: Serum /Animal 6 (1:1000)

Detection Antibody: goat aRabbitHRP 1:20.000

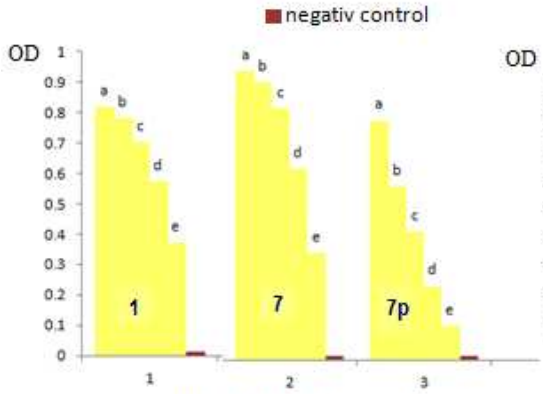


Fig.4.4.a: Indirect ELISA: Reaction of **celeriac**

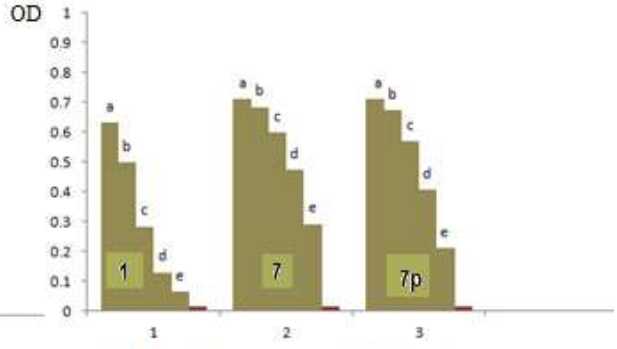


Fig.4.4.b: Indirect ELISA: Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333** µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

With antibodies from the first group of immunization
1: Serum /Animal 1(1:1000)
2: Serum /Animal 7(1:1000)
3: purified antibody from Animal 7 (1 µg/ml,) well: 0,05 µg)

Detection Antibodv: goat aRabbitHRP 1:20.000

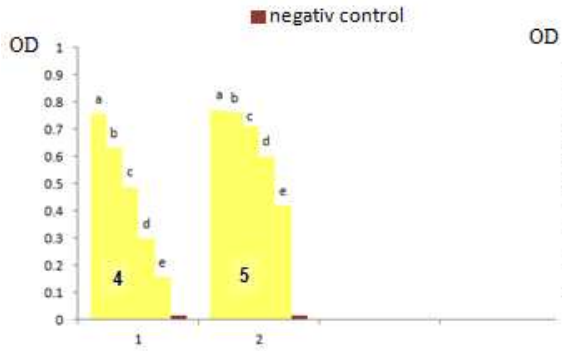


Fig.4.5.a: Indirect-ELISA: Reaction of Celeriac

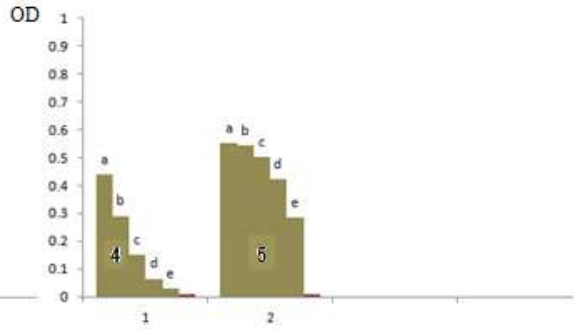


Fig.4.5.b: Indirect-ELISA: Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333** µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

with antibodies from the first group of immunization
1: Serum /Animal4(1:1000)
2: Serum /Animal 5(1:1000)

Detection Antibody: goat aRabbitHRP 1:20.000

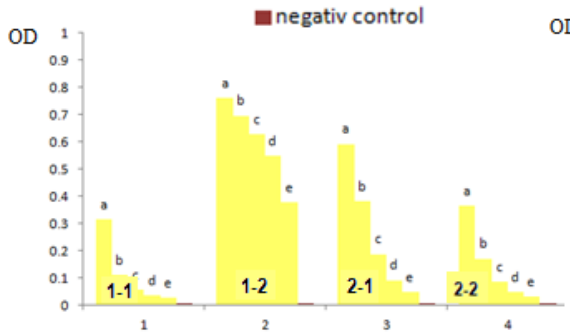


Fig.4.6.a: Indirect-ELISA: Reaction of **Celeriac**

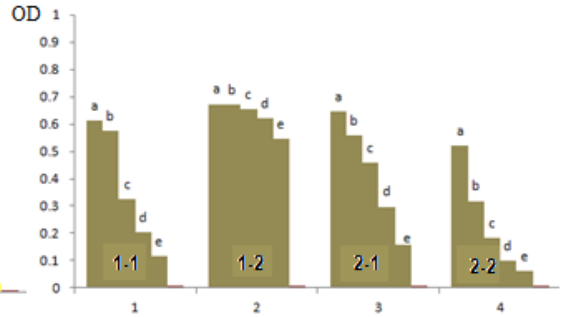


Fig.4.6.b: Indirect-ELISA Reaction of **Apig1**

Celeriac	**a=5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
Apig1	**a=1** µg/ml (well 0.05 µg)	**b=0.333** µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)

With antibodies of the second group of Immunization
1: Serum /Animal 1 (1:1000) Immunisation with Apig1-1
2: Serum /Animal 2 (1:1000) Immunisation with Apig1-2
3: Serum /Animal 3 (1:1000) Immunisation with Apig2-1
4: Serum /Animal 4 (1:1000) Immunisation with Apig2-2

Detection Antibody: goat aRabbitHRP 1:20.000

The Indirect-ELISA tests confirmed a successful immunization and purification of Api g 1 specific antibodies from the sera. The crude sera and the purified-antibodies could bind with the recombinant Protein Api g 1 and also with the celeriac protein extract.

4.2.2 Immunoblotting experiments to examine the specifity of sera and purified-antibodies

Antibodies gained after purification with an antigen (Api g 1) affinity column as well as the corresponding immune sera were tested by immunobloting. The aim was to identify their specific binding affinity to recombinant Api g 1 protein and to celeriac respectively. According to the literature the Api g 1 protein migrates as a single band with a molecular weight of 15 kDa[15]. The method was followed according to section 3.5.3 in Materials and Methods. In the Figures 4.7-4.14 the expected Band was marked by an arrow.

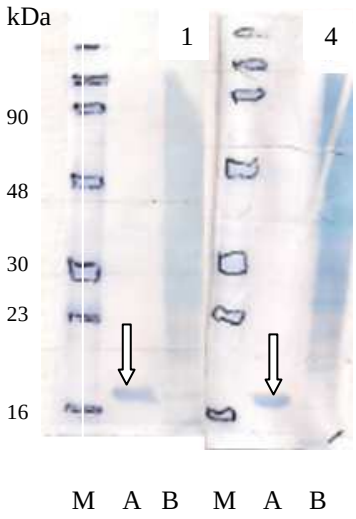


Fig.4.7: Western Blot of the first group of immunization
M) Marker Mark it blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

1: Binding antibody: Serum of Animal 1, 1:1000
4: Binding antibody: Serum of Animal 4, 1:1000
Detection antibody: goat-aRabbit-AP 1:1000

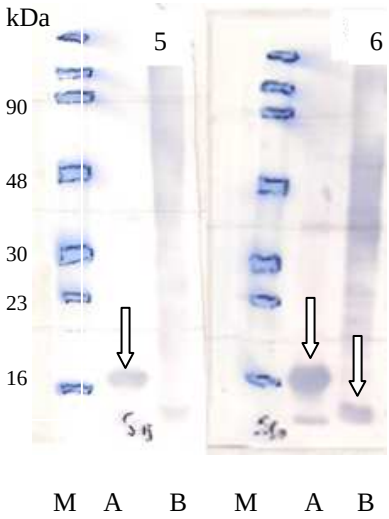


Fig.4.8: Western Blot of the first group of immunization
M) Marker Mark it blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

5: Binding antibody: Serum of Animal 5, 1:1000
6: Binding antibody: Serum of Animal 6, 1:1000
Detection antibody: goat aRabbit-AP 1:1000

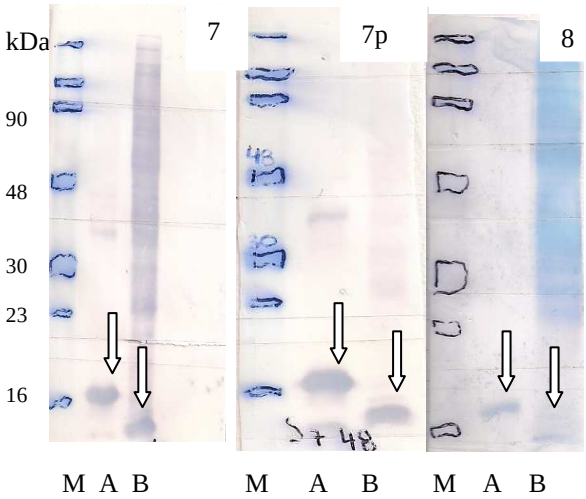


Fig.4.9: Western Blot of the first group of immunization
M) Marker Mark It blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

7: Binding antibody: Serum of Animal 7, 1:1000
7p: Binding antibody: purified antibody from Animal 7, 1µg/ml
8: Binding antibody: Serum of Animal 8
Detection antibody: goat aRabbit-AP 1:1000

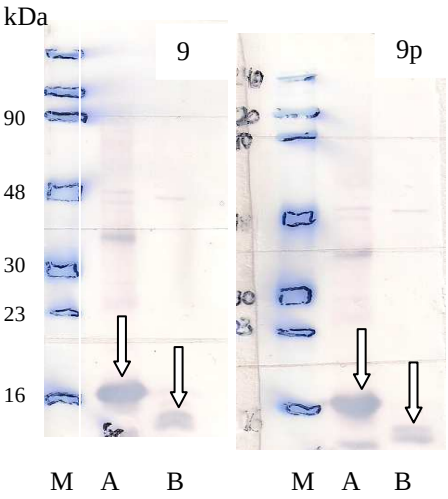


Fig.4.10: Western Blot: of the first group of immunization
M): Marker Mark It blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with

9: Binding antibody: Serum of Animal 9, 1:1000
9p: Binding antibody: purified antibody fr. Animal 9, 1µg/ml
Detection antibody: goat aRabbit-AP 1:1000

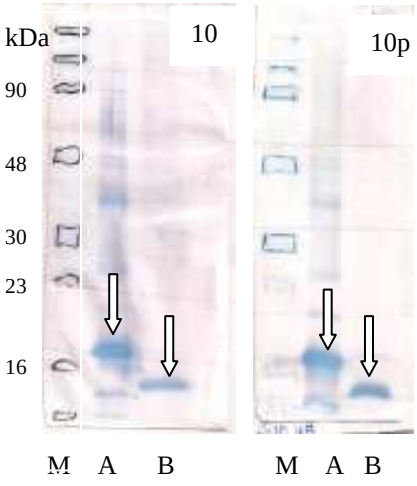


Fig.4.11: Western Blot: of the first group of Immunization
M) Marker Mark It blue
A) Apig1: 100 µg/ml (Load 1, 5 µg)
B) Celery 2mg/ml (Load 30 µg) with:

10: Binding antibody: Serum of Animal 10, 1:1000
10p: Binding antibody: purified antibody fr.Animal 10, 1µg/ml
Detection antibody: goat aRabbit-AP 1:1000

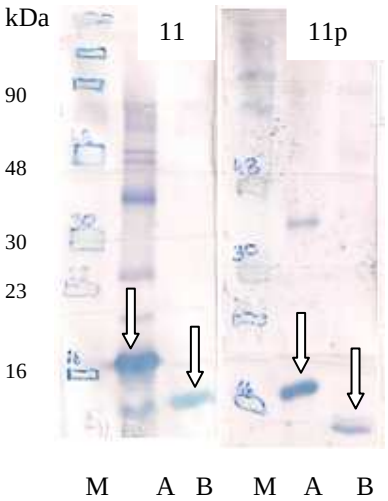


Fig.4.12: Western Blot: of the first group of immunization
M) Marker Mark It blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

11: Binding antibody: Serum of Animal 12, 1:1000
11p:Binding antibody: purified antibody fr. Animal 11, 1µg/ml
Detection antibody: goat aRabbit-AP 1:1000

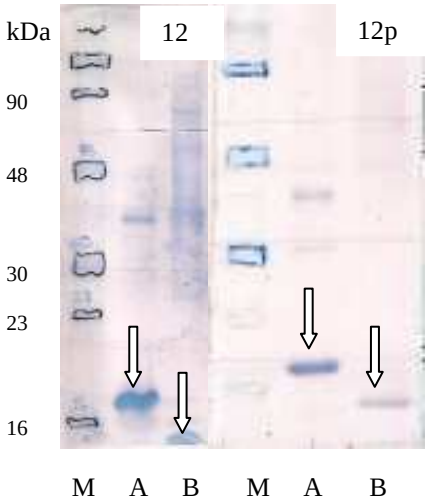


Fig.4.13: Western Blot: of the first group of immunization
M) Marker Mark It blue
A) Apig1: 100 µg/ml, (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

12: Binding antibody: Serum of Animal 12, 1:1000
12p: Binding antibody: purified antibody fr. Animal 12, 1µg/ml
Detection antibody: goat aRabbit-AP 1:1000

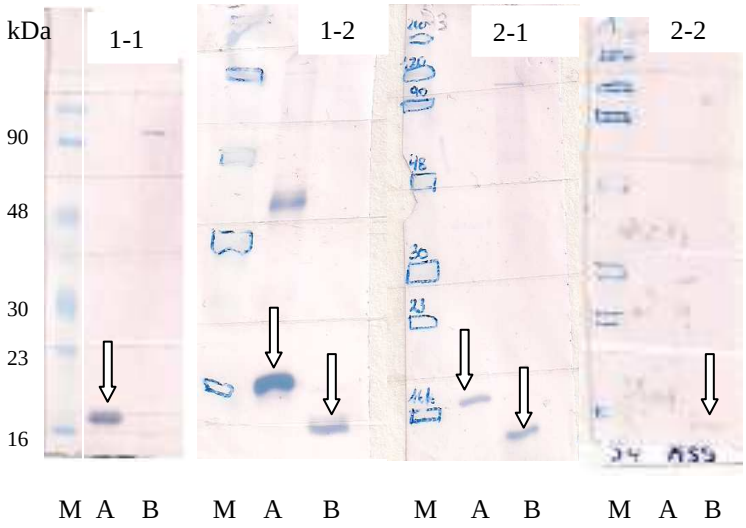


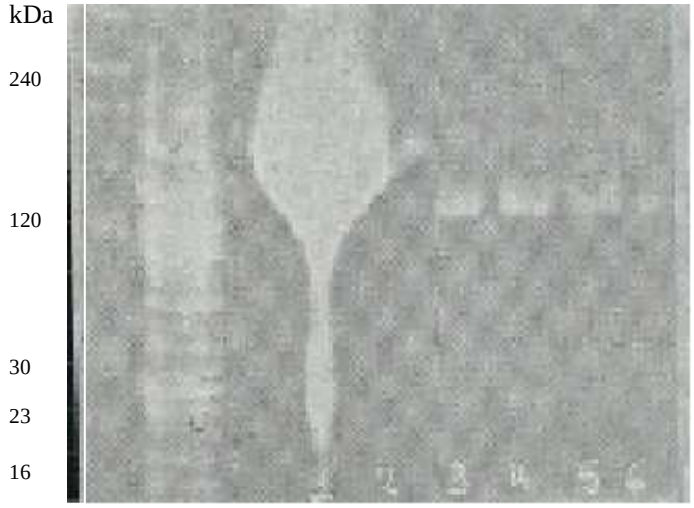
Fig.4.14: Western Blot: of the second group of immunization
M) Marker Mark It blue
A) Apig1: 100 µg/ml (Load 1, 5 µg)
B) Celeriac 2mg/ml (Load 30 µg) with:

1-1: Binding antibody: Serum of Immunisation with Apig1-1, 1:1000
1-2: Binding antibody: Serum of Immunisation with Apig1-2, 1:1000
2-1: Binding antibody: Serum of Immunisation with Apig2-1, 1:1000
2-2: Binding antibody: Serum of Immunisation with Apig2-2, 1:1000
Detection antibody: goat aRabbit-AP 1:1000

The results presented in Figure 4.7-4.14 show a single band migrating close to the marker band of 16 kDa which corresponds to the Api g 1 protein. The immunoblot confirmed that the above ELISA values (Fig. 4.1.a-4.5.b) of the first immunization/purification group were mainly due to specific bindings to the protein Api g 1. From the second immunization group the sera of antiApi1-2 showed a high binding capacity and preference to bind specifically with the Api g 1 which was the reason why this antibody was chosen to serve as detection antibody in some of the following experiments. As the immunization agent was only a small peptide it was furthermore presumed that the possibility of this sera to cross react with other proteins of similar plants would be rare.

4.3 The cross reactivity of antibody antiApi1-2

The serum of the animal immunized with peptide aa121-140 of the Api g 1.01 isoform got purified by an antigen- immunoaffinity column HYDRA as described in section 3.5.4 in Materials and Methods. The eluted fractions were examined via SDS-PAGE (Fig.4.15) in order to detect which fraction contained the antibody. The electrophoresis showed that the antibody was included in Fractions 2, 3, 5 which were then pooled. An amount of antibody was into the wash fraction and couldn´t be selected.



M Flowthrough. Wash/Fr.2/Fr.3/Fr.4/Fr.5/Fr.6

Fig.4.15: 12% SDS-PAGE of the eluted fractions of an affinity chromatography of serum obtained after immunization with peptide aa121-140 of the Api g1 isoform. (M:Marker, Fr: Fraction)

The gained antibody antiApi1-2 was covalently coupled to biotin in order to be easily detectable by addition of StreptavidinHRP.

The method is described in section 3.5.5 in Materials and Methods. The biotinilated antibody was tested by Indirect-ELISA to examine its cross-reactivity with 5 μg/ml carrot and parsley. All assays were carried out in triplicate. The negative control samples included all the reactants except the antigen. In Fig 4.16 the results of the Enzyme-linked immunosorbent assay are shown.

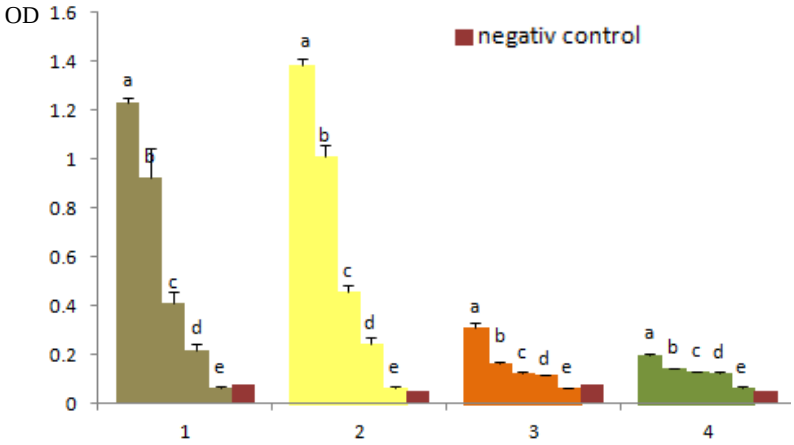


Fig.4.16: Indirect-ELISA: Reaction of:

1.Apig1	**a= 1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)
2. Celleriac	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
3. Carrot	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
4. Parsley	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)

with 0.5 µg/ml (well 0.025 µg) antiApi1-2Biotin and 0, 5 µg/ml (well 0.025 µg) HRP Streptavidin

The antibody antiApi1-2 showed a clearly binding preference to celeriac and Api g 1-protein. At the initial and equal concentration of 5 µg/ml for celery, carrot, parsley, the antibody showed five times higher signals to celeriac than to carrot. When comparing with parsley the signals were 12 times higher. Therefore this antibody was selected for further experiments in Sandwich-ELISA systems.

4.4 Optimization procedure for an Api g 1 specific Sandwich-ELISA

At an Indirect-ELISA the antibody serves as a binding partner of the antigen and also as a detection agent. In contrast to that, a more sensitive and specific test is the Sandwich-ELISA by which the antigen is in a first step captured by a specific antibody and detected in a second step by another specific antibody which might be labelled by a substrate converting enzyme such as HRP.

In order to develop a stable system, a celery specific Sandwich-ELISA had to be established. Therefore the recombinant protein Api g 1 was used for test establishment as it was presumed that it should be in a high concentration present in celeriac protein extracts, however be not as complex as a celery extract. As detection antibody should serve the antiApi1-2Biotin which had shown specificity and affinity in the previous experiments. Bound antiApi1-2 should be determined by a colorimetric reaction initiated by StreptavidinHRP binding to the covalently attached Biotin anchor. The concentrations for this specific application had to be determined experimentally. For

this reason the antiApi1-2-Biotin was applied in the first four rows at concentrations of 1 µg/ml and in the last four rows at 0,5 µg/ml. Also the concentration of applied StreptavidinHRP varied from 1 µg/ml in the first and fifth row to 0,5 µg/ml in the second and sixth row, to 0,25 µg/ml in the third and seventh row to 0,125 µg/ml in the fourth and eighth row. The results are shown in the Figures below (Fig.: 4.17-4.26)

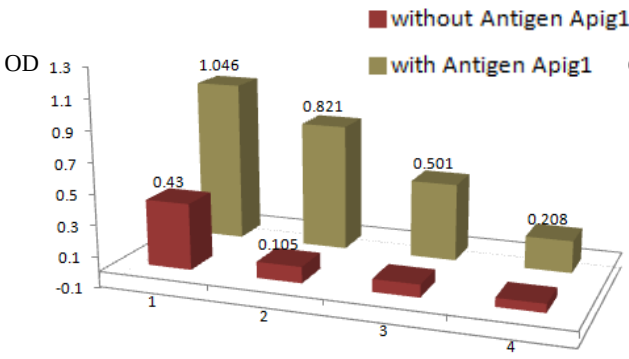


Fig.4.17: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 7,** 1 µg/ml (well: 0, 05 µg)
Antigen: Apig1 1 µg/ml(well:0.05 µg)
Detection antibody: **1 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP,
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

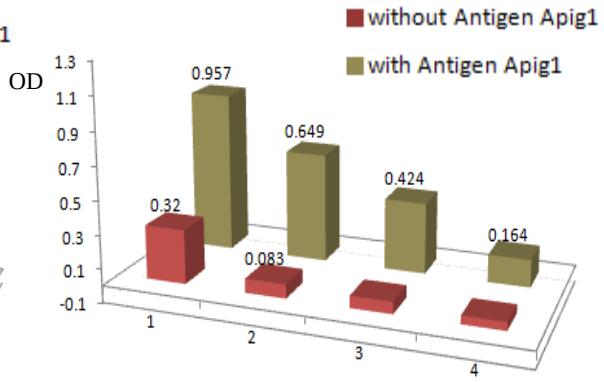


Fig.4.18: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 7**, 1 µg/ml (well: 0.05 µg)
Antigen: Apig1, 1 µg/ml (well:0,05 µg)
detection antibody **0,5 µg/ml antiApi1-2Biotin**, conjugated with
1) 1 µg/ml StreptavidinHRP,
2) 0,5 µg/ml StreptavidinHRP,
3) 0,25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

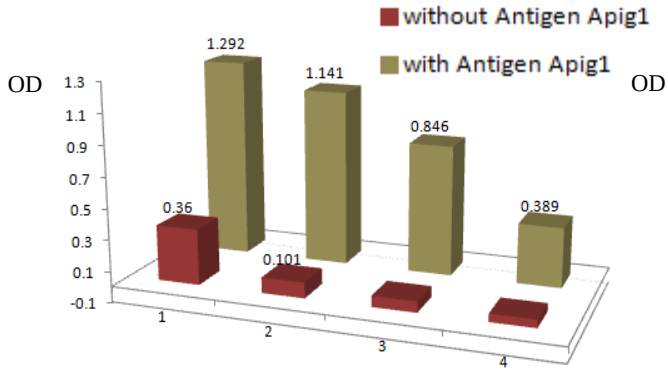


Fig.4.19: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 9** 1 µg/ml(well:0.05 µg)
Antigen: Apig1, 1 µg/ml(well:0.05 µg)
Detection antibody: **1 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0,5 µg/ml StreptavidinHRP,
3) 0,25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

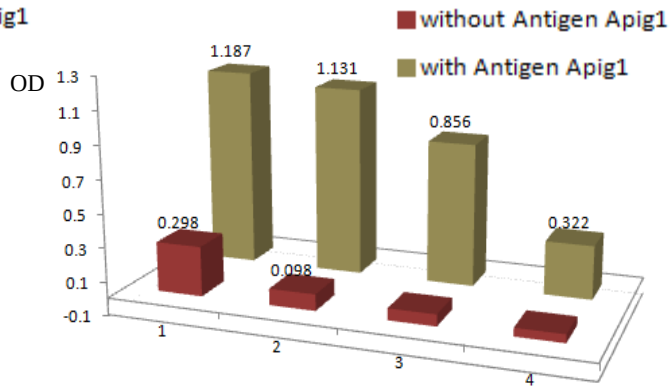


Fig.4.20: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 9** 1 µg/ml (well: 0, 05 µg)
Antigen: Apig1, 1 µg/ml(well:0.05 µg)
Detection antibody: **0, 5 µg/ml antiApi1-2Biotin**, conjugated with
1) 1 µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

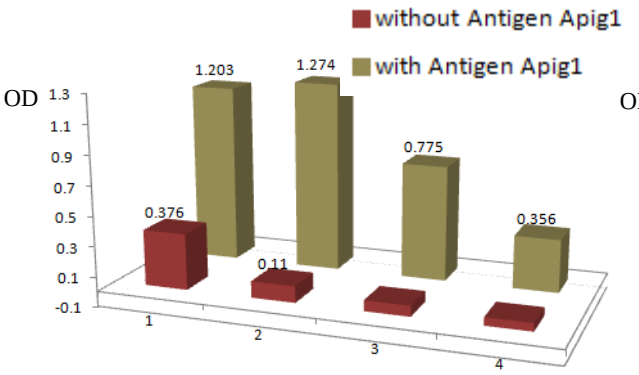


Fig.4.21: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 10**
1 µg/ml(well:0.05 µg)
Antigen: Apig1, 1 µg/ml(well:0.05 µg)
Detection antibody: **1 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP,
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

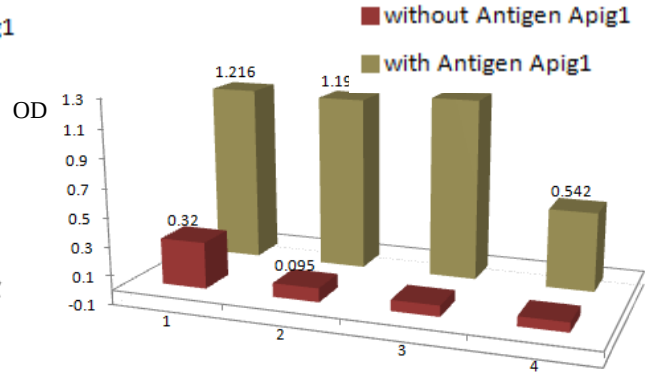


Fig.4.22: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 10**
1 µg/ml (well: 0, 05 µg)
Antigen: Apig1, 1 µg/ml (well:0.05 µg)
Detection antibody: **0.5 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP,
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

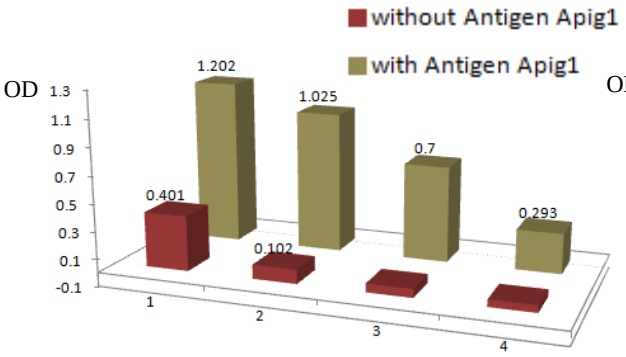


Fig.4.23: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 11**
1 µg/ml (well: 0.05 µg)
Antigen: Apig1, 1 µg/ml (well: 0.05 µg)
Detection antibody: **1 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ ml StreptavidinHRP
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

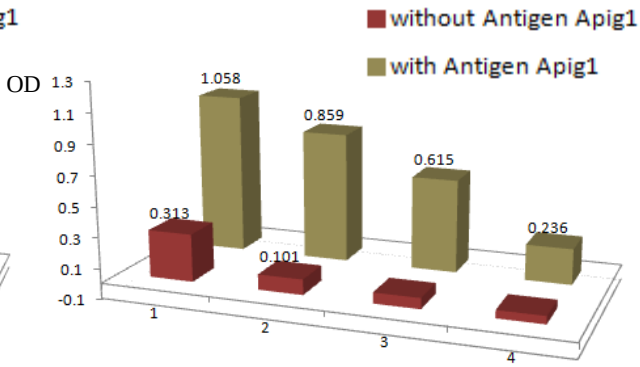


Fig.4.24: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 11**
1 µg/ml (well: 0.05 µg)
Antigen: Apig1, 1 µg/ml (well: 0, 05 µg)
Detection antibody: **0.5 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0,5 µg/ml StreptavidinHRP,
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

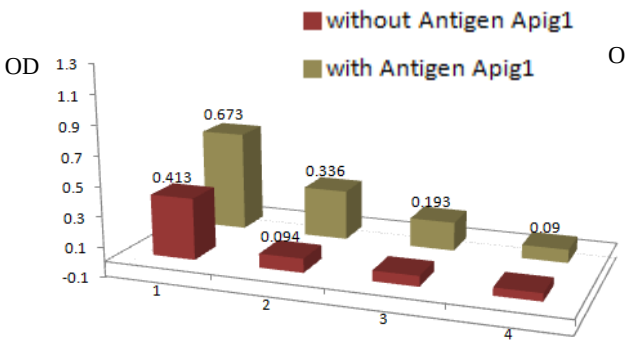


Fig.4.25: Sandwich-ELISA:
Capture antibody: purified antibody from **Animal 12**
1 µg/ml (well: 0.05 µg)
Antigen: Apig1, 1 µg/ml (well: 0.05 µg)
Detection antibody: **1 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

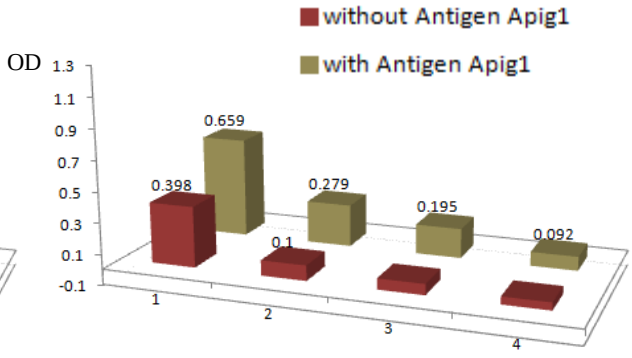


Fig.4.26: Sandwich-ELISA:
Capture antibody:purified antibody from **Animal 12**
1 µg/ml (well: 0.05 µg)
Antigen: Apig1, 1 µg/ml(well: 0.05 µg)
Detection antibody: **0.5 µg/ml antiApi1-2Biotin**, conjugated with
1) 1µg/ml StreptavidinHRP,
2) 0, 5 µg/ml StreptavidinHRP,
3) 0, 25 µg/ml StreptavidinHRP
4) 0,125 µg/ml StreptavidinHRP

The Sandwich-ELISA seems to work optimal when a concentration of 0,5 µg/ml StreptavidinHRP was applied. All the antibodies showed that when high levels of 1 µg/ml StreptavidinHRP were added in absence of antigen this was always related with high background reactivity. Not all the capture antibodies used gave the same high signals. The signals observed with purified antibodies of Animal Nr. 9, 10, 11 showed the best results and were therefore selected as capture antibodies for further experiments with celeriac.

4.5 Sandwich-ELISA test with celeriac

In order to develop a system which detects celery, the optimal conditions of the experiment with the recombinant Api g 1 (see chapter 4.4.) were adopted to celeriac. Purified antibody of Animal 10 (10p) which previously showed high signals was used as capture antibody and antibody antiApi1-2Biotin which showed in section 4.3 binding preference to celeriac was used as detection antibody. The concentration of 0,5 µg/ml StreptavidinHRP was applied. All assays were carried out in triplicate. The negative control samples included all the reactants except the antigen. In the following Fig.4.27 the results of the Sandwich-ELISA with celeriac, carrot, parsley and recombinant Api g 1 are shown.

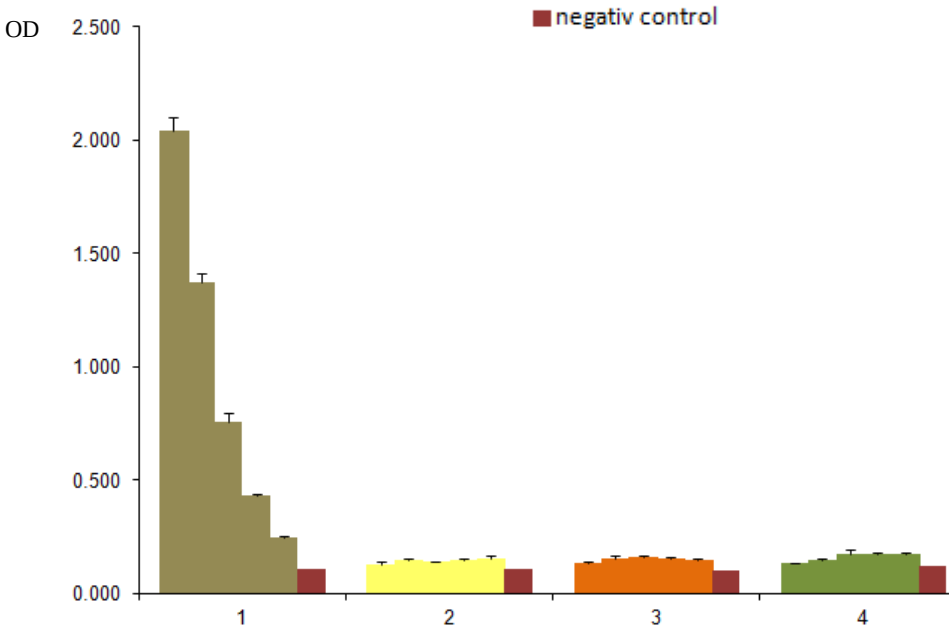


Fig. 4.27: Sandwich-ELISA:

Capture antibody: purified antibody from **Animal 10,** 2 µg/ml (well: 0.1 µg).
Antigen:

1.Apig1	**a= 1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)
2. Celleriac	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
3. Carrot	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
4. Parsley	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)

Detection antibody: antiApi1-2 Biotin 0.5 µg/ml (well 0.025 µg), Streptavidin conjugated HRP: 0, 5 µg/ml (well 0.025 µg)

In a series of experiments it was visible that the Sandwich-ELISA (Fig.: 4.27) could clearly detect the recombinant Api g 1. All colorimetric reactions that were 3 x stronger than the reaction of carrot in the Sandwich-ELISA were deemed to be positive. It was obvious that the system could determine the allergen protein Api g 1 nevertheless Api g 1 in its native form into celeriac protein extract was not detectable by this method.

4.6 Binding reactivity of antiApi1-2Biotin with celeriac and tenfold higher concentration of carrot and parsley

The biotinilated antibody antiApi1-2 was used at an Indirect-Elisa test in order to seek out if the purified antibody can distinguish between celeriac and carrot/parsley when tenfold higher concentration of the latter was applied. In a real situation of prepacked food the cross reacting ingredients carrot and parsley are mostly present in higher

concentrations as celery. Therefore tests were designed to examine if higher concentrations can influence the outcome. All assays were carried out in triplicate. The negative control samples included all the reactants except the antigen. At Fig.4.28 the results are presented.

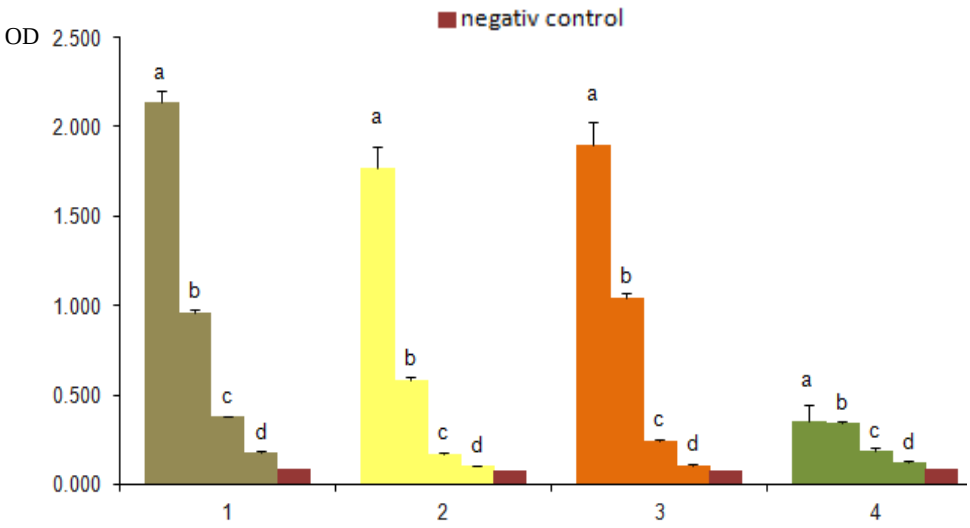


Fig.4.28: Indirect-ELISA: Reaction of :

1.Apig1	**a= 1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)
2. Celleriac	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
3. Carrot	**a= 5**0 µg/ml (well 2.50 µg)	**b=16.6** µg/ml (well 0.833 µg)	**c=5.53** µg/ml (well 0.277 µg)	**d=1.84** µg/ml (well 92.6 ng)	**e=0.61** µg/ml (well 30.09 ng)
4. Parsley	**a= 50** µg/ml (well 2.50 µg)	**b=16.6** µg/ml (well 0.833 µg)	**c=5.53** µg/ml (well 0.277 µg)	**d=0,1.84** µg/ml (well 92.6 ng)	**e=0.61** µg/ml (well 30.09 ng)

with antiApi1-2Biotin 0.5 µg/ml(well 0.025 µg) and HRP Streptavidin 0,5 µg/ml (well 0.025 µg)

The results showed that high concentration of carrot (50 µg/ml) influences the results in such a manner that it is not possible to distinguish between celeriac and carrot. Parsley didn´t seem to interfere.

4.7 Immunoblotting to define cross reactivity

After having tested the biotinylated antibody antiApi1-2, the remaining aim was to identify a purified antibody that does not show cross reactivity to carrot. For this reason immunoblots were performed. Celeriac and carrot protein extract were applied onto a 12% SDS Gel. All possible antibodies from the first and second immunization were checked. The results are shown in Fig.: 4.29-4.30.

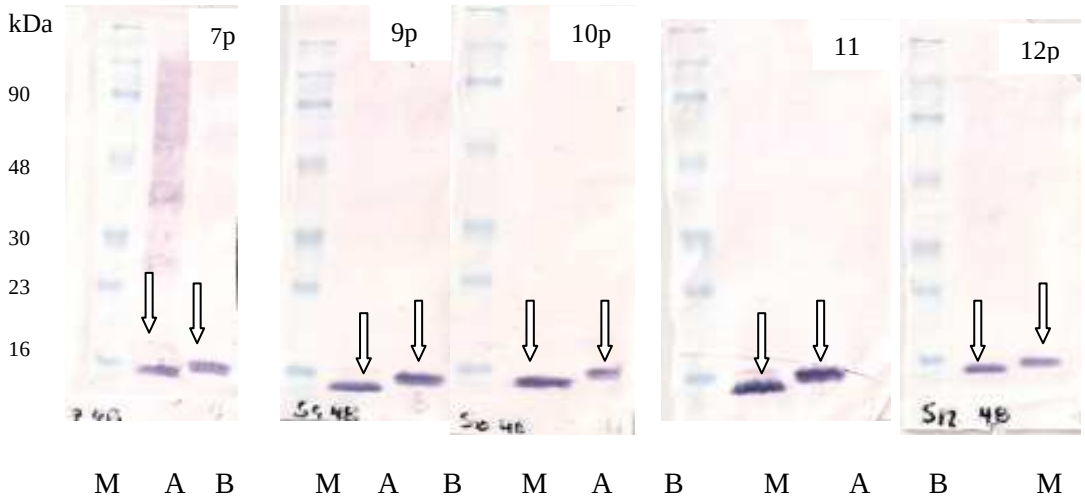


Fig.4.29: Western Blot:
A) Celeriac 2mg/ml (Slot 30µg) B) Carrot 2mg/ml (Slot 30µg) M): Marker with Api g 1 specific antibodies gained after Purification on HYDRA-Api g 1 immunoaffinity column

7p: Binding antibody: purified antibody from animal 7: 1 µg/ml
9p: Binding antibody: purified antibody from animal 9: 1 µg/ml
10p: Binding antibody: purified antibody from animal 10: 1 µg/ml
11p: Binding antibody: purified antibody from animal 11: 1 µg/ml
12p: Binding antibody: purified antibody from animal 12: 1 µg/ml
Detection antibody: goat aRabbit-AP 1:1000

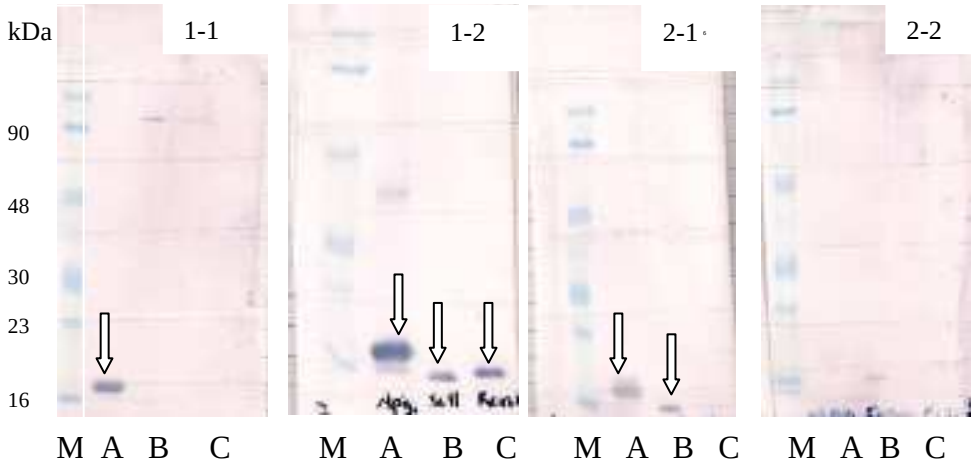
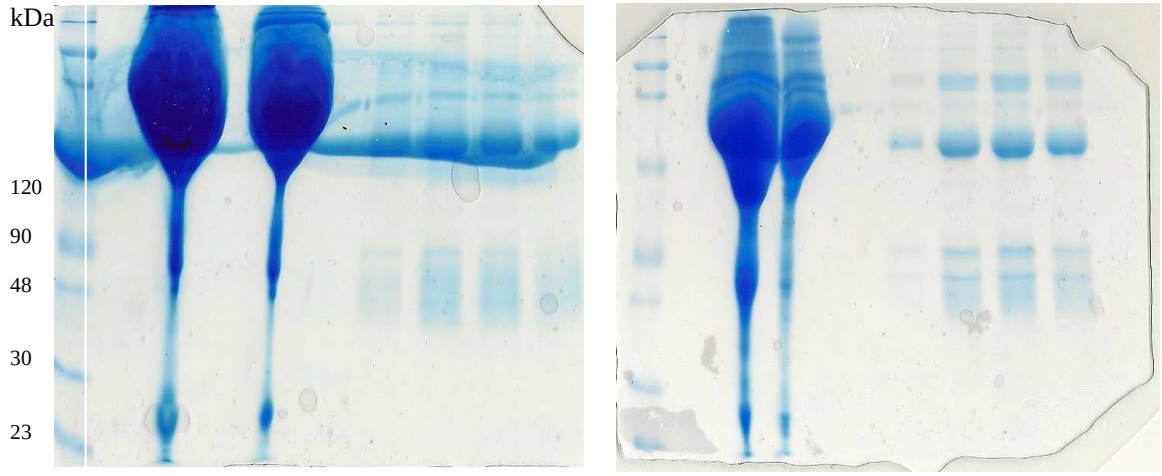


Fig.4.30: Western Blot:
A) Apig1 2mg/ml (Slot 30µg) B) Celeriac 2mg/ml (Slot 30µg)
C) Carrot 2mg/ml (Slot 30µg) M) Marker
with
1-1: Binding antibody: Serum Api1-1 1:1000
1-2: Binding antibody: Serum Api1-2 1:1000
2-1: Binding antibody: Serum Api2-1 1:1000
2-2: Binding antibody: Serum Api2-2 1:1000
Detection antibody: goat aRabbit-AP 1:1000

Next to celery's Api g 1 protein, all the antibodies from the first immunization seem to detect only one carrot protein, the DauC1 which gave the characteristic Band at 16 kDa. At the Figures 4.29 and 4.30 the corresponding Bands are marked by an arrow. From the second group of immunization the favorable sera-antibody antiApi1-2 showed affinity to carrot´s protein, a fact that was already known from the previous ELISA results. Sera-antibodies gained after immunization with peptide 1-1 and 2-1 from the Api g 1.01 and Api g 1.02 protein sequence respectively didn't show the characteristic DauC1 band, while sera-antibody gained from the immunization with peptide 2-2 of the Api g 1.02 didn't show dominant Bands at all.

4.8 Purification of Sera-antibodies by celery specific cross reactivity

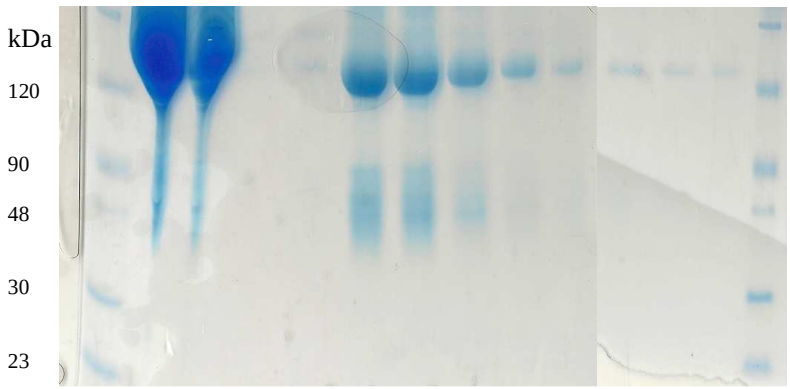
With the purpose of gaining antibodies that are specific for celery but not cross reacting with the DauC1 of carrot as well as suitable for a Sandwich-ELISA , a new affinity matrix was prepared. NHS-activated sepharose was incubated with a celeriac protein extraction in order to get immobilized onto the column. Sera-antibodies of Animal Nr.1, 4, 5, 6, 7, 8, were purified onto this column. The purification procedure is described in 3.5.7. 12% SDS-PAGE Electrophoresis was performed in order to find the fractions which contained the purified antibodies. In Fig. 4.31-4.35 the selected fractions are bold labeled.



M Flowthr. Wash Fr.1 **Fr.2 Fr3 Fr.4 Fr.5**
Animal 1

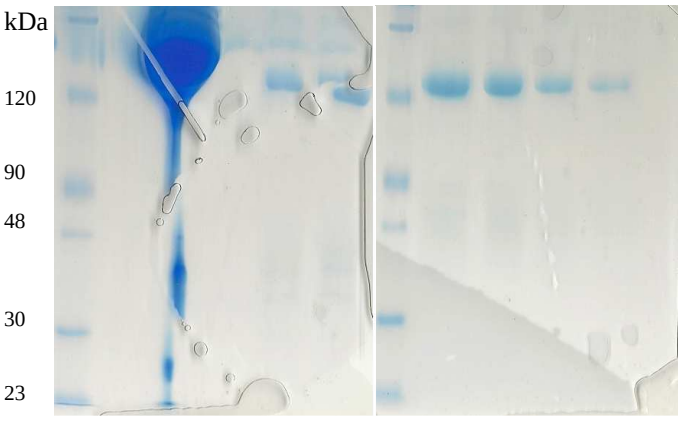
M Flowthr/Wash/Fr.1/Fr.2/**Fr.3/Fr.4/Fr.5/**Fr.6
Animal 6

Fig.4.31: SDS-PAGE 12% after an affinity chromatography to check which fragments include the celeriac specific antibodies. (M:Marker, Flowthr:Flowthrough, Fr: Fraction)



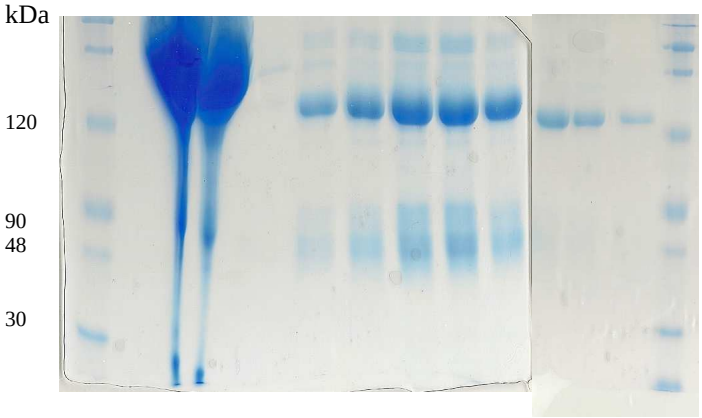
M /Flowthr/Wash/Fr.1/Fr.2/ **Fr3/ Fr.4/Fr.5/Fr6/**Fr7/ Fr8/Fr9/Fr10/M
Animal 5

Fig.4.32: SDS-PAGE 12% after an affinity chromatography to check which fragments include the celeriac specific antibodies. (M:Marker, Flowthr:Flowthrough, Fr: Fraction)



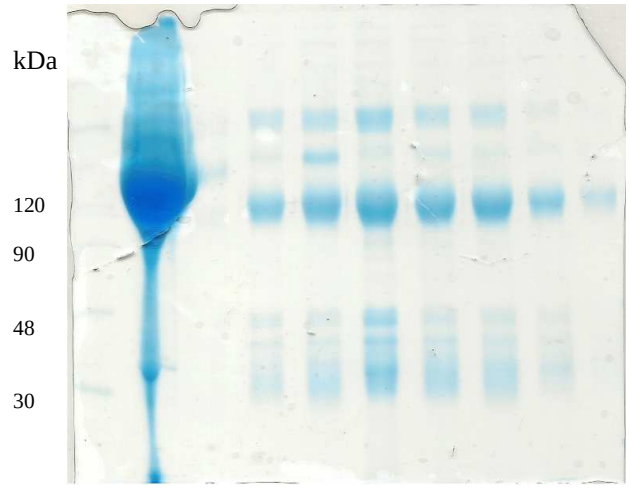
M / Wash/ Fr.1/ **Fr.2 Fr3**/ M/ **Fr.4/Fr.5/ Fr.6/** Fr.7/Fr.8/
Animal 4

Fig.4.33: SDS-PAGE 12% after an affinity chromatography to check which fragments include the celeriac specific antibodies (M:Marker, Flowthr:Flowthrough, Fr: Fraction)



M/ Flowthr/Wasch/Fr.1/**Fr.2/Fr.3/Fr.4/Fr.5/Fr.6/Fr.7/Fr.8/Fr9**/M
Animal 7

Fig.4.34: SDS-PAGE 12% after an affinity chromatography to check which fragments include the celeriac specific antibodies. (M:Marker, Flowthr:Flowthrough, Fr: Fraction)



M/Wasch/ Fr.1/**Fr.2/ Fr.3/ Fr.4/ Fr.5/ Fr.6/ Fr.7**/Fr.8
Animal 8

Fig.4.35: SDS-PAGE 12% after an affinity chromatography to check which fragments include the celeriac specific antibodies. (M:Marker, Flowthr:Flowthrough, Fr: Fraction)

The celeriac specific antibodies of each animal were further pooled, dialyzed against PBS and the protein concentration was determined at OD 280 nm.

4.9 Examination of the binding ability of the celeriac specific antibodies

In order to proof the binding ability of the antibodies gained after the procedure described above (4.8), Indirect-ELISA tests were performed. Antigen recombinant protein Api g 1, celeriac, carrot, parsley protein extract were tested. All assays were carried out in duplicate. The negative control samples included all the reactants except the antigen. The results are presented at Fig.4.36.

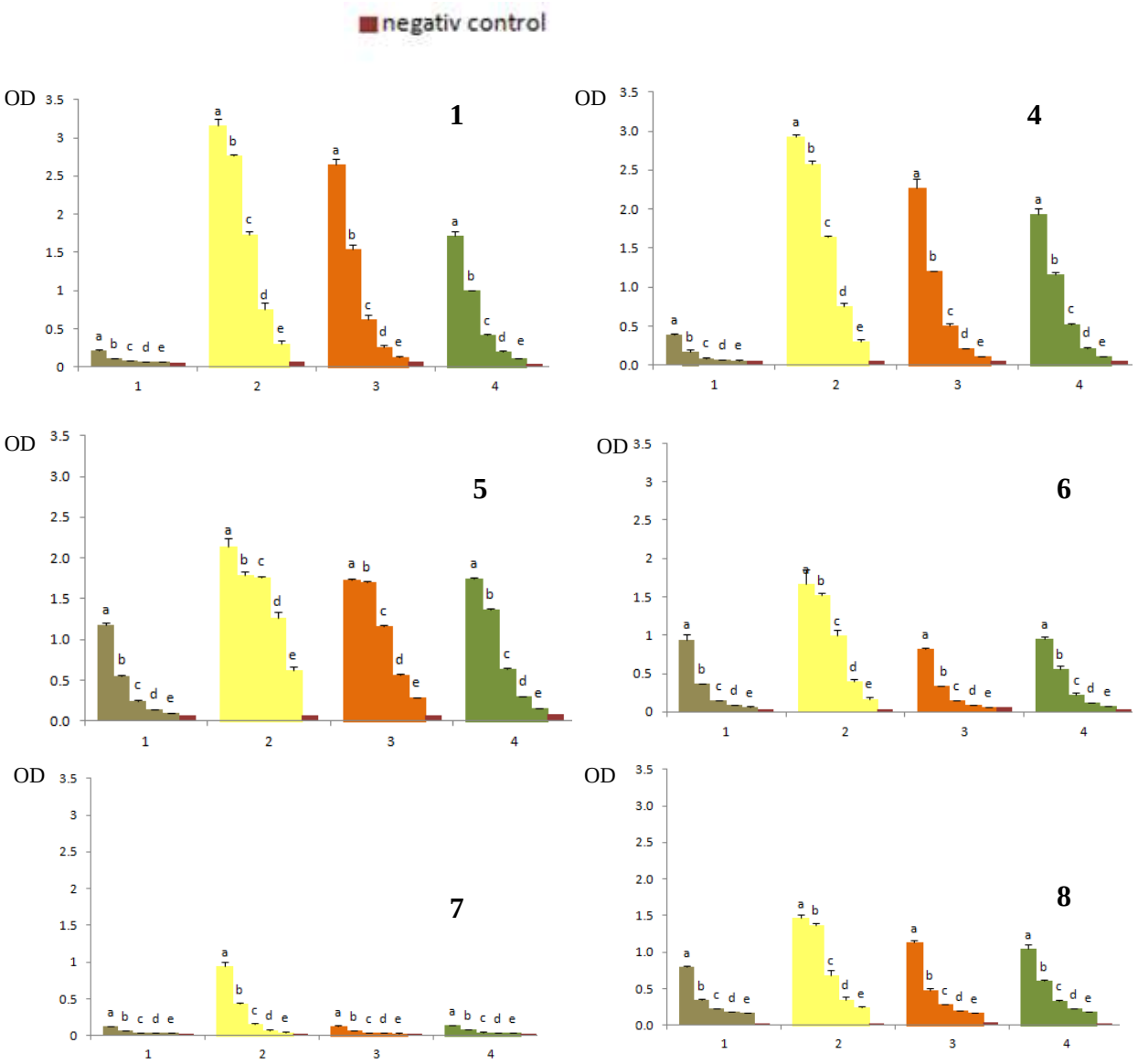


Fig.4.36: Indirect-ELISA:

Antigen

1.Api g 1	**a= 1** µg/ml (well 0.05 µg)	**b=0.333**µg/ml (well 16,666 ng)	**c=0,111** µg/ml (well 5.555 ng)	**d=37** ng/ml (well 1,852 ng)	**e=12.33** ng/ml (well 0.617 ng)
2. Celleriac	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
3. Carrot	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)
4. Parsley	**a= 5** µg/ml (well 0.250 µg)	**b=1,66** µg/ml (well 83.33 ng)	**c=0,553** µg/ml (well 27.77 ng)	**d=0,184** µg/ml (well 9,26 ng)	**e=0.061** µg/ml (well 3.09 ng)

Binding antibody: Antibody (1 µg/ml: well 0.05 µg), gained after purification onto a **sepharose-celeriac** chromatography column from
1: Animal 1, 4: Animal 4, 5: Animal 5
6: Animal 6, 7: Animal 7, 8: Animal 8

Detection Antibody: goat aRabbitHRP 1:20.000

The purified antibodies of animals Nr.1, Nr.4, Nr.5, Nr.6, Nr.8 showed a high reactivity to all their binding partners except to the protein Api g 1. Antibodies from Animal Nr.7 showed low signals at all. The antibodies were further tested by immunobloting. The results are shown in the following Fig.4.37.

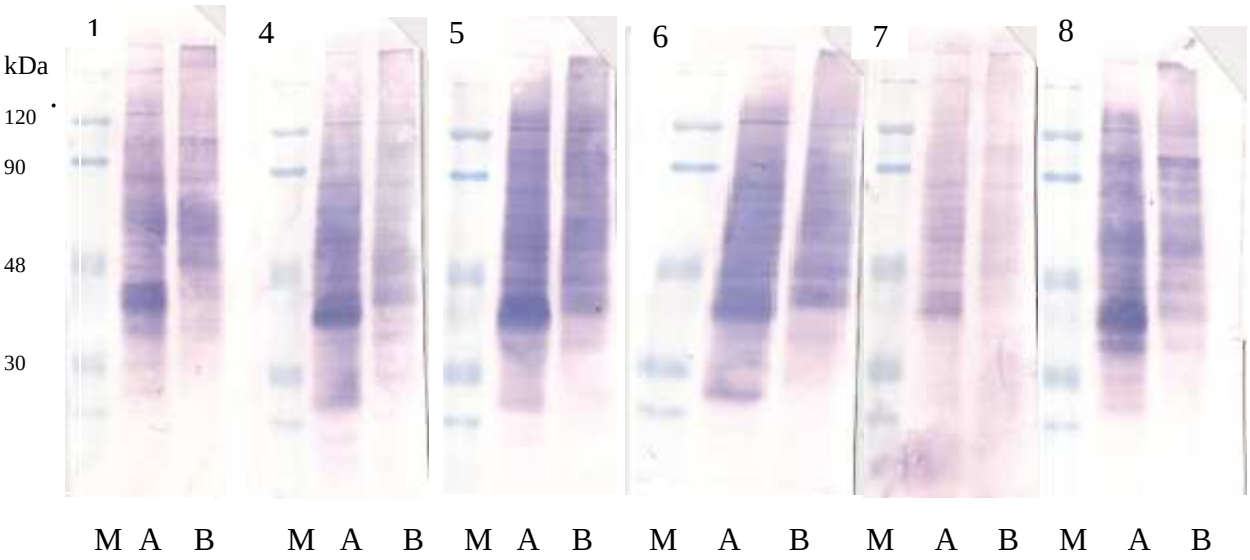


Fig.4.37: Western Blot:
A) Celeriac 2mg/ml (Slot:30 µg) B) Carrot 2mg/ml (Slot:30 µg)
with celeriac specific antibodies gained after Purification on NHS-sepharose-coupled with celeriac protein extract

1: Binding antibody: from Animal 1, 1µg/ml
4: Binding antibody: from Animal 4, 1µg/ml
5: Binding antibody: from Animal 5, 1µg/ml
6: Binding antibody: from Animal 6, 1µg/ml
7: Binding antibody: from Animal 7, 1µg/ml
8: Binding antibody: from Animal 8, 1µg/ml
Detecting antibody: goat aRabbit-AP 1:1000

The immunoblots gave evidence that the antibodies could bind with many celeriac proteins but also cross reacted with many carrot proteins, a fact that makes them unfavourable as captured antibodies in a Sandwich-ELISA system.

5. Discussion

The characterization of antibodies gained from the immunization of rabbits regarding their ability to bind with celery and the Api g 1 protein was the subject of this thesis. The results, the possible application of gained antibodies and future works that have to be done are discussed in this chapter.

5.1 Binding capacity of sera and purified-antibodies with celeriac/recombinant protein Api g 1 by the Indirect-ELISA method

After the immunization and the Api g 1 specific affinity chromatography, the immune sera and the specific antibodies were tested according to their ability to bind with celeriac protein extract and recombinant protein Api g 1. The Indirect-ELISA tests that were performed for this reason, confirmed a successful immunization and purification of Api g 1 specific antibodies from the sera. Both could bind with the recombinant Protein Api g 1 and also with the celeriac protein extract.

The different tendencies of the antibodies that had been observed are in accordance with the expectations. Animals Nr. 9/10, 11/12 were immunized with recombinant Api g 1 protein (see Table 4.1 in section 4.1.2) and with a peptide mixture, that contained parts of the Api g 1 protein sequence, respectively. Therefore their antibodies should be specific against these antigens and should show a higher binding capacity to Api g 1 than to celeriac. On the other hand, animals Nr.1, 4, 5, 6, 7, and 8, immunized against total celeriac extracts should correspond stronger to their immunogen. Therefore their higher signals with celeriac compared with the allergen protein Api g 1 were explainable and expected.

5.2 Examination of the specifity of sera and purified-antibodies by Immunoblotting

The antibodies that were purified with an Api g 1-affinity column as well as the corresponding crude sera, when tested by immonoblotting in order to estimate their specific binding affinity to Api g 1-protein and to celeriac proteins respectively showed dominantly a band at 15 kDa. This was in accordance with the literature as the Api g 1 migrates as a single band with a molecular weight of 15 kDa[15]. The recombinant Api g 1 protein carried a His-tag which is the reason why the characteristic band occurred higher in the gel (around 16 kDa) than the native Api g 1 protein in celeriac.

In certain cases the purified antibodies showed two bands in the Api g 1 samples which might be a sign of impurities in the extract of the recombinant protein. The corresponding immune sera as expected were not as specific and showed binding affinity to many other proteins of celeriac extract.

When comparing the Western Blot of purified antibody from animal Nr. 7 with those of animals Nr.9/10, Nr.11/12 there was in line with the ELISA experiment described above, a difference in the bands´ pattern to observe. This might be due to the different

immunization agents used, celeriac extract, Api g 1-protein or a Api g 1 specific peptide mixture respectively.

Sera of the second group of immunization, clearly revealed the bands that correspond to the Api g 1 protein without binding preference to other proteins. Sera of antiApi1-2 showed a high binding capacity and preference to bind specifically with the Api g 1 and for this reason it had been chosen as detection antibody.

5.3 The cross reactivity of the antibody antiApi1-2Biotin

According to the literature [6], the protein sequence of the Api g 1-protein shows 81% identity to the carrot`s major allergen DauC1 and 61% to two PR-proteins in parsley. The results of the Indirect-ELISA test to examine the cross-reactivity of the antiApi1-2Biotin with carrot and parsley, shown in Fig.:4.16, gave evidence that the antibody possesses a clear binding preference to celeriac and Api g 1-protein and does not cross react with other proteins of similar plants. At the initial and equal concentration of 5 μg/ml for celeriac, carrot, parsley, the antibody antiApi1-2Biotin showed five times higher signals to celeriac than to carrot. When comparing the signals with those of parsley they were 12 times higher. Therefore a clear differentiation might be possible and this antibody was selected for further experiments in Sandwich-ELISA systems.

5.4 Establishment of a Sandwich-ELISA system with celeriac

The results of the pretest with recombinant protein Api g 1 revealed that the concentration of antibody antiApi1-2Biotin was not of importance (Fig.4.17-4.26). A variation of its concentration gave equally high signals. On the contrary the concentration of StreptavidinHRP was very significant. High levels of 1 μg/ml StreptavidinHRP were related with high background reactivity. The concentration of 0,5 μg/ml StreptavidinHRP was therefore chosen. Also at this stage of experiments some capture antibodies were selected as they gave higher signals. Antibodies of Animal Nr. 9, 10, 11 showed the best results. The optimal conditions for a functional Sandwich-ELISA system to determine Api g 1 were successfully established and selected for further experiments with celery.

In a series of experiments, with celery as antigen, to observe was that the system could not determine Api g 1 in its native form into celeriac protein extract (Fig. 4.27). An explanation for the above result might be that the linear epitopes, used for immunization, gave immune reagents detecting the bacterially expressed recombinant Api g 1 in a perfect way, however the native protein allergen present in the celeriac extract might be folded in such a manner that its epitope was not available for binding with the antibodies used in the Sandwich-ELISA system.

5.5 Evaluation of Indirect-ELISA

A system based on a Sandwich-ELISA would be of great interest as it would be more stable and specific as the Indirect-ELISA. However with the given tools it was not possible to establish a celery specific Sandwich-ELISA system. Therefore the

applications of a detection kit based on an indirect system were further examined. In a real situation of prepacked food the cross reacting ingredients carrot and parsley are mostly present in higher concentration as celery. In this regard tests were designed to check if higher concentrations of other cross reacting ingredients influence the result. Celeriac was applied onto the plate at a concentration of 5 μg/ml but carrot and parsley were added at concentrations of 50 μg/ml.

In this setting the Indirect-ELISA showed that the antibody antiApi1-2Biotin strongly reacted to carrot immobilized in high concentrations. Therefore such a test would not distinguish between celeriac and carrot. In contrast the antibody didn´t show binding to parsley.

In a further series of experiments performing immunoblots, all the antibodies were tested in order to obtain an image of the number and the identity of the proteins in carrot that cross-reacted (Fig.:4.7-4.14). As a result, all the antibodies showed a specific band with an apparent MW of 16 kDa in carrot extracts which corresponds to the DauC1 protein, the major carrot allergen according to the literature [10]. Concluding, all the antibodies seem to recognize besides the reactivity with celery's Api g 1 protein, only one carrot protein, the DauC1.

Investigating the antibodies of the second group of immunization, gained from animals immunized with peptides parts of the Api g 1.01 and of the Api g 1.02 protein, one could clearly make out that they showed different results in immunoblots. In detail antiApi1-1 showed only reactivity with the recombinant Api g 1 protein and no reactivity with celeriac and carrot. The results were in accordance with the expectations. Based on information from databanks, the peptide (aa 1-12) from the Api g 1.01 sequence, shows only slight similarity to carrot´s protein DauC1 (data shown at Annex 10). Therefore the sera-antibody gained after immunization with this immunogen didn't show a reaction with carrot´s protein extract.
AntiApi1-2 showed a full range of bands, showing reactivity with the Api g1-protein, with celeriac and carrot protein extract. This result is in accordance with the results of the Indirect-ELISA test where antiApi1-2 bound to carrot when high amounts were used.
In Annex 10 it is shown that the peptide (aa121-140) from the Api g 1.01 protein sequence has high similarity to carrots Protein DauC1 and to some parsley`s proteins (PR Family). This explains the immunoblot results which revealed the characteristic bands with Api g 1 protein, with celeriac and carrot protein extract.
AntiApi2-1 antibody showed only reactivity with the Api g 1-protein and celeriac. The peptide (aa11-31) part of the Apig1.201 protein, shows less similarity to carrot`s, and parsley`s protein (data shown at Annex 10). This is in agreement with the immunoblot results which showed the corresponding band with recombinant protein Api g 1, with celeriac but no band with carrot.
AntiApi2-2 gave slight bands barely detectable with Api g 1 and celeriac.(Fig.:4.14)
One possible explanation of this slight reaction is the immunoglobulin´s concentration which might have been too low.

5.6 Purification of antibodies

In order to purify celery specific antibodies, celery proteins were immobilized onto NHS-activated-sepharose. Sera of six animals (Animal 1, 4, 5, 6, 7, 8) immunized with celery extract in different variations (see Table 3.1) were purified. The gained antibodies were further tested by Indirect-ELISA in order to find out their binding capacities to Api g 1- Protein, to celeriac, carrot and parsley. All of them showed a high reactivity to all their binding partners except to the protein Api g 1. This is explainable once they were specifically immunized with celery. However these antibodies do not show specific reactivity to celery. Therefore although their overall high binding capacity, they couldn`t be used as capture antibody in a Sandwich-ELISA test because of a lack of celery specificity. Performing immunoblots with these antibodies a wide spectrum of bands in celeriac extract was observed, which was expectable (Fig.:4.37). However they showed a broad banding pattern with carrot as well which is not favourable. For future experiments it should be examined if these antibodies may have further functions than acting as capture antibodies. Their application as detection antibodies will be considered.

5.7 Future prospects

Celery and carrot contain several proteins with IgE-binding activities. Their allergenic potential as well as biochemical and molecular characteristics have been reported in a number of publications, and can be found in protein databases. The major allergen of celeriac is the Api g 1, representing 20% of the total mass of celeriac protein content which might be the reason of it being well characterized and a good candidate for experimental investigation. However its protein sequence shows many similarities to other plant proteins, which increases the risk of cross reactions when immuno-tests are performed.

One of the future goals is to define regions of the protein sequence where the amino acid sequence doesn’t have strong similarities with those of other allergens. The second group of immunization with different peptides, parts of the Api g 1 sequence gave results going in such a direction.

Using the peptide (aa11-31) part of the Api g 1.201 protein as immunogen the sera-antibody gained showed less reactivity with Api g 1 and celeriac compared to antiApi1-2 antibody during Indirect-ELISA experiments.(Fig4.6a-4.6b) According to databank’s information its amino acid sequence comparison, shows less similarity to carrot`s, and parsley`s protein. The immunoblot showed a band with Api g 1, one with celeriac but no band with carrot (Fig.4.30). Based on this result some new experiments would be of interest, using this antibody as a capture antibody in Sandwich-ELISA systems
According to the Databanks, the sequence of peptide (aa121-138) of the Apig1.02 protein, shows neither similarity with parsley (three proteins) nor to carrot (protein DauC1) nor to celeriac`s protein Api g 1.01 (Annex 10). Indirect-ELISA results did not show signals as strong as the results with the previous antibodies. Immunoblot gave only very slight bands with Api g 1 and nearly no reactivity with celeriac. This

could be due to a too low antibody concentration. In this case increasing the antibody´s concentration might lead to more satisfying data. Another strategy could be to start new immunization with peptides discriminating stronger between celery allergen and corresponding proteins from related plants. In this regard it might be helpful to immunize other animal species than rabbits, which were used solely in these experiments, since the diet of rabbits include celery and therefore they might not be able to produce large amounts of antibodies against these immunogens.

In this project antiApi1-2 was chosen to serve as detector antibody. It was however not possible to establish a celery specific Sandwich-ELISA with this antibody. In this regard it might be possible that another candidate antibody that has already been gained in these immunizations may give a more specific result without cross reacting with carrot.

Also the fact that the Api g 1 protein is a thermo unstable protein which might be not detectable in processed food has to be considered. According to this information immunization with another celeriac major allergen has to be taken into account. In that case a small peptide of the protein sequence showing no or just little homology to other plant proteins has to be defined.

5.8 Application of antiApi1-2 antibody

The antibody antiApi1-2Biotin which was gained after the purification with an antigen affinity column showed high affinity to celeriac. However, as a critical point, it was observed that this antibody showed a pronounced cross reactivity with carrot at higher concentrations similar to those as they are present in food and spices. Parsley would not cause cross-reactivity problems and is not of important relevance.

The aim of the project was the development of a system which specifically detects celery protein in multi-component foods. In the presence of carrot at high concentrations such a test would not be able to specifically detect celery. The applicability of such a test is therefore momentarily limited to the prescreening of food products, however it cannot distinguish between carrot and celery in these products. In this regard positive results would need to be confirmed by methods such as PCR. Tests based on this antibody could be applied in food products in which carrot is definitely not included such as marinades for meat.

The Indirect-Elisa with the antibody antiApi1-2Biotin could be effectively employed to monitor the performance of industrial cleaning procedures of manufacturers dealing with celery products. The cleaning process in regard to contaminations of follow-up products is a very important step. The last washing solution should be free of celery traces and show no reactivity with the antiApi1-2. Even if the products have high concentration of carrot, the final washing water must be free of detectable traces, to be sure not having celery contamination.

6. Summary

The increasing number of people suffering from symptoms related to food allergies and the EFSA Directive 2007/68/EC makes European manufacturers sceptical about the allergen-management politics they have to follow. This lead some companies to include expensive and complicated detection methods into their routine analysis, while others turned to the over labelling of their products. Celery is one of the foods that causes allergy, especially in central Europe and is according to the above Directive labelling obligatory. In the present situation some PCR methods have been developed for the detection of celery allergens. Immunological detection methods, which are very common in food allergen detection, have not yet been developed for celery.

In the work presented, the aim was to establish such a detection system. In this regard rabbits were immunized in two different schemes with different immunization agents. Celery raw material, celery protein extract, recombinant celery allergen Api g 1, individual Api g 1.01 and Api g 1.02 specific peptides as well as an immunogenic Api g 1 specific peptide mix, were used to immunize the rabbits and to get specific antibodies against the immunogens. The antisera gained by these immunizations, were subjected to immunoaffinity purification using immobilized Api g 1 protein.

These purified polyclonal antibodies showed a good binding ability according to results from Indirect-ELISA assays, which were further confirmed by immunoblotting. The Western Blots revealed a dominant band at 15 kDa in celeriac protein extracts and at 16 kDa with recombinant Api g 1 due to its His-tag. Sera-antibodies were not specific. One of the purified antibodies (antiApi1-2), which was obtained by immunization with the peptide aa121-140, part of the Api g 1.01 protein, got conjugated with Biotin in order to be used as detection antibody.

Some allergens show cross reactivity with other plant proteins due to their protein sequence homology. In order to check if the above gained and conjugated antibody showed such a tendency, ELISA tests were performed with equal concentrations of celeriac, carrot, and parsley protein extracts. A clear differentiation was possible showing the specificity of the antibody. In order to establish a Sandwich-ELISA this antibody was combined with antibodies from the first immunization scheme, especially with the sera from animals 9/10 (immunized with recombinant Api g 1) and animals 11/12 (immunized with the Api g 1 specific peptide mix) acting as capture agents. Firstly the antigen recombinant protein Api g 1 was tested. In a second step a Sandwich-ELISA test with celeriac protein extract was examined. During the course of the project it got obvious that the Sandwich-ELISA established in that way was able to detect the recombinant Api g 1 protein specific and sensitive but not the native Api g 1 protein present in celery extract.

In order to understand how the Indirect-ELISA-test with the biotinilated antibody antiApi1-2 would work, in systems which carrot´s and parsley´s concentration were high, further tests were performed. At a 10-fold higher concentration of carrot and parsley protein extracts (50 µg/ml), this antibody showed a high degree of cross reactivity, a fact that would be problematic in a multicomponent food. By

immunoblots it could be shown that the only interfering factor was a protein at 16 kDa which corresponds to DauC1, the major protein allergen in carrot.

To gain antibodies that could be used to establish a stable Sandwich-ELISA, antisera of animals 1, 4, 5, 6, 7, 8, were subjected to purification with sepharose on which whole celery protein was immobilized. These animals were immunized against celeriac. During Indirect-ELISA-tests they showed less reactivity towards recombinant Api g 1, high reactivity towards celery protein but also against carrot protein. Performing immunoblotting these antibodies showed complex banding patterns in celery and carrot extracts and therefore could not be used as specific agents in Sandwich-ELISAs because of their obvious low specificity.

Summarizing the results, one can conclude that the antibody antiApi1-2 as shown in an Indirect-ELISA test could have some application. The detection of celery in a multicomponent food with high concentrations of carrot would not have a reliable specificity. Therefore this antibody might be used for complex foods that do not include carrot. In addition it would be useful to examine if the system can be applicable in the cleaning procedure of the food industry. Traces of detectable celery, even carrot, would indicate insufficient cleaning and would help manufacturers to optimize their validations and plan their cleaning steps more effectively.

In the future, improvements could be made by new immunization schemes. In order to minimize cross reactivity, new peptides of the antigen´s protein sequence, which do not have high sequence homology with other plant proteins, have to be carefully selected and used as immunization agents. Moreover it should be considered to immunize animal species which would correspond with an extensive immune response towards these celery allergens. New antibodies could be combined with the already gained antibodies in order to establish an improved Sandwich-ELISA test.

7. Annex

Annex 1

'ANNEX IIIa

Ingredients referred to in Article 6(3a), (10) and (11)

1. Cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or their hybridised strains) and products thereof, except:

   (a) wheat-based glucose syrups including dextrose (1);

   (b) wheat-based maltodextrins (1);

   (c) glucose syrups based on barley;

   (d) cereals used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.

2. Crustaceans and products thereof.

3. Eggs and products thereof.

4. Fish and products thereof, except:

   (a) fish gelatine used as carrier for vitamin or carotenoid preparations;

   (b) fish gelatine or Isinglass used as fining agent in beer and wine.

5. Peanuts and products thereof.

6. Soybeans and products thereof, except:

   (a) fully refined soybean oil and fat (1);

   (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, natural D-alpha tocopherol succinate from soybean sources;

   (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources;

   (d) plant stanol ester produced from vegetable oil sterols from soybean sources.

7. Milk and products thereof (including lactose), except:

   (a) whey used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages;

   (b) lactitol.

8. Nuts, i.e. almonds (*Amygdalus communis L.*), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoiesis (Wangenh.) K. Koch*), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia nuts and Queensland nuts (*Macadamia ternifolia*), and products thereof, except:

   (a) nuts used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.

9. Celery and products thereof. 

10. Mustard and products thereof.

11. Sesame seeds and products thereof.

12. Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO_2.

13. Lupin and products thereof.

14. Molluscs and products thereof.'

Annex 2

Protein sequences of Api g1.01 and Api g 1.02 [30] s

```
        10         20         30         40         50         60
MGVQTHVLEL TSSVSAEKIF QGFVIDVDTV LPKAAPGAYK SVEIKGDGGP GTLKIITLPD

        70         80         90        100        110        120
GGPITTMTLR IDGVNKEALT FDYSVIDGDI LLGFIESIEN HVVLVPTADG GSICKTTAIF

       130        140        150
HTKGDAVVPE ENIKYANEQN TALFKALEAY LIAN
```

Api g 1.01

```
        10         20         30         40         50         60
MGVQKTVVEA PSTVSAEKMY QGFLLDMDTV FPKVLPQLIK SVEILEGDGG VGTVKLVHLG

        70         80         90        100        110        120
EATEYTTMKQ KVDVIDKAGL AYTYTTIGGD ILVDVLESVV NEFVVVPTDG GCIVKNTIIY

       130        140        150
NTKGDAVLPE DKIKEATEKS ALAFKAVEAY LLANLQFLA
```

Api g 1.02

Annex 3:

The secondary structure of the Api g 1 .01[30]

☐	Chain	1 – 154	154	Major allergen Api g 1	PRO_0000154172

Secondary structure

Helix Strand Turn

Details...

☐	Beta strand	3 – 14	12	
☐	Helix	16 – 23	8	
☐	Helix	27 – 34	8	
☐	Helix	36 – 38	3	
☐	Beta strand	40 – 49	10	
☐	Beta strand	53 – 57	5	
☐	Beta strand	66 – 75	10	
☐	Turn	76 – 79	4	
☐	Beta strand	80 – 88	9	
☐	Helix	89 – 91	3	
☐	Turn	92 – 94	3	
☐	Beta strand	95 – 106	12	
☐	Beta strand	112 – 123	12	
☐	Helix	130 – 153	24	

Annex 4

Protein sequence of Api g 2.01[30]

```
MGVSKVAIAVAVMLMVVVINHPAVVEGLTCGQVTGKLGGCLGYL
KGGGYPSPACCGGVKGLNSLAKTPADRKQACACLKTLAGSVKGINYGAA
SALPGKCGIRIPYPISPSTDCSRVN
```

Api g 2.01

Protein sequence of Api g 3.01 [30]

```
        10         20         30         40         50         60
MAASTMALSS PALAGKAVKV APSSSELFGN GRVSMRKTVK APVSDSPWYG PDRVKYLGPF

        70         80         90        100        110        120
SGEAPSYLTG EFPGDYGWDT AGLSADPETF AKNRELEVIH SRWAMLGALG CVFPELLARN

       130        140        150        160        170        180
GVKFGEAVWF KAGSQIFSEG GLDYLGNPSL VHAQSILSIW ATQVILMGAV EGYRVAGGPL

       190        200        210        220        230        240
GEIVDPLYPG GSFDPLGLAE DPERSAELKV KELKNGRLAM FSMFGFFVQA IVTGKGPLEN

       250        260
LADHLADPVN NNAWAFATNF VPGK
```

Api g 3.01

Protein sequence of Api g 4.01 [30]

```
        10         20         30         40         50         60
MSWQAYVDDH LMCEVEGNPG QTLTAAAIIG HDGSVWAQSS TFPQIKPEEI AGIMKDFDEP

        70         80         90        100        110        120
GHLAPTGLYL GGAKYMVIQG EPNAVIRGKK GSGGVTIKKT GQALVFGVYD EPVTPGQCNV

       130
IVERLGDYLI DQGL
```

Api g 4.01

Protein sequence of Api g 5.01 [30]

```
        10         20         30         40         50         60
LPNPSGFVTC LSSISKSVYT PAINLKAVIA DPVAKTAVVQ AGATLGEVYY XIIYARVLWV

        70         80
GNTTQKLEWI RSLHDYQSSF FPFFSA
```

Api g 5.01

Annex 5

Similarities of Api g 1.01 with other major food allergens [30]

Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
★	**Major allergen Api g 1**	Apium graveolens (Celery)	154	100.0%	780	3.0×10^{-81}	
★	**Major allergen Dau c 1**	Daucus carota (Carrot)	154	81.0%	652	2.0×10^{-66}	
☆	**Pathogenesis-related protein**	Daucus carota (Carrot)	154	79.0%	641	3.0×10^{-65}	**DcPRP**
☆	**PRP-like protein**	Daucus carota (Carrot)	154	75.0%	627	1.0×10^{-63}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	75.0%	625	2.0×10^{-63}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	75.0%	623	4.0×10^{-63}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	75.0%	621	7.0×10^{-63}	
☆	**Pathogenesis-related protein**	Pimpinella brachycarpa	154	77.0%	617	2.0×10^{-62}	**PR1**
☆	**Pathogenesis-related protein-like protein 1**	Daucus carota (Carrot)	154	75.0%	615	4.0×10^{-62}	**DcPRPlike1**
☆	**PRP-like protein**	Daucus carota (Carrot)	154	74.0%	606	4.0×10^{-61}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	74.0%	602	1.0×10^{-60}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	74.0%	600	2.0×10^{-60}	
☆	**Pathogenesis-related protein-like protein 2**	Daucus carota (Carrot)	154	71.0%	597	4.0×10^{-60}	**DcPRPlike2**
★	**Pathogenesis-related protein B**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	61.0%	497	2.0×10^{-48}	**PCPR1-3**
★	**Pathogenesis-related protein A**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	61.0%	496	2.0×10^{-48}	**PCPR1-1 PCPR1-2**
☆	**Pathogenesis-related protein 1**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	60.0%	489	1.0×10^{-47}	
☆	**PR10-3**	Panax ginseng (Korean ginseng)	154	57.0%	444	2.0×10^{-42}	
☆	**PR10-1**	Panax ginseng (Korean ginseng)	155	56.0%	442	4.0×10^{-42}	
★	**Ribonuclease 1**	Panax ginseng (Korean ginseng)	154	56.0%	437	2.0×10^{-41}	
☆	**PR10-2**	Panax ginseng (Korean ginseng)	154	53.0%	407	5.0×10^{-38}	
☆	**Pathogensis-related protein 10**	Panax ginseng (Korean ginseng)	154	54.0%	407	5.0×10^{-38}	**PR-10**
☆	**Putative uncharacterized protein**	Vitis vinifera (Grape)	195	50.0%	403	1.0×10^{-37}	VIT_05s0077g01600
★	**Ribonuclease 2**	Panax ginseng (Korean ginseng)	153	53.0%	402	2.0×10^{-37}	
★	**Major allergen Api g 2**	Apium graveolens (Celery)	159	52.0%	402	2.0×10^{-37}	
☆	**Putative uncharacterized protein**	Vitis vinifera (Grape)	159	50.0%	399	4.0×10^{-37}	VITISV_017149
★	**Pathogenesis-related protein 2**	Petroselinum crispum (Parsley) (Petroselinum hortense)	158	51.0%	394	2.0×10^{-36}	**PR2**

Homology of protein sequence of Api g 1with Parsley´s Protein PR1-3

```
1     MGVQTHVLELTSSVSAEKIFQGFVIDVDTVLPKAAPGAYKSVE-IKGDGGPGTLKIITLP   59 P49372
      MGVQ   +E TSSVSAEK+F+G  +D+DT+LP+  PGA KS E ++GDGG GT+K++ L
1     MGVQKSEVEATSSVSAEKLFKGLCLDIDTLLPRVLPGAIKSSETLEGDGGVGTVKLVHLG   60 P19418

60    DGGPITTMTLRIDGVNKEALTFDYSVIDGDILLGFIESIENHVVLVPTADGGSICKTTAI  119 P49372
      D  P  TM  ++D ++K   T+ YS+IDGDILLGFIESI NH   VP ADGG   K+T I
61    DASPFKTMKQKVDAIDKATFTYSYSIIDGDILLGFIESINNHFTAVPNADGGCTVKSTII  120 P19418
```

Homology of protein sequence of Api g 1 with Parsley´s Protein PR1-1

```
1     MGVQTHVLELTSSVSAEKIFQGFVIDVDTVLPKAAPGAYKSVE-IKGDGGPGTLKIITLP   59 P49372
      MGVQ   +E TSSVSAEK+F+G  +D+DT+LP+  PGA KS E ++GDGG GT+K++ L
1     MGVQKSEVETTSSVSAEKLFKGLCLDIDTLLPQVLPGAIKSSETLEGDGGVGTVKLVHLG   60 P19417

60    DGGPITTMTLRIDGVNKEALTFDYSVIDGDILLGFIESIENHVVLVPTADGGSICKTTAI  119 P49372
      D  P  TM  ++D ++K   T+ YS+IDGDILLGFIESI NH   VP ADGG   K+T I
61    DASPFKTMKQKVDAIDKATFTYSYSIIDGDILLGFIESINNHFTAVPNADGGCTVKSTII  120 P19417

120   FHTKGDAVVPEENIKYANEQNTALFKALEAYLIAN   154 P49372
      F+TKGDAVVPEENIK+AN+QN  +FKA+EAYLIAN
121   FNTKGDAVVPEENIKFANDQNLTIFKAVEAYLIAN   155 P19417
```

Homology of protein sequence of Api g 1 with parsley´s protein PR2

```
1     MGVQTHVLELTSSVSAEKIFQGFVIDVDTVLPKAAPGAYKSVEI-KGDGGPGTLKIITLP    59 P49372
      MG  T  +E+ SSV A+ I++GF++D+D ++PK  P A KS+EI  GDGG GT+K +TL
1     MGAVTTDVEVASSVPAQTIYKGFLLDMDNIIPKVLPQAIKSIEIISGDGGAGTIKKVTLG    60 P27538

60    DGGPITTMTLRIDGVNKEALTFDYSVIDGDILLGFIESIENHVVLVPTADGGSICKTTAI   119 P49372
      +    T +  RID ++ EAL + YS+I+GD+LLG IESI +   +VPT DGG I K T I
61    EVSQFTVVKQRIDEIDAEALKYSYSIIEGDLLLGIIESITSKFTVVPT-DGGCIVKNTTI   119 P27538

120   FHTKGDAVVPEENIKYANEQNTALFKALEAYLIAN   154 P49372
      +   GDAV+PEEN+K A EQ+  +FKA+EAYL+AN
120   YTPIGDAVIPEENVKEATEQSGMVFKAIEAYLLAN   154 P27538
```

Homology of protein sequence of Api g 1 with carrot`s protein DauC1

```
1     MGVQTHVLELTSSVSAEKIFQGFVIDVDTVLPKAAPGAYKSVEIKGDGGPGTLKIITLPD    60 P49372
      MG Q+H LE+TSSVSAEKIF G V+DVDTV+PKAAPGAYKSVE+KGDGG GT++IITLP+
1     MGAQSHSLEITSSVSAEKIFSGIVLDVDTVIPKAAPGAYKSVEVKGDGGAGTVRIITLPE    60 O04298

61    GGPITTMTLRIDGVNKEALTFDYSVIDGDILLGFIESIENHVVLVPTADGGSICKTTAIF   120 P49372
      G PIT+MT+R D VNKEALT+D +VIDGDILLGFIESIE H+V+VPTADGGSI KTTAIF
61    GSPITSMTVRTDAVNKEALTYDSTVIDGDILLGFIESIETHLVVVPTADGGSITKTTAIF   120 O04298

121   HTKGDAVVPEENIKYANEQNTALFKALEAYLIAN   154 P49372
      HTKGDAVVPEENIK+A+ QNTALFKA+EAYLIAN
121   HTKGDAVVPEENIKFADAQNTALFKAIEAYLIAN   154 O04298
```

Homology of protein sequence of Api g 1 with Api g 2

```
1     MGVQTHVLELTSSVSAEKIFQGFVIDVDTVLPKAAPGAYKSVEI-KGDGGPGTLKIITLP    59 P49372
      MGVQ  V+E  S+VSAEK++QGF++D+DTV PK  P   KSVEI +GDGG GT+K++ L
1     MGVQKTVVEAPSTVSAEKMYQGFLLDMDTVFPKVLPQLIKSVEILEGDGGVGTVKLVHLG    60 P92918

60    DGGPITTMTLRIDGVNKEALTFDYSVIDGDILLGFIESIENHVVLVPTADGGSICKTTAI   119 P49372
      +    TTM  ++D ++K  L + Y+ I GDIL+  +ES+ N  V+VPT DGG I K T I
61    EATEYTTMKQKVDVIDKAGLAYTYTTIGGDILVDVLESVVNEFVVVPT-DGGCIVKNTTI   119 P92918

120   FHTKGDAVVPEENIKYANEQNTALFKALEAYLIAN   154 P49372
      ++TKGDAV+PE+ IK A E++   FKA+EAYL+AN
120   YNTKGDAVLPEDKIKEATEKSALAFKAVEAYLLAN   154 P92918
```

Annex 6

Similarities of the isoform Api g 1.02 with other major food allergens [30]

Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
★	**Major allergen Api g 2**	Apium graveolens (Celery)	159	100.0%	792	1.0×10^{-82}	
☆	**Major allergen isoform Dau c 1.0201**	Daucus carota (Carrot)	154	95.0%	741	9.0×10^{-77}	
☆	**PR10-1**	Panax ginseng (Korean ginseng)	155	70.0%	561	7.0×10^{-56}	
★	**Ribonuclease 1**	Panax ginseng (Korean ginseng)	154	70.0%	556	2.0×10^{-55}	
☆	**PR10-3**	Panax ginseng (Korean ginseng)	154	64.0%	523	2.0×10^{-51}	
★	**Pathogenesis-related protein 2**	Petroselinum crispum (Parsley) (Petroselinum hortense)	158	59.0%	503	3.0×10^{-49}	**PR2**
☆	**PR10-2**	Panax ginseng (Korean ginseng)	154	61.0%	503	3.0×10^{-49}	
☆	**Pathogensis-related protein 10**	Panax ginseng (Korean ginseng)	154	62.0%	503	3.0×10^{-49}	**PR-10**
★	**Ribonuclease 2**	Panax ginseng (Korean ginseng)	153	62.0%	498	1.0×10^{-48}	
★	**Pathogenesis-related protein B**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	63.0%	495	3.0×10^{-48}	**PCPR1-3**
★	**Pathogenesis-related protein A**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	62.0%	490	1.0×10^{-47}	**PCPR1-1 PCPR1-2**
☆	**Pathogenesis-related protein 1**	Petroselinum crispum (Parsley) (Petroselinum hortense)	155	56.0%	440	7.0×10^{-42}	
★	**Major allergen Api g 1**	Apium graveolens (Celery)	154	52.0%	402	2.0×10^{-37}	
☆	**Pathogenesis-related protein**	Pimpinella brachycarpa	154	51.0%	382	4.0×10^{-35}	**PR1**
★	**Major allergen Pru ar 1**	Prunus armeniaca (Apricot)	160	49.0%	379	8.0×10^{-35}	
★	**Major allergen Dau c 1**	Daucus carota (Carrot)	154	50.0%	379	8.0×10^{-35}	
☆	**Pathogenesis-related protein**	Daucus carota (Carrot)	154	49.0%	379	8.0×10^{-35}	**DcPRP**
☆	**PRP-like protein**	Daucus carota (Carrot)	154	50.0%	377	1.0×10^{-34}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	50.0%	377	1.0×10^{-34}	
☆	**PRP-like protein**	Daucus carota (Carrot)	154	50.0%	376	2.0×10^{-34}	
☆	**Bet v 1 related allergen**	Actinidia chinensis (Kiwi) (Yangtao)	159	51.0%	375	2.0×10^{-34}	**ypr-10**
☆	**Pathogenesis-related protein-like protein 2**	Daucus carota (Carrot)	154	48.0%	371	7.0×10^{-34}	**DcPRPlike2**
☆	**Putative uncharacterized protein**	Vitis vinifera (Grape)	159	48.0%	369	1.0×10^{-33}	VIT_05s0077g01660
☆	**Pathogenesis-related protein-like protein 1**	Daucus carota (Carrot)	154	50.0%	368	2.0×10^{-33}	**DcPRPlike1**
☆	**PRP-like protein**	Daucus carota (Carrot)	154	49.0%	368	2.0×10^{-33}	
☆	**Putative uncharacterized protein**	Vitis vinifera (Grape)	159	47.0%	367	2.0×10^{-33}	VITISV_025731

Homology of protein sequence of Api g 1.02 with parsley`s protein PR2

```
1     MGVQKTVVEAPSTVSAEKMYQGFLLDMDTVFPKVLPQLIKSVEILEGDGGVGTVKLVHLG    60 P92918
      MG   T VE  S+V A+ +Y+GFLLDMD + PKVLPQ IKS+EI+ GDGG GT+K V LG
1     MGAVTTDVEVASSVPAQTIYKGFLLDMDNIIPKVLPQAIKSIEIISGDGGAGTIKKVTLG    60 P27538

61    EATEYTTMKQKVDVIDKAGLAYTYTTIGGDILVDVLESVVNEFVVVPTDGGCIVKNTTIY   120 P92918
      E +++T +KQ++D ID   L Y+Y+ I GD+L+ ++ES+ ++F VVPTDGGCIVKNTTIY
61    EVSQFTVVKQRIDEIDAEALKYSYSIIEGDLLLGIIESITSKFTVVPTDGGCIVKNTTIY   120 P27538

121   NTKGDAVLPEDKIKEATEKSALAFKAVEAYLLAN   154 P92918
         GDAV+PE+ +KEATE+S + FKA+EAYLLAN
```

Homology of protein sequence of Api g 1.02 with parsley`s protein PR1-3

```
1     MGVQKTVVEAPSTVSAEKMYQGFLLDMDTVFPKVLPQLIKSVEILEGDGGVGTVKLVHLG    60 P92918
      MGVQK+ VEA S+VSAEK+++G  LD+DT+ P+VLP  IKS E LEGDGGVGTVKLVHLG
1     MGVQKSEVEATSSVSAEKLFKGLCLDIDTLLPRVLPGAIKSSETLEGDGGVGTVKLVHLG    60 P19418

61    EATEYTTMKQKVDVIDKAGLAYTYTTIGGDILVDVLESVVNEFVVVP-TDGGCIVKNTTI   119 P92918
      +A+ + TMKQKVD IDKA   Y+Y+ I GDIL+  +ES+ N F  VP  DGGC VK+T I
61    DASPFKTMKQKVDAIDKATFTYSYSIIDGDILLGFIESINNHFTAVPNADGGCTVKSTII   120 P19418

120   YNTKGDAVLPEDKIKEATEKSALAFKAVEAYLLAN   154 P92918
      +NTKGDAV+PE+ IK A +++   FKAVEAYL+AN
121   FNTKGDAVVPEENIKFANDQNLTIFKAVEAYLIAN   155 P19418
```

Homology of protein sequence of Api g 1.02 with parsley`s protein PR1-1

```
1     MGVQKTVVEAPSTVSAEKMYQGFLLDMDTVFPKVLPQLIKSVEILEGDGGVGTVKLVHLG    60 P92918
      MGVQK+ VE  S+VSAEK+++G  LD+DT+ P+VLP  IKS E LEGDGGVGTVKLVHLG
1     MGVQKSEVETTSSVSAEKLFKGLCLDIDTLLPQVLPGAIKSSETLEGDGGVGTVKLVHLG    60 P19417

61    EATEYTTMKQKVDVIDKAGLAYTYTTIGGDILVDVLESVVNEFVVVP-TDGGCIVKNTTI   119 P92918
      +A+ + TMKQKVD IDKA   Y+Y+ I GDIL+  +ES+ N F  VP  DGGC VK+T I
61    DASPFKTMKQKVDAIDKATFTYSYSIIDGDILLGFIESINNHFTAVPNADGGCTVKSTII   120 P19417

120   YNTKGDAVLPEDKIKEATEKSALAFKAVEAYLLAN   154 P92918
      +NTKGDAV+PE+ IK A +++   FKAVEAYL+AN
121   FNTKGDAVVPEENIKFANDQNLTIFKAVEAYLIAN   155 P19417
```

Homology of protein sequence of Api g 1.02 with carrot`s protein DauC1

```
1     MGVQKTVVEAPSTVSAEKMYQGFLLDMDTVFPKVLPQLIKSVEILEGDGGVGTVKLVHLG    60 P92918
      MG Q   +E  S+VSAEK++ G +LD+DTV PK  P   KSVE+ +GDGG GTV+++ L
1     MGAQSHSLEITSSVSAEKIFSGIVLDVDTVIPKAAPGAYKSVEV-KGDGGAGTVRIITLP    59 O04298

61    EATEYTTMKQKVDVIDKAGLAYTYTTIGGDILVDVLESVVNEFVVVPT-DGGCIVKNTTI   119 P92918
      E +  T+M  + D ++K  L Y  T I GDIL+  +ES+    VVVPT DGG I K T I
60    EGSPITSMTVRTDAVNKEALTYDSTVIDGDILLGFIESIETHLVVVPTADGGSITKTTAI   119 O04298

120   YNTKGDAVLPEDKIKEATEKSALAFKAVEAYLLAN   154 P92918
      ++TKGDAV+PE+ IK A  ++   FKA+EAYL+AN
120   FHTKGDAVVPEENIKFADAQNTALFKAIEAYLIAN   154 O04298
```

Annex 7

Similarities of Api g 3 with other major food allergens [30]

Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
★	Chlorophyll a-b binding protein, chloroplasti...	Apium graveolens (Celery)	264	100.0%	1,384	1.0×10^{-151}	LHC0
☆	Chlorophyll a/b binding protein of LHCII type...	Panax ginseng (Korean ginseng)	266	93.0%	1,299	1.0×10^{-141}	CAB
☆	Light harvesting chlorophyll a/b-binding prot...	Nicotiana sylvestris (Wood tobacco) (South American tobacco)	265	92.0%	1,293	1.0×10^{-140}	Lhcb1*4
☆	Putative uncharacterized protein	Populus trichocarpa x Populus deltoides	264	90.0%	1,285	1.0×10^{-139}	
☆	Light-harvesting complex II protein Lhcb1	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	264	90.0%	1,285	1.0×10^{-139}	Lhcb1-2 POPTRDRAFT
☆	Light-harvesting complex II protein Lhcb1	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	264	90.0%	1,284	1.0×10^{-139}	Lhcb1-1 POPTRDRAFT
☆	Light harvesting chlorophyll a/b-binding prot...	Nicotiana sylvestris (Wood tobacco) (South American tobacco)	265	90.0%	1,283	1.0×10^{-139}	Lhcb1*2
★	Chlorophyll a-b binding protein 21, chloropla...	Nicotiana tabacum (Common tobacco)	265	91.0%	1,282	1.0×10^{-139}	CAB21
☆	Light-harvesting complex II protein Lhcb1	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	264	89.0%	1,281	1.0×10^{-139}	Lhcb1-3 POPTRDRAFT
☆	Light harvesting chlorophyll a/b-binding prot...	Nicotiana sylvestris (Wood tobacco) (South American tobacco)	265	90.0%	1,277	1.0×10^{-138}	Lhcb1*1
★	Chlorophyll a-b binding protein 40, chloropla...	Nicotiana tabacum (Common tobacco)	267	90.0%	1,275	1.0×10^{-138}	CAB40
☆	Putative chloroplast chlorophyll a/b-binding ...	Carya cathayensis	267	89.0%	1,275	1.0×10^{-138}	ABC
☆	Light harvesting chlorophyll a/b-binding prot...	Nicotiana sylvestris (Wood tobacco) (South American tobacco)	265	90.0%	1,275	1.0×10^{-138}	Lhcb1*3
☆	Chloroplast chlorophyll A/B binding protein	Canarium album	264	89.0%	1,274	1.0×10^{-138}	
★	Chlorophyll a-b binding protein 1B, chloropla...	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)	265	89.0%	1,273	1.0×10^{-138}	CAB1B
☆	Chlorophyll a/b binding protein	Solanum tuberosum (Potato)	265	89.0%	1,270	1.0×10^{-137}	Lhcb1-1
☆	Chlorophyll a/b binding protein	Solanum tuberosum (Potato)	265	89.0%	1,270	1.0×10^{-137}	Lhcb1-2
☆	Light-harvesting chlorophyll a/b-binding prot...	Lactuca sativa (Garden lettuce)	266	91.0%	1,270	1.0×10^{-137}	cab
☆	Chlorophyll a/b binding protein	Solanum tuberosum (Potato)	265	89.0%	1,269	1.0×10^{-137}	Lhcb1-4
☆	Light-harvesting chlorophyll a/b protein	Lemna gibba (Swollen duckweed)	266	90.0%	1,267	1.0×10^{-137}	
☆	Chlorophyll a/b binding protein	Solanum tuberosum (Potato)	265	89.0%	1,266	1.0×10^{-137}	Lhcb1-5
☆	Chlorophyll a-b binding protein 3C-like	Solanum tuberosum (Potato)	267	89.0%	1,266	1.0×10^{-137}	
☆	Light harvesting chlorophyll a/b-binding prot...	Nicotiana sylvestris (Wood tobacco) (South American tobacco)	267	90.0%	1,266	1.0×10^{-137}	Lhcb1*6
☆	Chloroplast chlorophyll A/B binding protein	Mangifera indica (Mango)	264	89.0%	1,265	1.0×10^{-137}	MCAB
☆	Chlorophyll A/B binding protein, putative	Ricinus communis (Castor bean)	265	89.0%	1,265	1.0×10^{-137}	RCOM_1214080
☆	Major chlorophyll a/b binding protein LHCb1.2	Spinacia oleracea (Spinach)	267	88.0%	1,265	1.0×10^{-137}	lhcb1.2

Homology of protein sequence (shown in grey) of Api g 3 with tomato´s protein CAB1B.

```
1     MAASTMALSSPALAGKAVKVAPSSSELFGNGRVSMRKTV-KAPVSDSPWYGPDRVKYLGP   59 P92919
      MAA+TMALSSP+ AG+AVK++PS+SE+ GNGR++MRK V K+  S SPWYGPDRVKYLGP
1     MAAATMALSSPSFAGQAVKLSPSASEISGNGRITMRKAVAKSAPSSSPWYGPDRVKYLGP   60 P07370

60    FSGEAPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIHSRWAMLGALGCVFPELLAR  119 P92919
      FSGE+PSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIH RWAMLGALGCVFPELLAR
61    FSGESPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIHCRWAMLGALGCVFPELLAR  120 P07370

120   NGVKFGEAVWFKAGSQIFSEGGLDYLGNPSLVHAQSILSIWATQVILMGAVEGYRVAGGP  179 P92919
      NGVKFGEAVWFKAGSQIFSEGGLDYLGNPSLVHAQSIL+IWA QV+LMGAVEGYR+AGGP
121   NGVKFGEAVWFKAGSQIFSEGGLDYLGNPSLVHAQSILAIWACQVVLMGAVEGYRIAGGP  180 P07370

180   LGEIVDPLYPGGSFDPLGLAEDPERSAELKVKELKNGRLAMFSMFGFFVQAIVTGKGPLE  239 P92919
      LGE+VDPLYPGGSFDPLGLAEDPE  AELKVKE+KNGRLAMFSMFGFFVQAIVTGKGPLE
181   LGEVVDPLYPGGSFDPLGLAEDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLE  240 P07370

240   NLADHLADPVNNNAWAFATNFVPGK   264 P92919
```

Annex 8

Similarities of Api g 4 with other major food allergens [30]

Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
★	**Profilin**	Apium graveolens (Celery)	134	100.0%	713	2.0×10^{-73}	
☆	**Profilin**	Petroselinum crispum (Parsley) (Petroselinum hortense)	134	94.0%	683	5.0×10^{-70}	**PRF4**
☆	**Profilin**	Petroselinum crispum (Parsley) (Petroselinum hortense)	134	93.0%	680	1.0×10^{-69}	**PRF2**
☆	**Profilin**	Petroselinum crispum (Parsley) (Petroselinum hortense)	134	92.0%	668	3.0×10^{-68}	**PRF1**
☆	**Profilin**	Petroselinum crispum (Parsley) (Petroselinum hortense)	134	91.0%	667	3.0×10^{-68}	**PRF3**
★	**Profilin**	Daucus carota (Carrot)	134	91.0%	659	3.0×10^{-67}	
☆	**Profilin**	Olea europaea (Common olive)	134	82.0%	620	9.0×10^{-63}	**Ole e 2**
★	**Profilin-2**	Olea europaea (Common olive)	134	82.0%	618	2.0×10^{-62}	**PRO2**
☆	**Profilin**	Olea europaea (Common olive)	134	82.0%	618	2.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	82.0%	618	2.0×10^{-62}	**Ole e 2**
☆	**Profilin**	Corylus avellana (European hazel) (Corylus maxima)	133	82.0%	615	4.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	82.0%	615	4.0×10^{-62}	**Ole e 2**
★	**Profilin-3**	Olea europaea (Common olive)	134	81.0%	614	5.0×10^{-62}	**PRO3**
☆	**Profilin**	Ricinus communis (Castor bean)	133	81.0%	614	5.0×10^{-62}	RCOM_1048910
☆	**Profilin**	Corylus avellana (European hazel) (Corylus maxima)	133	82.0%	614	5.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	614	5.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	614	5.0×10^{-62}	
☆	**Profilin**	Corylus avellana (European hazel) (Corylus maxima)	133	82.0%	613	6.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	613	6.0×10^{-62}	**Ole e 2**
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	613	6.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	613	6.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	81.0%	613	6.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	80.0%	612	8.0×10^{-62}	
☆	**Profilin**	Olea europaea (Common olive)	134	82.0%	612	8.0×10^{-62}	

Homology of protein sequence of Api g 4 with Profilin from Carrot

```
1     MSWQAYVDDHLMCEVEGNPGQTLTAAAIIGHDGSVWAQSSTFPQIKPEEIAGIMKDFDEP   60  Q9XF37
      MSWQ YVDDHLMCEV+GNPGQ L+AAAIIGHDGSVWAQSSTFP+ KPEEI GIMK+FDEP
1     MSWQIYVDDHLMCEVDGNPGQQLSAAAIIGHDGSVWAQSSTFPKFKPEEITGIMKNFDEP   60  Q8SAE6

61    GHLAPTGLYLGGAKYMVIQGEPNAVIRGKKGSGGVTIKKTGQALVFGVYDEPVTPGQCNV  120  Q9XF37
      GHLAPTGLYLGG KYMVIQGEP AVIRGKKGSGGVTIKKTGQALVFGVYDEPVTPGQCN+
61    GHLAPTGLYLGGIKYMVIQGEPIAVIRGKKGSGGVTIKKTGQALVFGVYDEPVTPGQCNL  120  Q8SAE6

121   IVERLGDYLIDQGL   134  Q9XF37
      IVERLGDYLI+QGL
121   IVERLGDYLIEQGL   134  Q8SAE6
```

Homology of protein sequence of Api g 4 with Profilin PRO2 of olive

```
1     MSWQAYVDDHLMCEVEGNPGQTLTAAAIIGHDGSVWAQSSTFPQIKPEEIAGIMKDFDEP   60  Q9XF37
      MSWQAYVDDHLMC++EG+ G  LTAAAI+GHDGSVWAQS+TFPQ KPEE+ GIM DF+EP
1     MSWQAYVDDHLMCDIEGHEGHRLTAAAIVGHDGSVWAQSATFPQFKPEEMNGIMTDFNEP   60  O24170

61    GHLAPTGLYLGGAKYMVIQGEPNAVIRGKKGSGGVTIKKTGQALVFGVYDEPVTPGQCNV  120  Q9XF37
      GHLAPTGL+LGG KYMVIQGE  AVIRGKKGSGG+TIKKTGQALVFG+Y+EPVTPGQCN+
61    GHLAPTGLHLGGTKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGIYEEPVTPGQCNM  120  O24170

121   IVERLGDYLIDQGL   134  Q9XF37
      +VERLGDYL++QGL
121   VVERLGDYLLEQGL   134  O24170
```

Annex 9

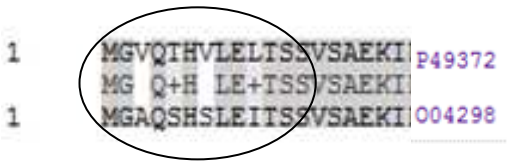
Similarities of Api g 5 with other major food allergens [30]

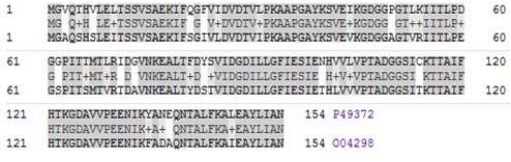
Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
★	**Allergen Api g 5**	Apium graveolens (Celery)	86	100.0%	437	2.0×10^{-41}	
☆	**Putative uncharacterized protein**	Panax ginseng (Korean ginseng)	153	72.0%	122	5.0×10^{-5}	
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	531	48.0%	96	5.5×10^{-2}	POPTRDRAFT_550604
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	57.0%	95	7.1×10^{-2}	POPTRDRAFT_590917
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	57.0%	95	7.1×10^{-2}	POPTRDRAFT_785162
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	54.0%	93	1.2×10^{-1}	POPTRDRAFT_789090
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	54.0%	93	1.2×10^{-1}	POPTRDRAFT_593088
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	534	52.0%	92	1.6×10^{-1}	POPTRDRAFT_582590
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	485	55.0%	91	2.1×10^{-1}	POPTRDRAFT_946873
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	228	63.0%	91	2.1×10^{-1}	POPTRDRAFT_939422
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	63.0%	91	2.1×10^{-1}	POPTRDRAFT_753730
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	526	57.0%	91	2.1×10^{-1}	POPTRDRAFT_1070599 POPTRDRAFT_839640
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	57.0%	91	2.1×10^{-1}	POPTRDRAFT_753689
☆	**F12K21.9**	Arabidopsis thaliana (Mouse-ear cress)	715	58.0%	89	3.5×10^{-1}	At1g34575
☆	**FAD-binding and BBE domain-containing protein**	Arabidopsis thaliana (Mouse-ear cress)	527	58.0%	89	3.5×10^{-1}	At1g34575 AT1G34575
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	535	52.0%	89	3.5×10^{-1}	POPTRDRAFT_785160
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	532	60.0%	89	3.5×10^{-1}	POPTRDRAFT_772209
☆	**Putative uncharacterized protein**	Arabidopsis lyrata subsp. lyrata (Lyre-leaved rock-cress)	367	55.0%	88	4.6×10^{-1}	ARALYDRAFT_473604
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	531	52.0%	88	4.6×10^{-1}	POPTRDRAFT_597710
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	501	50.0%	88	4.6×10^{-1}	POPTRDRAFT_270233
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp. trichocarpa)	533	52.0%	88	4.6×10^{-1}	POPTRDRAFT_753691
☆	**Berberine bridge enzyme-like protein**	Arabidopsis thaliana (Mouse-ear cress)	530	42.0%	86	7.9×10^{-1}	**T13K14.20** AT4g20860 At4g20860 AT4G20860
☆	**At1g30710**	Arabidopsis thaliana (Mouse-ear cress)	531	55.0%	86	7.9×10^{-1}	**T5I8.16** At1g30710 AT1G30710
☆	**FAD-binding domain-containing protein**	Arabidopsis lyrata subsp. lyrata (Lyre-leaved rock-cress)	531	55.0%	86	7.9×10^{-1}	ARALYDRAFT_890626
☆	**Predicted protein**	Populus trichocarpa (Western balsam poplar) (Populus balsamifera subsp.	514	58.0%	86	7.9×10^{-1}	POPTRDRAFT_233054

Annex 10

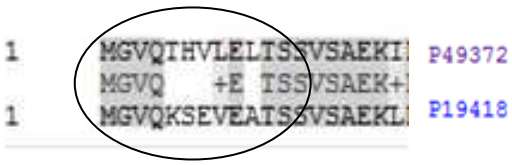
Peptides region which were used for the immunization ID 1959 (Serum Api 1-1)

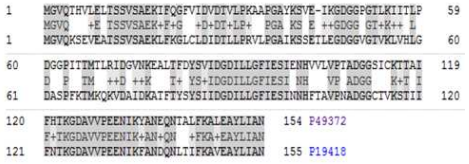
Celery-Carrot Similarity Apig1.01-**DauC1**.(On the right site the whole sequence, on the left enlarged only the region of the peptide used for the immunization)



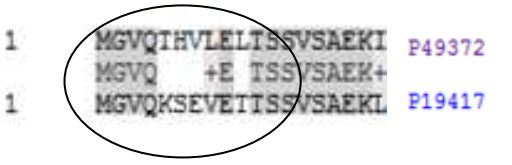


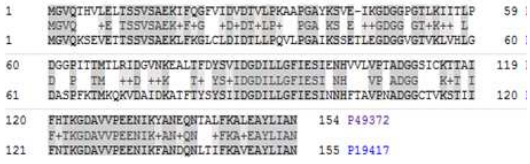
Celery-Parsley Similarity Apig1.01-**PR13**



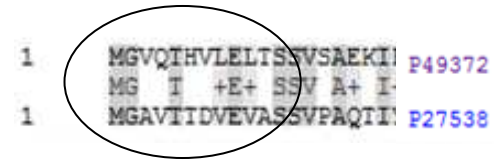


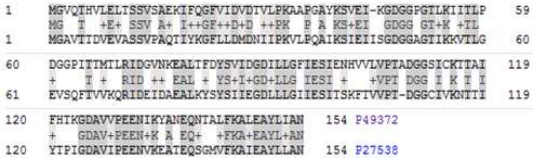
Celery-Parsley Similarity Apig1.01-**PR11**



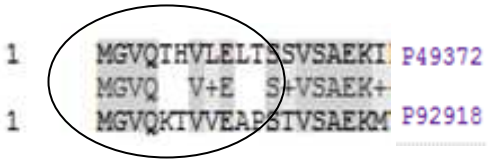


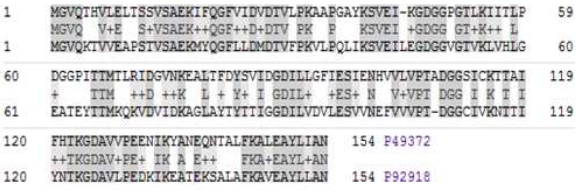
Celery-Parsley Similarity Apig1.01-**PR2**





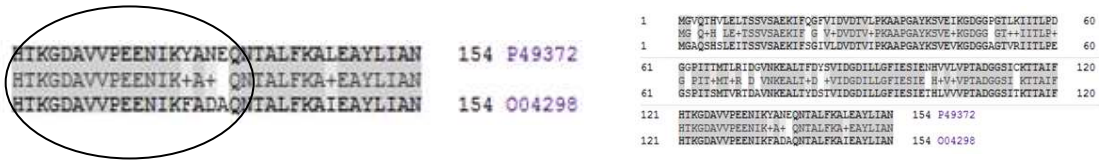
Similarity Apig1.01-**Apig1.02**



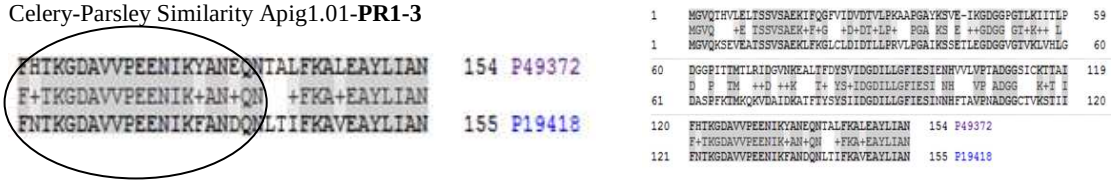


Peptides region which was used for the immunization ID 1959 (Serum Api 1-2)

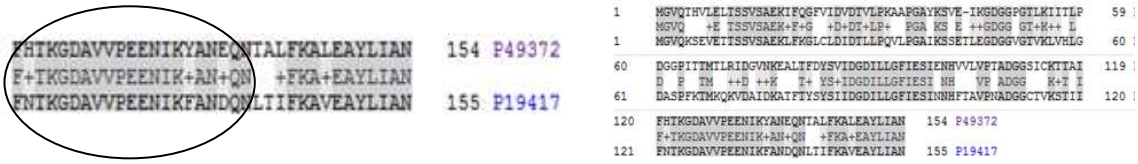
Celery-Carrot Similarity Apig1.01-**DauC1.** .(On the right site the whole sequence, on the left enlarged only the region of the peptide used for the immunization)



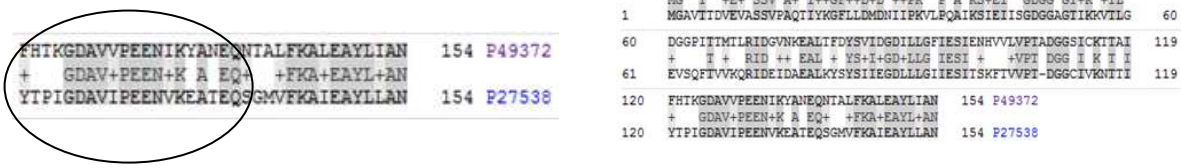
Celery-Parsley Similarity Apig1.01-**PR1-3**



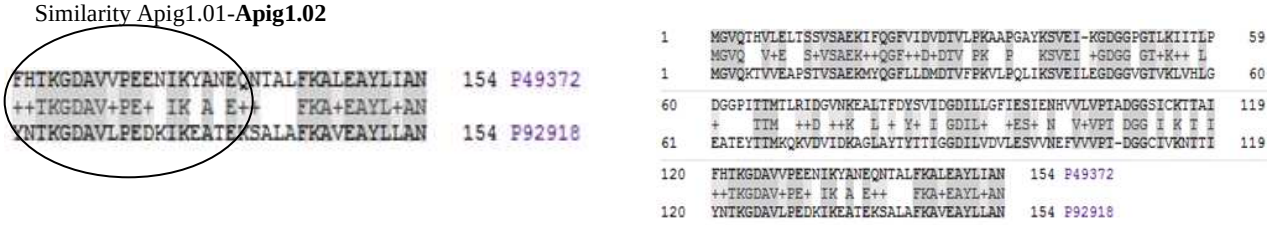
Celery-Parsley Similarity Apig1.01-**PR1-1**



Celery-Parsley Similarity Apig1.01-**PR2**

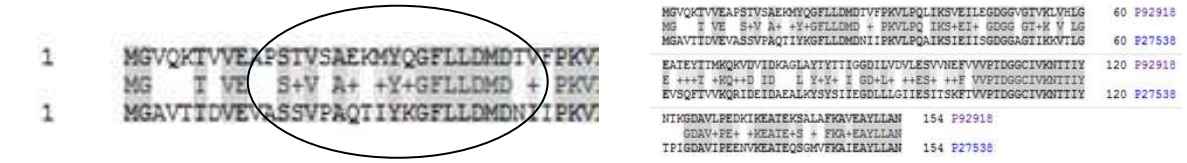


Similarity Apig1.01-**Apig1.02**

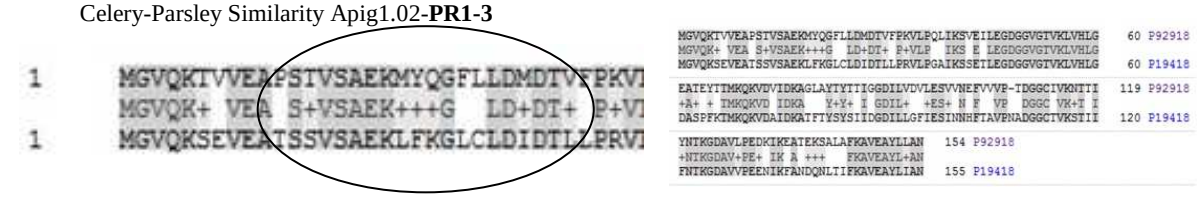


Peptides region which was used for the immunization ID 1959 (Serum Api 2-1)

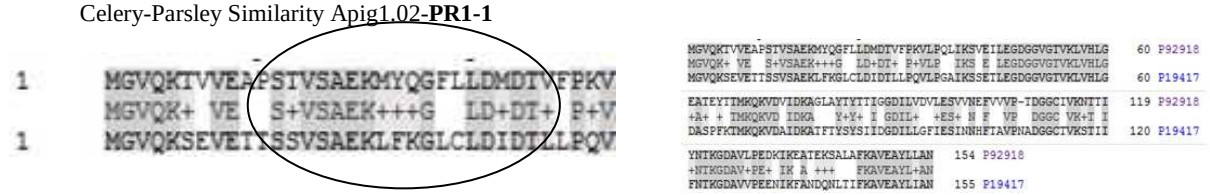
Celery-Parsley Similarity Apig1.02-**PR2.**



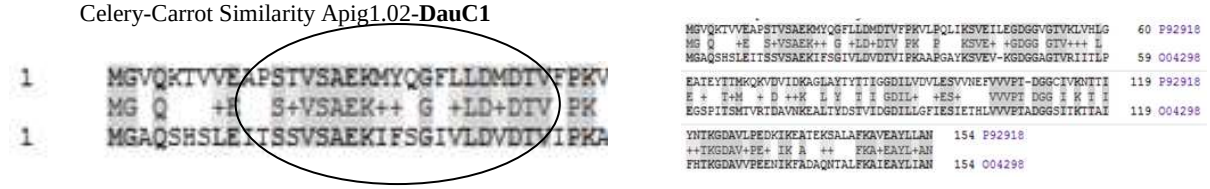
Celery-Parsley Similarity Apig1.02-**PR1-3**



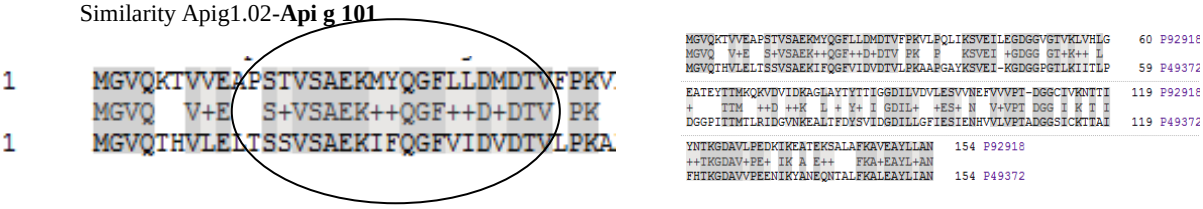
Celery-Parsley Similarity Apig1.02-**PR1-1**



Celery-Carrot Similarity Apig1.02-**DauC1**

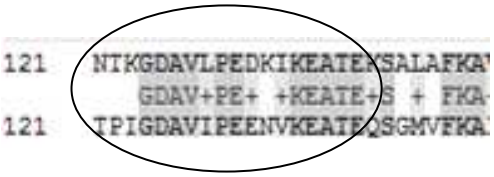


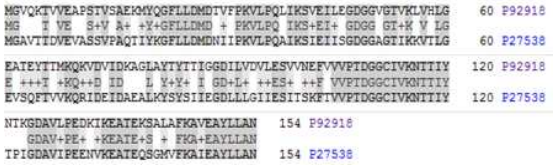
Similarity Apig1.02-**Api g 101**



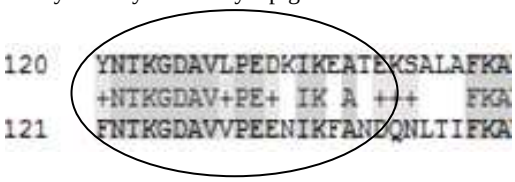
Peptides region which was used for the immunization ID 1959 (Serum Api 2-2)

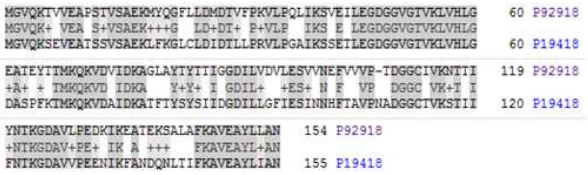
Celery-Parsley Similarity Apig1.02-**PR2.** (On the right site the whole sequence, on the left enlarged only the region of the peptide used for the immunization)



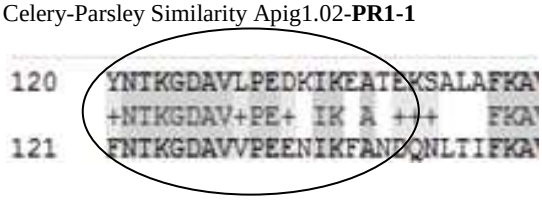


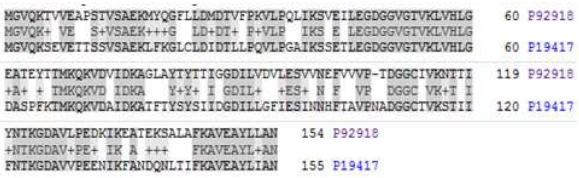
Celery-Parsley Similarity Apig1.02-**PR1-3**



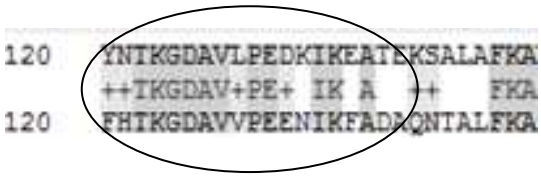


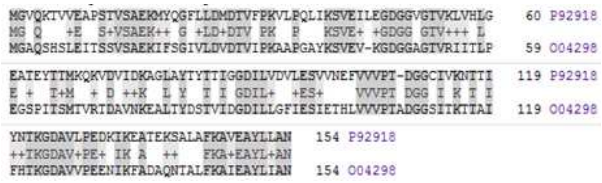
Celery-Parsley Similarity Apig1.02-**PR1-1**



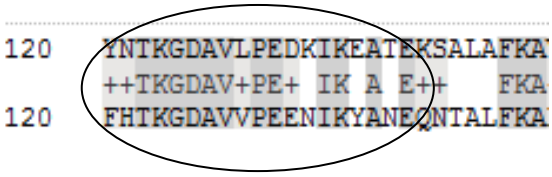


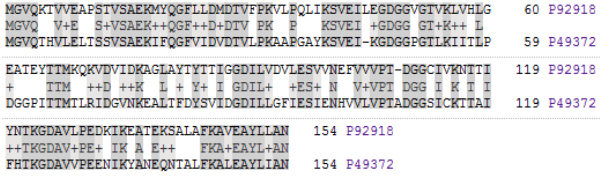
Celery-Carrot Similarity Apig1.02-**DauC1**





Similarity Apig1.02-**Apig1.01**





Annex 11

Protein Marker MarkItblue used for SDS-PAGE

Figure: 5 µl of Mark-It Blue were resolved on a 4-20% gradient gel. The unstained gel was documented using a flat-bed scanner in reflection mode and the data were corrected for the loss of contrast.

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