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DIPLOMARBEIT

Molekulare und immunologische Charakterisierung von Profilin aus Weizen (*Triticum aestivum*)

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Molecular and immunological characterisation of profilin from wheat (*Triticum aestivum*)

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I. INTRODUCTION

1. Hypersensitivity

In 1906 Clemens von Pirquet coined the term allergy. He described allergy as an altered capacity of the body to react to a foreign substance. This rather broad definition was replaced by a more accurate one: an allergy is a disease following a response by the immune system to an otherwise innocuous antigen [1].
Allergies fall into a group of immune responses called hypersensitivity reactions (tab. 1).

1.1 Coombs and Gell classification

Coombs and Gell divided Hypersensitivity reactions into four groups:

	Type I	Type II		Type III	Type IV		
Immune reactant	IgE	IgG, IgM		IgG, IgM	T_H1 cells	T_H2 cells	CTL
Antigen	Soluble antigen	Cell- or matrix associated antigen	Cell surface receptor	Soluble antigen	Soluble antigen	Soluble antigen	Cell-associated antigen
Effector mechanism	Mast-cell activation	Complement, F_cR^+ cells	Antibody alters signalling	Complement, Phagocytes	Macrophage activation	IgE production, Eosinophil activation, Mastocytosis	Cytotoxicity
Example of hypersensitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Penicillin- and other drug allergy	Chronic urticaria	Serum sickness, Arthus reaction	Contact dermatitis, tuberculin reaction	Chronic asthma, chronic allergic rhinitis	Contact dermatitis

Tab. 1: Coombs and Gell classification of hypersensitivity reactions [1]

1.2 Allergy (Type I Hypersensitivity)

During the first encounter between immune system and allergen, allergen specific IgE antibodies are produced. These antibodies occupy the $F_c\varepsilon R1$ receptor on mast cells and basophil granulocytes. If the allergen is able to crosslink specific IgEs during the second encounter, the mast cells (basophils) degranulate and the allergic reaction takes its course. The pathophysiological mechanism of allergy falls into three stages: sensitisation, early phase- and late phase reaction.

1.2.1 Sensitisation

Is the allergen able to enter the body and encounter an antigen presenting cell (e.g. dendritic cell), this cell will take up and process the allergen. This is followed by the

presentation of parts of the allergen to a naive T-cell ($CD4^+$), which differentiates into a T_H2 cell. This T_H2 cell starts to produce interleukin 4 (IL-4), a cytokine that forces B-cells to differentiate into an antibody producing plasma cell. After class switch from IgM to IgE, the antigen specific antibodies (IgE) enter circulation and spread throughout the body to bind the high affinity receptors (FcεRI) on mast cells (basophils). During this process no symptoms occur [1].

1.2.2 Early phase reaction

If the immune system of the sensitised person encounters the allergen a second time, the bound IgEs on mast cells (basophils) crosslink via the allergen and trigger a cellular signal cascade. This leads to the degranulation of mast cells (basophils). In this process, preformed mediators, such as histamine, tryptases, kininogenase and ECF-A (Eosinophil Chemotactic Factor for Anaphylaxis) are released. Within a short period of time (30 minutes) these molecules lead to symptoms such as conjunctivitis, rhinitis, broncho-constriction, gastrointestinal reactions or even to an anaphylactic shock [1].

1.2.3 Late phase reaction

Not all mediators are stored in the granulae of mast cells (basophils), some are newly formed. Potent mediators like leukotrienes (e.g. B_4, C_4, D_4), prostaglandin D_2 and PAF are newly synthesized and drive the inflammatory reaction even further. Two to twenty four hours after exposure to the allergen, macrophages, T_H2-cells, eosinophils, and neutrophils join the reaction. Eosinophils and neutrophils release hydrolytic enzymes, which lead to necrosis [2].
If the allergic person is not exposed to the allergen source permanently, the symptoms will subside in time [1].

2. Food allergy

2.1 Prevalence and classification

In first world countries 2-4% [3] of adults and 6-8% [4] of children suffer from food allergy. IgE mediated food allergies have to be distinguished from other adverse food reactions caused by non allergic mechanisms. The most prominent non-allergic adverse food reaction is food intolerance towards lactose. For the correct diagnosis

of food allergy it is essential to distinguish between the different types of adverse food reactions. For this reason the European Academy of Allergology and Clinical Immunology (EAACI) developed a classification system (fig. 1).

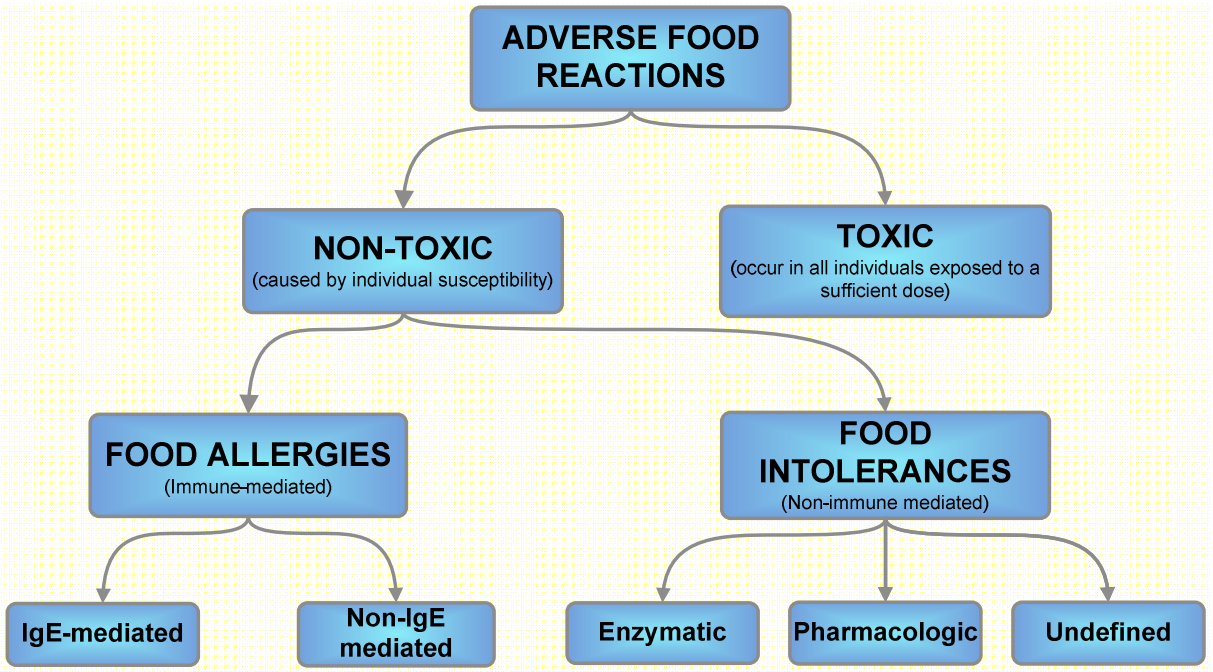


Fig. 1: EAACI classification of adverse food reactions [5].

Adverse food reactions can be divided into toxic and non toxic reactions. Toxic reactions affect every person exposed to the culprit food (e.g. S*almonella* poisoning). Non-toxic reactions are divided into food allergies and food intolerances. Food intolerances can be either of an enzymatic nature (e.g. lactose intolerance) or of pharmacological origin. Food allergies are either IgE mediated or non-IgE mediated. The most common IgE mediated food allergies are directed against proteins from cow's milk and hen's egg, especially in children [5]. Other prominent sources of allergens are fish, legumes, meats and grains [5]. Fortunately, 85% of affected children outgrow their allergies [6].

Two forms of IgE mediated food allergies have to be distinguished. In class I food allergy the sensitisation process occurs in the gastrointestinal tract due to the resistance to gastric digestion of these allergens [7]. This kind of food allergy dominates in infants.

Class II food allergies mainly occur in adults and develop as a consequence of an allergic sensitisation to inhalant allergens, such as birch pollen. The immunological basis is IgE cross reactivity, which might be clinically relevant or irrelevant [8].

2.2 Symptoms

Foods may induce a variety of symptoms in allergic persons, and, notably some symptoms may equally have an allergic or a non allergic cause. There may be a clear temporal relationship between the intake of the food and the development of the allergic symptoms, but symptoms may also develop after hours making the relation with the ingested food less clear [5].
Organs affected (organ systems) by an allergic reaction are the skin, the eyes, the respiratory tract, the gastrointestinal tract and the circulatory system (tab.2). Surprisingly the skin is the most affected organ, followed by the eyes and respiratory tract, the gastrointestinal tract and the circulatory system are the least affected organ system.

2.2.1 Oral allergy syndrome (OAS) & skin

The most frequent complex of symptoms of food allergy in adults is the OAS. It involves IgE- mediated contact urticaria of lips, oral mucosa, and pharynx [9,10]. In most cases, the clinical course is mild with symptoms limited to the oropharynx and resolving within minutes. In some cases the reaction is more severe, leading towards a generalized anaphylactic reaction [9]. The classical OAS is associated with sensitization towards heat/pepsin labile plant proteins in patients with pollen-related food allergy. In this case, crossreacitivity between homologous plant-derived proteins in pollen and vegetable foods is the basis of OAS. About 75% of birch pollen allergic patients may experience OAS after ingestion of raw fruits like apples, nuts, carrot, celery and kiwi [11]. In this case allergens responsible for OAS are Bet v 1 homologues, the major birch pollen allergen. Also, patients sensitised to profilin experience OAS. In these subjects tomato, orange and melon are additional offending foods [12].
The skin is frequently involved in IgE mediated food allergy [5]. Cutaneous symptoms may vary from pruritus, urticaria, and angioedema to mobilliform rashes. Acute urticaria is the most common skin disorder in adult patients with food allergy [14]. Urticaria appears within minutes of ingesting the offending food and may last for

several hours [5]. Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease commonly associated with the presence of IgE specific for airborne and/or food allergens. AD is commonly associated with food allergy in children but rarely in adults [5].

SKIN (44.7%)	EYES&RESPIRATORY TRACT (23.7%)	GASTROINTESTINAL TRACT (21.7%)	CIRCULATORY SYSTEM (9.9%)
Urticaria	Rhinoconjunctivitis	Nausea	Anaphylaxis
Eczema	Larynx edema	Vomiting	
Oral allergy syndrome	Asthma	Diarrhoea	

Tab. 2: Symptoms in food allergy [13]

2.2.2 Respiratory disorders

Respiratory symptoms such as rhinoconjuctivitis and bronchospasm occur in food allergic patients following the ingestion of the offending food in association with gastrointestinal and skin disorders, but are rarely present as the only symptom [14]. Respiratory symptoms elicited by inhalative allergens usually occur without affecting other organ systems [5].

2.2.3 Gastrointestinal disorders

Symptoms which fall into this category are nausea, vomiting, gastric retention, intestinal hypermotility, abdominal pain and diarrhoea [14]. Reactions develop within minutes or several hours after the ingestion of the offending food. Allergens causing this kind of symptoms are heat- and pepsin stable and therefore able to reach the gastrointestinal tract as intact proteins.

2.2.4 Anaphylaxis

Anaphylaxis is defined as a severe, life-threatening, generalized or systemic hypersensitivity reaction [5]. It is the most dramatic allergic reaction and always requires emergency treatment [5]. Anaphylaxis is caused by the abrupt and massive release of mediators from mast cells (basophils) throughout the body. The gastrointestinal tract, the skin, the respiratory tract and the cardiovascular system are affected. Patients react within minutes after the ingestion of the offending food. The reactions can be fatal, if not treated properly.

2.3 Food allergens

2.3.1 Protein families

The majority of plant food allergens originates from few protein families (fig.2). The most prominent are the prolamin- and cupin superfamilies, profilins and Bet v 1 related proteins. Proteins are part of the same family, if their sequence identity is ≥30% and/or if there are functional or structural similarities [8].

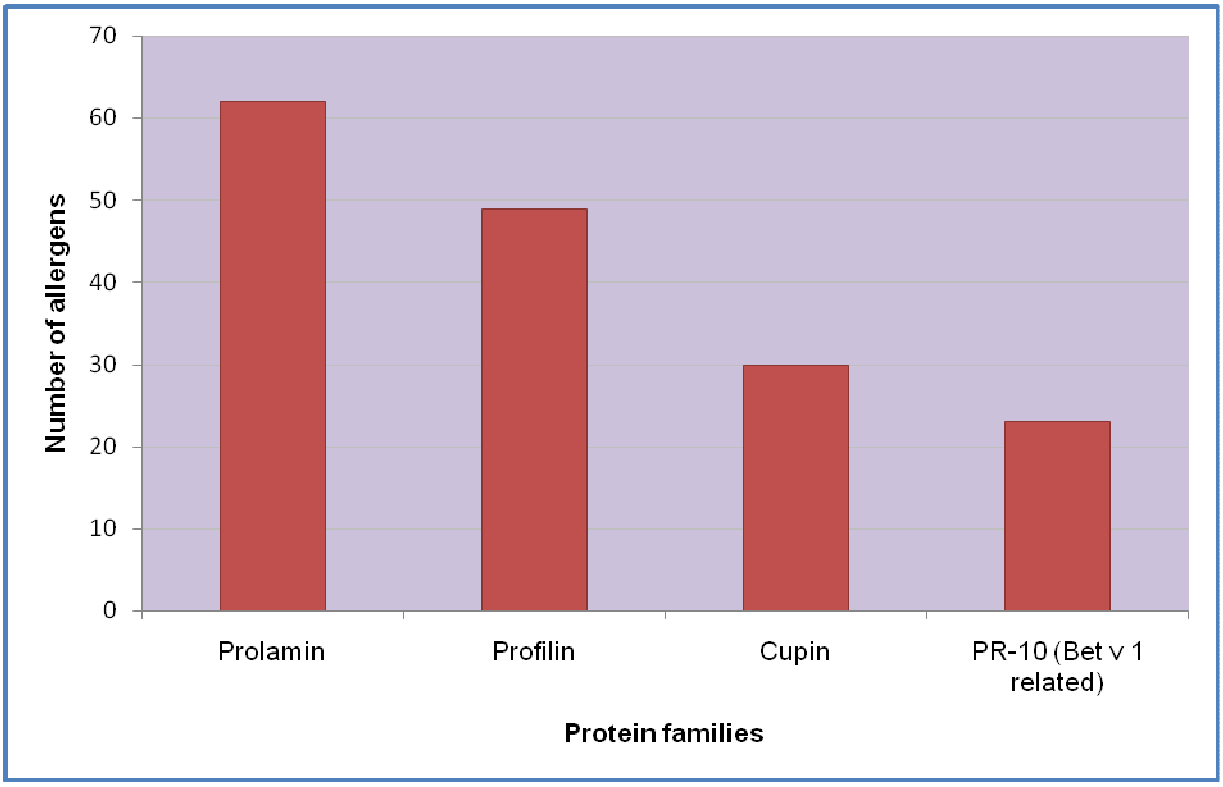


Fig. 2: The four most prominent protein families of plant food allergens

(AllFam Database: http://www.meduniwien.ac.at/allergens/allfam/)

Both, the prolamin- and the cupin superfamiliy contain allergens found in nuts (e.g. peanut, cashew nut) and seeds (e.g. yellow mustard seed, sesame seeds) [8].
Profilin is found in every higher organism [12], this protein elicits usually mild symptoms like oral allergy syndrome (OAS). Profilin sequences are highly conserved among plants, with 70% to 85% identical amino acid residues in sequences of different species [8].
Bet v 1 (***Bet****ula* ***v****errucosa*) related proteins, which cause allergic symptoms are found mostly in the pathogenesis related family 10 (PR-10). Expression of these proteins is induced by stress (e.g. pathogen infection or physical stress) [8].

2.3.2 Profilin

Profilins are monomeric proteins with a molecular weight between 12 and 15 kDa, which are found in all eukaryotic cells (tab.3).

Profilin was first described in 1977 [15] as an actin binding protein, which sequesters actin monomers and inhibit actin filament growth in vitro. Profilin's function also includes remodelling the actin cytoskeleton, regulating actin microfilament assembly and dynamics along with other proteins like ADF or fimbrin [16].

Suppression of profilin gene expression affects cell growth, morphogenesis, and seedling growth, highlighting the important physiological role of profilin mediated actin dynamics [17, 18].

Profilin also plays a role in signal transduction. In any signal-cytoskeleton response system, the final signalling targets are most likely proteins, associated to the cytoskeleton, which control organization and dynamics [19].

Profilin's relevance in allergy was discovered in 1991, when a highly cross reactive minor birch pollen allergen, Bet v 2 (***Bet****ula* ***v****errucosa*), was identified as profilin [20]. Since then, numerous allergenic profilins were described from pollen, fruits, vegetables, spices, seeds, nuts, and latex [21, 22].

Allergic patients whose sera contain profilin-specific IgE commonly show allergic symptoms towards a large number of unrelated plants. However, positive in vitro testing, only gives information about the state of sensitisation, not about the (clinical) relevance of the tested allergen.

Despite extensive cross reactivity among plant profilin and to the human homologue as well, it seems that a large proportion of IgE reactivities to profilins is clinically irrelevant [23, 24]. However, a major role (clinical relevance) for profilin in plant food allergy has been determined for melon (*Cucumis melo*) [25] orange (*Citrus sinensis*) [26], tomato (*Solanum lycopersicum)*, and banana (*Musa paradisiaca*) [27].

Allergen name	Source	Trivial name
Ana c 1	*Ananas comosus*	Ananas
Api g 4	*Apium graveolens*	Celery
Cit s 2	*Citrus sinensis*	Sweet orange
Dau c 1	*Daucus carota*	Carot
Sol l 1	*Solanum lycopersicum*	Tomato
Mus xp 1	*Musa x paradisiaca*	Banana
Tri a 12	*Triticum aestivum*	Wheat

Tab. 3: Examples of profilins, which play a role in food allergy (IUIS Database: http://www.allergen.org/Allergen.aspx)

2.3.3 Cross reactivity

Cross reactions are described as the recognition of epitopes present on an allergen by specific IgE of another allergen (homologous proteins). This is caused by highly conserved 3D structures and/or amino acid sequences of allergens, which result in highly similar epitopes (e.g. among different profilins or within Bet v 1 protein family). Allergen specific IgE antibodies are therefore not able to distinguish between these different antigens [28, 29]. So far, several pollen-food allergy syndromes have been described, such as birch-fruit [8,30], celery-mugwort-spice [31], latex-fruit [32, 33], mugwort-mustard [34], mugwort-peach [35], and ragweed-melon-banana syndrome [36], and the pan-allergens profilin [24, 37] and lipid transfer proteins (LTPs) [38]. The prerequisite for allergenic cross reactivity with clinical significance is a sequence identity of at least 50% [39].

3. Wheat

3.1 The history of wheat

Wheat has accompanied humans since remote times (as far back as 3000 to 4000 BC) in their evolution and development, evolving itself from wild grasses to its primitive form (emmer wheat) and from there, with human help, into the presently cultivated species. The more important modern wheat species are hexaploid bread wheat (*Triticum aestivum*) and tetraploid durum wheat (*Triticum turgidum L. Var. durum*), which are different from one another in genomic make-up, in grain composition and in food end-use quality attributes. Except for the very warm tropics, wheat adapts to all diverse climatic conditions prevailing in agricultural lands and, therefore, it is harvested in the world all year round. Its wide adaption to diverse environmental conditions, along with its unique characteristic of possessing a viscoelastic storage protein complex called gluten, are the main factors making wheat the most important food crop in the world [40].

Despite the many benefits humans draw from wheat, there are a few significant downsides to wheat consumption. Wheat can cause diseases like celiac disease, wheat allergy, bakers' asthma, and wheat dependant exercise induced anaphylaxis (tab.4).

3.2 Wheat related diseases

There are three major diseases caused by wheat consumption or wheat flour inhalation (tab.4). A fourth disease is the very rare wheat-dependant exercise induced anaphylaxis (WDEIA).

Wheat related diseases	Antibody/ Exposure route	Prevalence	Symptoms
Celiac disease	IgA/ingestion	1% in Europe	diarrhoea, steatorrhoea weight loss, fatigue, anaemia
Wheat allergy	IgE/ingestion	4% sensitization in 3 y old children [41]	urticaria, eczema, dermatitis,
Bakers‘ asthma	IgE/inhalation	15% of bakers, millers,...	Asthma, rhinits
WDEIA	IgE/Ingestion	unknown	anaphylaxis

Tab. 4: characterisation of wheat related diseases

3.2.1 Wheat allergens

So far 6 allergens are listed in the IUIS allergen database (tab.5).

Tri a 12 (***Tri****ticum* ***a****estivum*) belongs to the profilin family. Profilins are monomeric proteins with a molecular weight between 12 and 15 kDa, which are found in all eukaryotic cells.

Tri a 14 is part of a group of proteins called nonspecific lipid transfer proteins (LTPs). Its molecular weight is 9 kDa and LTPs were identified as relevant allergens in plant foods, mainly Rosaceae fruits, and pollens, being particulary important in the Mediterranean area. It was reported that wheat LTP plays a major role in bakers’ asthma, an occupational disease affecting up to 15% of bakers and other cereal flour handling professions [42].

Tri a 18 is an IgE-binding protein consisting of four hevein-like domains. Wheat germ agglutinin (Tri a 18) was identified as an allergen relevant in bakers’ asthma [43].

Tri a 19 plays a major role in wheat-dependant, exercise-induced anaphylaxis [44]. Omega-5 gliadin‘s molecular weight is 65 kDa.

Tri a 25, wheat thioredoxin is considered to be responsible for symptoms in bakers' asthma. It is suspected to cross react with thioredoxins from maize and grass pollen [45].

Tri a 26, Gluten is the protein component of wheat flour. HMW glutenin from wheat was described as a major allergen in wheat-dependant exercise-induced anaphylaxis. Also, gluten is the causative agent of celiac disease [46].

Name	Function	Disease	Tissue
Profilin Tri a 12	actin binding protein	Bakers' asthma Wheat allergy	seed & pollen
LTP Tri a 14	lipid transfer protein	Bakers' asthma	seed
Hevein like protein Tri a 18	agglutinin	Bakers' asthma	seed
Omega-5-Gliadin Tri a 19	seed storage protein	WDEIA	seed
Thioredoxin Tri a 25	protein disulfid reductase	Bakers' asthma	seed
Glutenin Subunit, Tri a 26	seed storage protein	Celiac disease WDEIA	seed

Tab.5: Wheat allergens listed in the IUIS Database (http://www.allergen.org/Allergen.aspx)

3.2.2 Celiac disease

The most prominent wheat related disease is celiac disease (CD). CD represents an enteropathy, which is caused by ingestion of wheat proteins in genetically predisposed individuals [47, 48]. CD patients exhibit IgA antibody and T cell reactivity mainly against a complex fraction of alcohol soluble proteins in wheat seeds, termed gliadins (table 5) [49], and develop autoreactive IgA antibodies against the endomysial autoantigen tissue transglutaminase and reticulin [46, 50, 51]. Around 1% of the European population suffer from CD, the symptoms include (bloody) diarrhoea, weight loss and anaemia. The only therapy available is to avoid wheat containing foods altogether.

3.2.3 Wheat food allergy

IgE mediated food allergy, another genetically determined hypersensitivity of the gut, can also be caused by dietary intake of wheat proteins [52, 53]. As in other IgE-mediated food allergies, symptoms of wheat allergy become apparent within 30 minutes after ingestion of the culprit food with cutaneous, respiratory and gastrointestinal manifestations, which can lead to anaphylaxis [54]. These reactions are clinically distinct from other adverse reactions to wheat proteins, including bakers' asthma, celiac disease and WDEIA [55, 56, 57]. Although there are some similarities, differences are present in wheat specific allergens, patterns of exposure, immunologic mechanism and pathophysiology [58].The only available therapy so far is avoidance of the allergen source.

3.2.4 Bakers' Asthma

Bakers' asthma is one of the most common forms of occupational asthma, affecting up to 15% of bakers, millers, and pastry factory workers [59, 60]. The disorder is mainly caused by an IgE mediated allergic response to inhalation of cereal flours. The major causative allergens of the bakers' asthma condition are proteins derived from wheat and rye flours, although baking additives, such as fungal α-amylase, are also important [59, 61]. Symptoms are, as the name indicates, asthma, but also rhinitis. So far no therapy but avoidance of the allergen source is available.

3.2.5 Wheat dependant exercise induced anaphylaxis (WDEIA)

Wheat dependant exercise induced anaphylaxis is a severe IgE mediated allergic reaction provoked by the combination of wheat ingestion with intensive physical exercise over the following hours [62]. WDEIA is a distinct form of wheat allergy, which is elicited by allergens different from wheat allergy or bakers' asthma. Symptoms include generalized urticaria, hypotension and shock [62].

4. Allergy diagnosis

4.1 Current diagnostic tools

4.1.1 In vivo tests

Skin prick tests (SPT) are used to screen for food specific IgE [63]. SPT are easily performed and show results within 15 minutes. The diagnostic accuracy depends on the quality of allergen extracts used. For many foods, commercial extracts for SPT

show a low sensitivity resulting in a high rate of false-negative results. This is caused by the low abundance or the lack of stability of several allergens to endogenous enzymatic processes taking place in plant food extracts [64].

4.1.2 Food challenges

The oral challenge is a diagnostic test, which provides strong evidence of a food allergy, and allows the clinician to recommend a correct elimination diet. However, in patients with a history of anaphylaxis after the ingestion of an isolated food, challenges are not the method of choice [64].

Food challenges can be performed open, single blinded, or double blinded. The double blind placebo controlled food challenge (DBPCFC) is considered to be the gold standard for the diagnosis of food allergy. In this test placebo and allergen are administered in a random order. Both, patient and health care professional do not know when the placebo or allergen is applied. The problem with DBPCFC is the absence of a uniform procedure manual, which makes comparison of studies difficult [64].

4.2 In vitro tests

4.2.1 Assays for specific IgE antibodies

So far the measurement of allergen specific IgE in the blood is the most useful marker in clinical diagnostics.

Typical assays for specific IgE antibodies are CAP/RAST, ELISA and EAST and RAST [5, 65].

An antibody assay will generally not be able to discriminate between genuine sensitization and serological cross reaction to a given structure [5].

4.3 Recombinant allergens

Improving diagnostic tests for food allergy with recombinant manufactured allergens is vital for an accurate diagnosis in patients suffering from food related symptoms. Most allergens involved in food allergy are plant-derived proteins, which are easily degraded by physical and/or chemical strain during the extraction process. This does not only result in difficulties during the preparation of food extracts but also implies that certain allergens within an extract may be degraded during the storage for longer time periods [66].

Examples for successfully produced allergens are:
Recombinant Api g 1: the major allergen from celery, was used for skin tests. The results indicated that this protein allowed accurate in vivo diagnosis of celery allergy in areas where birch trees are common [67].
Recombinant Cor a 1.0401: Sera of patients with an allergy to hazelnut were investigated either with the recombinant allergen or with hazelnut extract. EAST with recombinant Cor a 1 yielded a sensitivity of 95% compared to 70% of the commercially available CAP/RAST system, which is based on a total food extract [68].

5. Aim of the study

The aim of this study was to clone the cDNA sequence of Tri a 12, the Bet v 2 homologue from wheat (*Triticum aestivum*), to express it as a non-fusion protein in *E. coli*, and to purify it to near homogeneity.
Recombinant Tri a 12 was characterised regarding its molecular and immunological features. Furthermore, as this study is a part of the EC-funded project EUROPREVALL (“The Prevalence, Cost and Basis of Food Allergy across Europe”), rTri a 12 will be used for CAP establishment (Phadia, Uppsala, Sweden), CHIP production (VBC Genomics, Vienna, Austria), and histamine release assays (RefLab, Copenhagen, Denmark). With the establishment of novel component-resolved diagnostics epidemiologic studies across Europe will be undertaken within this project.

II. MATERIALS & METHODS

1. Cloning

1.1 Overview

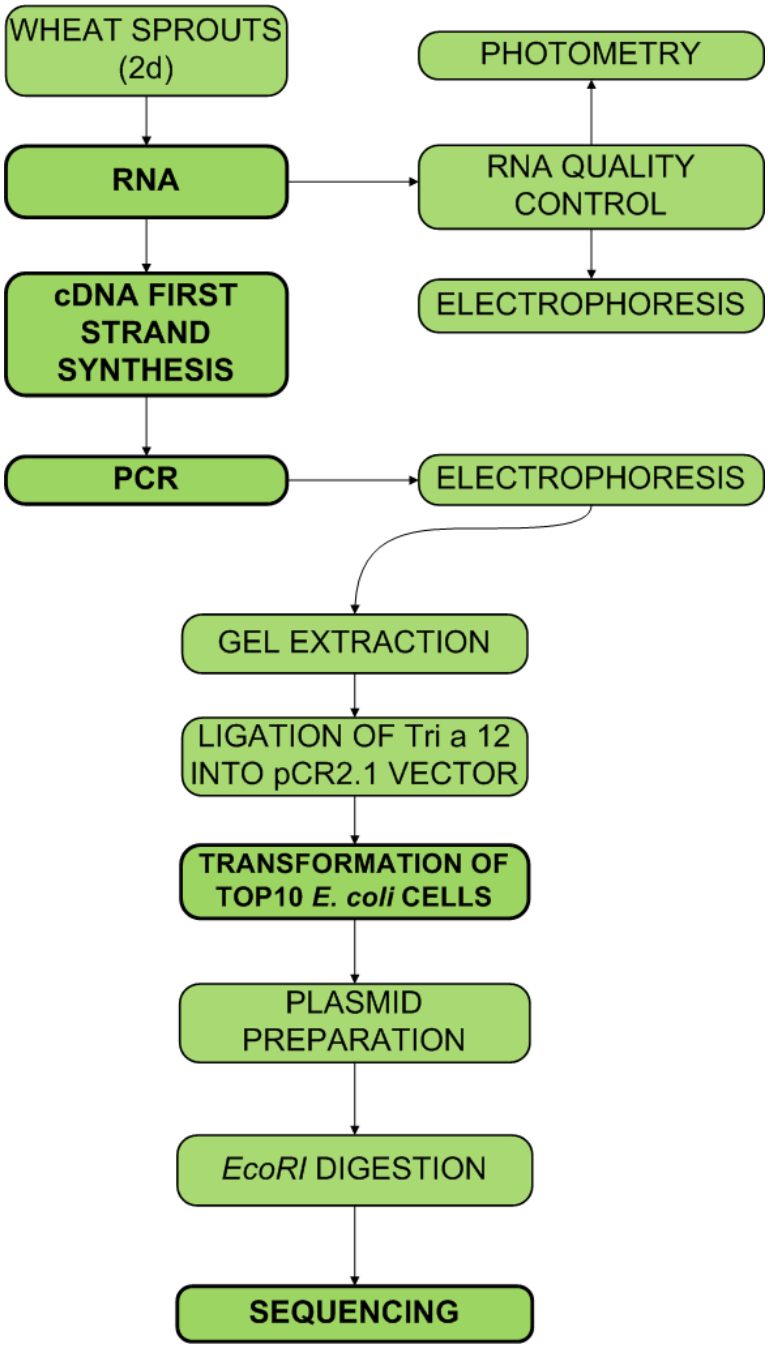


Fig.3: Cloning Overview

1.2 Buffers & Solutions

DEPC/MilliQ H_2O

500 µl DEPC per litre MilliQ-H_2O, incubate in lamina flow overnight

10x FA gel buffer (RNA)

200 mM MOPS
50 mM sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

1x FA gel running buffer

100 ml 10x FA gel buffer
20 ml 37% formaldehyde
880 ml DEPC/MilliQ-H_2O

5x TBE gel running buffer

445 mM TRIS pure
445 mM boric acid
10 mM EDTA-Na_2

SOC medium

0.5% yeast extract
2.0% peptone
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
20 mM MgSO4
20 mM glucose

LB medium

0.5% yeast extract
1% peptone
1% NaCl

LB agar

LB medium
15 g agar/l

1.3 Plant material

Triticum aestivum cv.Edison was chosen, because of its widespread use in Lower Austria [RWA Raiffeisen Ware Austria AG; Vienna, Austria].

Material:

- *T. aestivum cv. Edison* corn
- Petri dish
- MilliQ water
- Sterile scalpel
- Liquid nitrogen

Protocol:

Ten grams of wheat corn were incubated in a Petri dish (filled with water) for 48 hours. After two days the wheat sprouts were harvested by separation from the corn (with a sterile scalpel) before they were frozen in liquid nitrogen.

1.4 RNA isolation

To obtain stable RNA a ribonuclease free environment was provided. All lab equipment was heat sterilzed. All solutions were treated with DEPC to inactivate ribonucleases (by binding histidine residues).

Material:

- Wheat sprouts (2 days old)
- Liquid nitrogen
- Mortar and pestle
- Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA)
- β–mercaptoethanol

Protocol:

All centrifugation steps were carried out at 16,100 g.

Wheat (*T. aestivum*) samples were collected after 2 days. Wheat sprouts (2 d old, 110 mg) were shock frozen in liquid nitrogen and ground to a fine powder using pre-chilled mortar and pestle. Five micro litres β–mercaptoethanol were added to 500 µl lysis solution. Then this mixture was added to 110 mg ground sprout material. This mixture was transferred to an Eppendorf tube. After one minute of thorough vortexing, the sample was incubated at 56℃ for 5 minutes. To separate cellular debris, the sample was centrifuged for 3 minutes. The resulting supernatant was pipetted into a filtration column (seated in a 2 ml tube), and centrifuged for one minute. Afterwards, 250 µl binding solution were added to the flow through. After mixing gently, the sample was transferred onto the binding column (seated in a 2 ml collection tube) and spun for 1 minute. The flow through was discarded.

Washing the sample included three steps: 500 µl wash solution I were transferred into the column. After 1 minute of centrifugation and discarding of the flow through, 500 µl wash solution II were added and the column was

centrifuged for 30 seconds. The flow through was discarded. Wash solution two was applied twice (same procedure). To dry the column, it was spun for 1 minute. Elution was executed in a new (sterile) 2 ml collection tube. Fifty micro litres elution solution was pipetted directly onto the centre of the binding matrix. After incubation for 1 minute at RT, the column was spun for 1 minute. The flow through was stored at -20℃.

1.5 RNA Quality Control

1.5.1 Photometry

Photometric control of the isolated RNA was performed to assess its quality.

Material:

- HELLMA precision cell (made of Quartz SUPRASIL^{R}; Light path 10 mm; Centre 10 mm; Type Nr. 105.202-QS)
- DEPC/MilliQ-H_2O
- Wheat (*T. aestivum*) total RNA
- HITACHI U-1800 Spectrophotometer (Tokyo, Japan)

Protocol:

RNA concentration was determined spectrophotometrically by a wavelength scan from 200 nm to 300 nm. Pure preparations of RNA usually have an $OD_{260nm/280nm}$ ratio of 2[69]. Samples were diluted in DEPC water 1 : 40 (test volume 100 µl).

For calculating the RNA yield the following formula was used:

$$OD_{260nm} \textbf{ x } \text{dilution factor } \textbf{x } 40 \textbf{ = } \mu g/ml \text{ [69]}$$

1.5.2 RNA agarose gel electrophoresis

During agarose gel electrophoresis RNA (or DNA) is separated and visualized. An electric current is applied to the agarose gel, which leads to the migration of negatively charged RNA (DNA) molecules according to their size. Ethidium bromide is added to the agarose gel to visualize the RNA (DNA). This dye

intercalates the RNA (DNA) and by irradiating the agarose gel with ultra violet light the RNA (DNA) becomes visible.

Material:

- Agarose
- 10x FA gel buffer
- DEPC/MilliQ water
- 37% formaldehyde
- Ethidium bromide (10 mg/ml)
- RiboRuler RNA ladder, low range (fig.4;Fermentas; St. Leon-Roth, Germany)
- Total RNA extract
- Loading dye solution for RNA electrophoresis (Fermentas; St. Leon-Roth, Germany)
- 1x FA running buffer

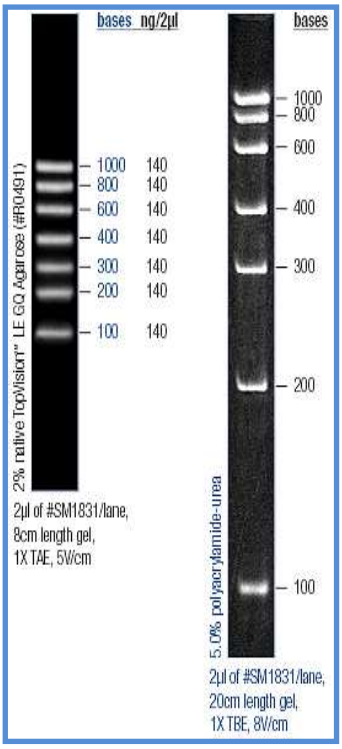


Fig. 4: RNA marker (Copyright: www.fermentas.com)

Protocol:

The gel was prepared by mixing 1.2 g agarose, 10 ml 10x FA gel buffer and DEPC-MilliQ water (total volume: 100 ml). This mixture was heated in a microwave oven and after cool down to 65℃, 1.8 ml 37% formaldehyde and 1µl ethidium bromide were added. The solution was poured onto the gel support and solidification was finished, the gel was equilibrated for 30 minutes in 1x FA buffer. Total RNA extract (5 µl) was mixed with 2x loading dye solution (5 µl) and incubated at 65℃ for 5 minutes . After the gel was transferred to the gel chamber, 10 µl sample and 3 µl RNA marker were loaded (running conditions: 65 V/35.5 mA).

1.6 Complementary DNA first strand synthesis (RT-PCR)

Reverse transcriptase PCR is a form of PCR using a RNA dependant DNA polymerase to transcribe mRNA to cDNA.

Material:

- T25NN (oligodT) for cDNA synthesis:
  5’ – GGAGAAGGATTTTTTTTTTTTTTTTTTTTTTT – 3’; 100pmol/µl
- total RNA (607 mg/ml)
- DEPC water
- 5x buffer for M-MuLV Reverse Transcriptase
- RiboLock ribonuclease inhibitor (40 U/µl)
- dNTPmix (10 mM each)
- M-MuLV reverse transcriptase (20 U/µl)

Reaction was carried out in a Primus 96^{plus} thermal cycler (MWG Biotech; High Point, NC, USA)

RiboLock ribonuclease Inhibitor, 5x buffer for M-MuLV RT and M-MuLV reverse transcriptase were purchased from Fermentas (St. Leon-Roth, Germany)

Protocol:

Reaction mix (20µl total reaction volume):

- 7 µl DEPC water
- 3 µl total RNA
- 1 µl T25NN

Reaction mix was incubated at 70℃ for 5 minutes in a thermal cycler. After denaturing the following reagents were added:

- 4 µl 5x buffer for M-MuLV reverse transcriptase
- 1 µl Ribolock ribonuclease inhibitor
- 2 µl dNTPmix

After incubation at 37℃ (thermal cycler) for 5 min utes

- 2 µl M-MuLV reverse transcriptase were added

Finally, the complete reaction mix was incubated at 37℃ (thermal cycler) for 60 minutes. The reaction was terminated by an incubation at 70℃ for 10 minutes. cDNA was stored at -20℃.

1.7 PCR

The polymerase chain reaction is a method for amplifying DNA in vitro. It uses the heat stable DNA polymerase from *Thermus aquaticus*. High temperature is used to denature the template DNA into two single stranded molecules. Two small oligonucleotides are used as primers. These small molecules anneal to their respective strands. Following primer annealing, primer extension results in a copy of the original DNA template. This cycle can be repeated as often as desired, but usually thirty cycles suffice.

1.7.1 Primer Design

To design appropriate primers, sequences from the genbank database were retrieved. *Triticum aestivum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) was chosen as a housekeeping gene to check the integrity of the total RNA extracted (see chapter 1.4). Housekeeping genes are constitutively expressed genes, which give information about the quality of the template (c)DNA.

DNA – Sequences

1.) *Triticum aestivum* profilin isoform 3
Genbank Database accession number: X89827
>gi|1008444:51-473 *T.aestivum* mRNA for profilin (clone TaPRO3)

forward primer: 5’ – ATGTCGTGGAAGGCGTACGTCGA – 3’ (rTri a 12 IF3 for)
reverse primer: 5’ – AGTAACCCTGATCGATCAGGTAATC – 3’ (rTri a 12 IF3 rev)

2.) *Triticum aestivum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl)
Genbank Database accession number: AY328025
>gi|32966579:60-1493 *Triticum aestivum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) mRNA, complete cds; chloroplast gene for chloroplast product

forward primer : 5’ – TGGGGTTCCGCCCGAAGAAGCA – 3’

reverse primer : 5’ – ACTTGGATACCATGAGGCGGGCCT – 3’

All primers were purchased from MWG BIOTECH (High Point, NC, USA).
Primers were diluted in MilliQ water to a working concentration of 5 pmol/µl.

1.7.2 PCR reaction

Material:

- MilliQ water
- Wheat cDNA
- Primers (5 pmol/µl)
- 25 mM $MgCl_2$
- 10 mM dNTPmix
- Taq polymerase buffer
- Taq polymerase (5 U/µl)

*Taq p*olymerase (buffer), dNTP mix and $MgCl_2$ were purchased from Fermentas (St. Leon-Roth, Germany).
Reaction was carried out in a Primus 96^{plus} thermal cycler (MWG Biotech, High Point, NC, USA).

Protocol:

PCR reaction mix (50 µl total reaction volume)

- 22.5 µl MilliQ-H_2O
- 5 µl Taq polymerase buffer
- 5 µl cDNA
- 5 µl Primer forward (rTri a 12 IF3 for)
- 5 µl Primer reverse (rTri a 12 IF3 rev)
- 4 µl $MgCl_2$
- 3 µl dNTPmix
- 0.5 µl Taq polymerase

1.7.3 PCR program:

- 95℃ 3’

- 95℃ 1’
- 60℃ (T_A) 1’
- 72℃ (T_X) 2’

} 35 x

- 72℃ 10’
- 4℃ ∞

T_x extension temperature

T_A annealing temperature

1.8 Agarose gel electrophoresis

To determine the success of the PCR experiment, an agarose gel electrophoresis was performed (for method background see chapter 1.5.2).

Material:

- 2.4 g agarose
- 200 ml 1xTBE Buffer
- 30 µl Ethidium bromide (10 mg/ml)
- 5 µl DNA Marker (fig.5; 100 bp ladder plus)
- 6x Loading Dye solution
- 20 µl PCR product

DNA Marker (100 bp ladder plus) and 6x Loading Dye solution were purchased from Fermentas (St. Leon-Roth, Germany)

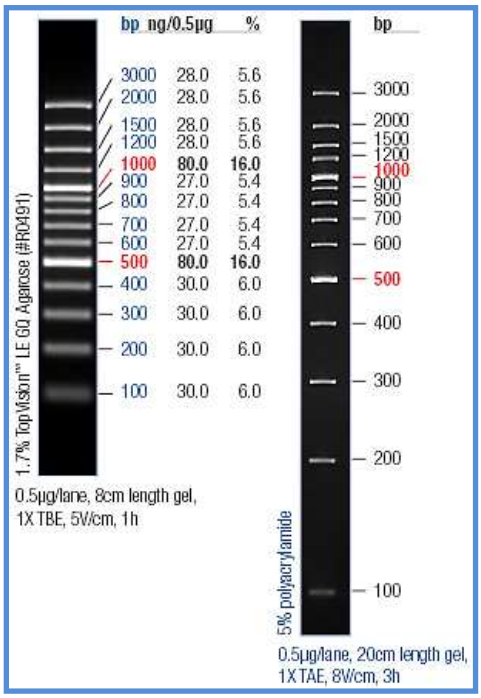


Fig. 5: DNA marker (Copyright: www.fermentas.com)

Protocol:

Agarose and TBE buffer were mixed and heated in a microwave oven. Before the gel was poured, 30 µl Ethidium bromide were added. After the gel hardened, the gel chamber was filled with 1x TBE running buffer. After 10µl sample were mixed with

2µl 6x loading dye, the gel was transferred to the gel chamber.
Running conditions: 130 V/60 mA

1.9 Gel extraction

QIAEX II gel extraction kit is designed to extract and purify DNA from any agarose gel without phenol extraction or ethanol precipitation. DNA molecules are adsorbed to QIAEX II silica particles in the presence of high salt. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide are removed during washing steps. The pure DNA is eluted with 20 µl water.

1.9.1 QIAGEN QIAEX II – Gel Extraction Kit

Material:

- Agarose gel
- Sterile scalpel
- Gel extraction kit (Qiagen; Hilden, Germany)

Protocol:

The DNA band of interest was excised from the agarose gel with a sterile scalpel. After weighing the fragment, 3 volumes of Buffer QX1 were added. After resuspending the QIAEX II particles, 30 µl were added to the DNA/QX 1 mixture. To solubilise the agarose and bind the QIAEX II particles to the DNA, the reaction mix was incubated at 50℃ for 10 minut es.
The procedure was continued with a centrifugation at 16,100 g for 30 seconds. The pellet was washed with 500 µl QX 1 and centrifuged again (see above). The supernatant was removed and the pellet washed twice with buffer PE to remove remaining salt contaminations. Following the last washing step, the supernatant was discarded and the pellet dried until it turned white (40 minutes).
To obtain the DNA, 20 µl of aqua ad injectabilia were added to the dry pellet. After resuspension and incubation (5’ RT) a last centrifugation step followed (16,100 g). The DNA containing supernatant was transferred to a new 1.5 ml tube.

1.9.2 PCR of gel extraction products (Gelex PCR)

To obtain more PCR product, a new PCR experiment with the extracted DNA was performed. The same conditions as in the first PCR experiment (chapter 1.7) were employed, except for a 1℃ higher anneali ng temperature (61℃), and instead of 5 µl cDNA,1 µl DNA from the gel extraction was used.

1.9.3 Agarose gel electrophoresis of Gelex PCR

The same conditions as in the agarose gel electrophoresis of the PCR product (chapter 1.8) were used. After excising the correct band from the agarose gel, a gel extraction procedure (chapter 1.9.1) yielded a sufficient amount of DNA, encoding profilin.

1.10 Cloning: ligation & transformation

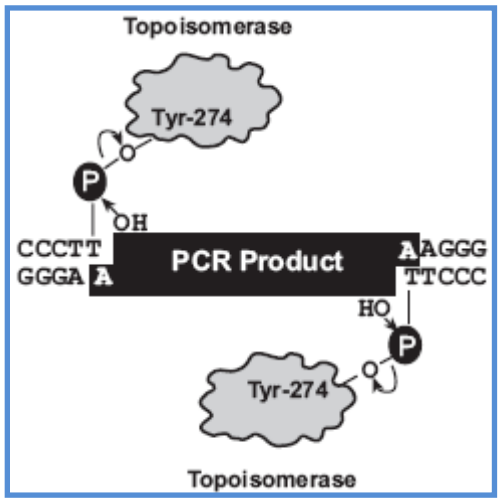


Fig.6: function of topoisomerase (Copyright: Invitrogen, Carlsbad, CA, USA)

To shuttle the purified PCR product to *E. coli* cells it has to be combined with a suitable plasmid vector (pCR2.1-TOPO, Invitrogen; Carlsbad, CA, USA). This linearized vector contains single 3´-thymidine overhangs (fig.6), whereas the 3´ ends of the PCR product are made of single deoxyadenosine overhangs. This allows PCR inserts to ligate with the vector. Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand [70]. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′

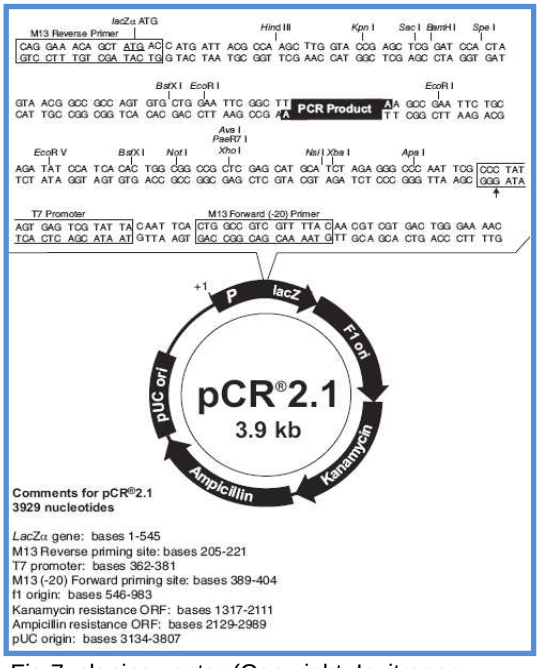


Fig.7: cloning vector (Copyright: Invitrogen, Carlsbad, CA, USA)

hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase [71].
The vector contains part of the lacZ Operon. This allows for a blue/white colony selection after the transformation.

1.10.1 Ligation

Material:

- PCR product
- Salt solution, 1.2 M NaCl + 0.06 M $MgCl_2$
- pCR2.1 vector (fig.7; Invitrogen; Carlsbad, Ca, USA)

Reactions were carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech, High Point, NC, USA).

Protocol:

Four micro litres PCR product were added to 1 µl salt solution and 1 µl pCR2.1 vector. After 15 minutes of incubation at 22℃, the ligation was completed.

1.10.2 Transformation

Material:

- Ligation product (PCR product/pCR2.1 vector)
- TOP10 chemically competent *E. coli* cells (Invitrogen; Carlsbad, Ca, USA)
- SOC medium
- LB medium
- Ampicillin (100mg/ml)
- XGal (20mg/ml)

Protocol:

Three micro litres ligation product were added to 50 µl of One Shot TOP10 chemically competent *E. coli* cells. After 15 minutes on ice and 30 seconds on 42℃, 250 µl SOC medium were added. Samples were in cubated at 37℃ for 1

hour. Petri dishes were prepared with 40 µl ampicillin and 80 µl XGal enriched LB agar medium.
The transformed bacteria were centrifuged at 16,100 g for 30 seconds. One hundred and fifty micro litres of the supernatant were discarded. After the pellet was resupended, 100 µl bacterial culture were plated on one Petri dish (LB-amp agar), on another 50 µl, and as a negative control 100 µl bacterial culture containing an empty vector on a third plate. The Petri dishes were incubated at 37℃ over night.

1.11 Colony screening

Material:

- Autoclaved toothpicks
- Bacterial colonies
- LB medium
- Ampicillin (100 mg/ml)
- Streptromycin (20 mg/ml)

Protocol:

Autoclaved toothpicks were used to transfer white colonies (LacZ operon destroyed by insert) to bacterial tubes, filled with 4 ml LB medium, 4 µl ampicillin (100 mg/ml), and 4 µl streptomycin (20 mg/ml). The rationale was, that the vector contained the LacZ operon, which would colour the colonies blue, if the transformation was unsuccessfull. The vector also holds an ampicillin resistance (serves as a selection marker). The bacterial strain TOP10 contains a streptomycin resistance. So it can be ensured, that only nonblue, streptomycin^r, ampicillin^r bacteria grow in culture tubes containing LB with ampicillin and streptomycin.

1.12 Plasmid DNA preparation

After the resuspended (buffer A1) bacteria were treated with SDS/alkaline lysis buffer (A2) the plasmid was liberated. Buffer A3 neutralizes the lysate and allows the binding of plasmid DNA to the silica membrane of the purification column. Salts, metabolites, and other contaminations are removed by washing with buffer A4. Pure plasmid DNA is eluted with slightly alkaline buffer AE.

Material:

- Nucleospin Plasmid kit (Macherey-Nagel, Düren, Germany)
- Bacterial culture (containing plasmid)

Protocol:

All centrifugation steps were carried out at 11,000 g.

Escherichia coli TOP10 LB culture (4x1 ml) was transferred in a 1,5 ml tube and centrifuged for 30 seconds. The supernatant was discarded. The pellet was resuspended in 250 µl of Buffer A1. To facilitate cell lysis, 250 µl buffer A2 were added. After the tube was inverted 8 times, incubated for five minutes at RT, 300 µl buffer A3 were added and the solutions mixed by inverting the tubes.

To clarify the lysate, it was centrifuged for 10 minutes. A separation column was placed in a 2 ml collecting tube and the supernatant of the previous step was loaded. After centrifugation for 1 minute the DNA bound to the column. The flow through was discarded.

After 600 µl of buffer A4 (washing buffer) were added to the column, it was centrifuged for 1 minute. The flow through was discarded. After drying the silica membrane (in the column) by centrifuging for 2 minutes, the DNA was eluted. The column was transferred to a new 1.5 ml tube and 50 µl of buffer AE (elution buffer) were added. After incubation for 1 minute at room temperature and a centrifugation for 1 minute, the elution step was completed.

1.13 *EcoRI* Digestion

Purified plasmid DNA (pCR2.1-TOPO+wheat profilin insert) was digested with the restriction enzyme *EcoRI,* prior to DNA sequencing. Restriction enzymes cut double stranded DNA at unique positions (restriction sites). This results in DNA molecules with either blunt- or sticky ends. If both vector and insert, are cut with the same restriction enzyme(s) it is possible to ligate the two molecules, resulting in a new combined DNA molecule.

The pCR2.1 vector contains *EcoRI* restriction sites near both ends of the insert at positions 284 & 302 (fig.7)

Material:

- MilliQ water
- 10x fast digest buffer
- DNA (from plasmid preparation)
- Fast digest enzyme *EcoRI*

10x fast digest buffer and fast digest enzyme *EcoRI* were purchased from Fermentas (Hilden, Germany)
Reaction was carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech, High Point, NC, USA).

Protocol:
Reaction mix (20 µl total reaction volume):

- 15 µl MilliQ water
- 2 µl 10x fast digest buffer
- 2 µl DNA
- 1 µl fast digest Enzyme *EcoRI*

After mixing thoroughly, the digestion reaction was performed at 37℃ for 15 minutes. The success of the digestion was confirmed by an agarose gel electrophoresis (see chapter 1.8).

1.14 Sequencing

To determine the sequence of the cloned gene, DNA sequencing method developed by Frederick Sanger was used [72]. This method is based on DNA synthesis in the presence of dideoxy nucleotide triphosphates, which differ from normal deoxynucleotides in their lack of a 3’ hydroxyl group. During a PCR the respective dideoxy nucleotide triphosphates can be incorporated into a growing chain, but when incorporated they terminate synthesis due to the lack of a 3’ hydroxyl group [73].
For every analysis, four reaction tubes are prepared (A, C, G, T). Each tube receives a different dideoxynucleotide (ddATP, ddCTP, ddGTP, ddTTP), together with the normal deoxynucleotide triphosphates, DNA polymerase, and

fluorescence labelled primers. A dideoxy nucleotide triphosphate will be incorporated randomly. This results in the synthesis of truncated DNA fragments of various lengths. These fragments can be visualized by electrophoresis, if a labelled primer (e.g. infra red dye) is used for the PCR reactions.

The sequencing procedure is divided into the sequencing-PCR, electrophoresis and data analysis.

1.14.1 Sequencing PCR

Material:

- SequiTherm EXCEL II Long Read L-C Kit (Epicentre Biotech, Madison, WI, USA)
- DNA template, amount needed was calculated using the following formula:

400 fmol **x** 660 g/Mol **x** length of insert (bp) **=** fg insert required for sequencing

[69]

- Primers:

  T7: IRD-800 (infra red dye) modified:
  5’ – TAATACGACTCACTATAGGG – 3’
  M13:
  5’ – CAGGAAACAGCTATGACC – 3’
  primer concentration: 5 pmol/µl

All reactions are carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech,High Point, NC, USA)

5’- CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGG
AATTCGCCCTTAAGGGC**GAATTC**TGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCC
TATAGTGAGTCGTATTA – 3’

Fig.8: sequence of pCR2.1-TOPO (EcoRI restriction sites bold; T7 primer printed in black, M13 primer printed in red)

Protocol:

Sequencing mix (17 µl total reaction volume)

- 7.2 µl 3.5xSequiTherm EXCEL II sequencing buffer
- 3.8 µl deionized water
- 3 µl DNA template (conc.: 22.8 ng/µl)
- 2 µl T7 or M13 primer (conc.: 5 pmol)
- 1 µl SequiTherm EXCEL II DNA polymerase (5 U/µl)

2 µl of each termination mix (ddGTP, ddATP, ddTTP, ddCTP) were pipetted into a 0,25 ml PCR tube. To each termination mix 4 µl sequencing mix were added.

PCR program:

- 95℃ 5’ (template denaturation step)

- 95℃ 30’’
- 50℃ 15’’ } 30x
- 70℃ 1’

- 4℃ ∞

Afterwards 3 µl stop solution were added.

1.14.2 Sequencing gel electrophoresis (41 cm gel)

Material:

- Urea (99,5% p.a.; ROTH, Karlsruhe, Germany)
- MilliQ water
- 5xTBE
- DMSO (100% Dimethyl Sulfoxide; Sigma Aldrich, St. Louis, MO, USA)
- Long Ranger gel solution
- Temed (100%; ROTH, Karlsruhe, Germany)
- 10% APS

Protocol:
Sequencing gel composition (37.5 ml total volume):

- 15.75 g Urea (7M)
- 22.175 ml MilliQ water
- 7.5 ml 5xTBE
- 1.125 ml DMSO (Sigma; 100%)
- 4.5 ml Long Ranger Gel Solution
- 37.5 µl Temed (ROTH; 100%)
- 262.5 µl 10% APS

After pouring the sequencing gel and waiting for 1.5 h to allow polymerization, the gel was mounted on the DNA-sequencer (DNA Sequencer model 4000L, LICOR, Lincoln, NE, USA). One micro litre of sample was loaded into each slot. The sequencing procedure was performed overnight. LICOR Base ImagIR Data Collection V2.31 software was used for sequence analysis.

1.14.3 Data analysis

Data was analyzed with the LICOR Image Analysis V4.0 and the LICOR ALIGN IR V2.0 software.
For analysis on the amino acid level the following software was used:

- EMBOSS Transeq
- ClustalX

The sequences retrieved from sequencing were analyzed further and compared to database entries (DNA & Protein). DNA sequences were translated using the EMBOSS TransSeq software. Alignments were done with ClustalX.

2. Expression

2.1 Overview

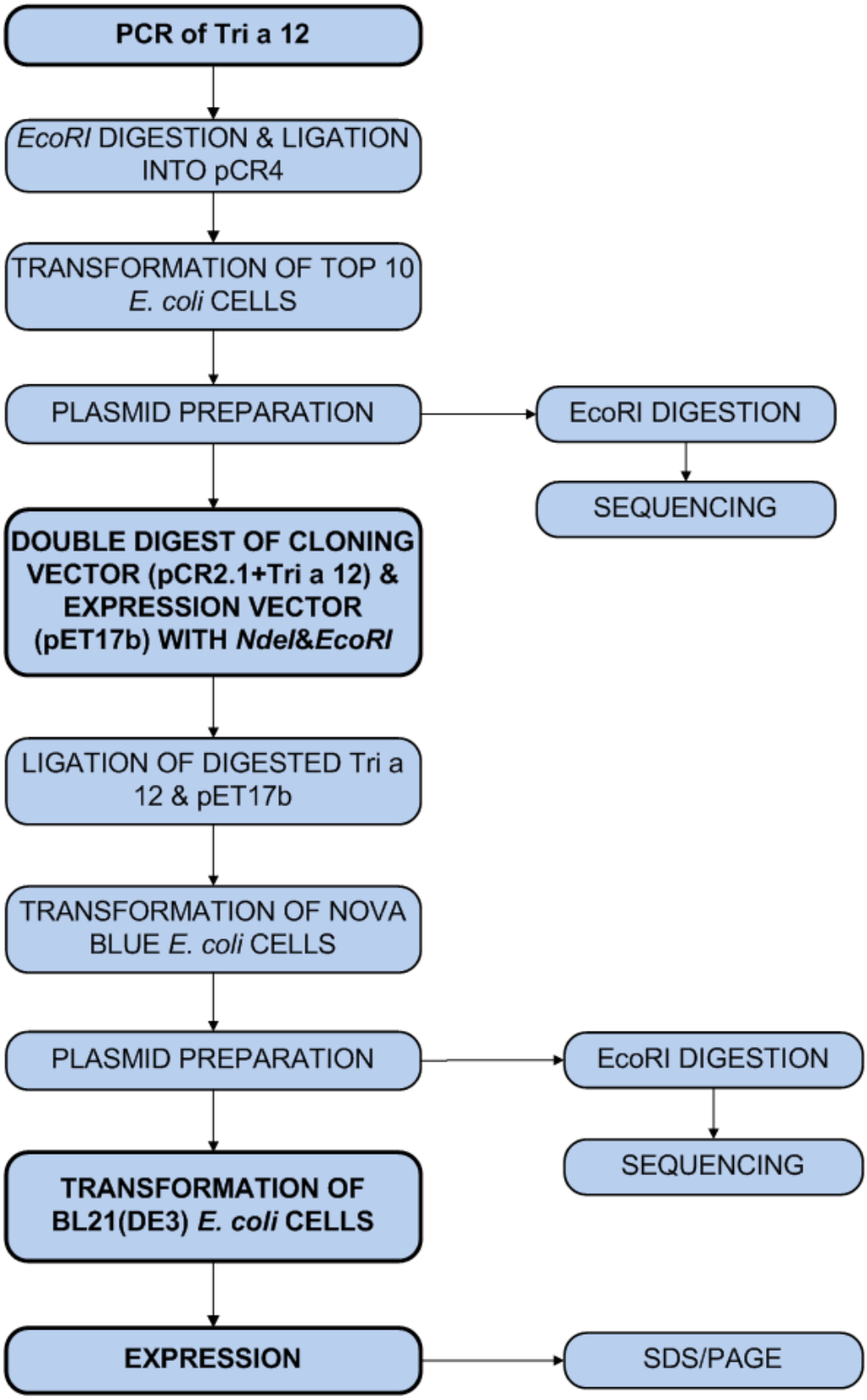


Fig.9: Overview expression

2.2 Buffers & Solutions

SOC medium

0.5%	yeast extract
2.0%	peptone
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
20 mM	MgSO4
20 mM	glucose

LB medium

0.5%	yeast extract
1%	peptone
1%	NaCl

LB agar

LB medium
15 g Agar/l

SEM buffer

10 mM	PIPES
15 mM	$CaCl_2 \cdot 2H_2O$
250 mM	KCl (pH 6.7)
55 mM	$MnCl_2 \cdot 2H_2O$

Lysis buffer

50 mM	NaH_2PO_4
300 mM	NaCl
10 mM	imidazol

2.3 Expression System

2.3.1 Restriction sites-PCR

NdeI and EcoRI restriction sites were added to wheat profilin encoding DNA using PCR. Subsequently, wheat profilin DNA was ligated into expression vector pET17b. The resulting expression plasmid was used to transform competent *E. coli* BL21(DE3).

Material:

- Forward primer: 5’ – GGG*CATATG*TCGTGGAAGGCGTAC – 3’ (rTri a 12 exp for)
- Reverse primer: 5’ – CCC*GAATTC*TTAGTAACCCTGATCGA – 3’ (rTri a 12 exp rev)
  restriction sites in italics; primer concentration: 5 pmol/l.
- MilliQ water
- PCR cocktail (5 µl *Taq* polymerase buffer + 2 µl 10mM dNTP mix + 3 µl 25 mM $MgCl_2$)
- Plasmid preparation DNA (clone 3R-pCR2.1)
- 25 mM $MgCl_2$
- 10 mM dNTP mix
- *Taq* polymerase (5 U/µl)

- Template DNA (rTri a 12 in pCR2.1)

Taq polymerase (buffer), dNTP mix and $MgCl_2$ were purchased from Fermentas (St. Leon, Germany).
Reaction was carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech, High Point, NC, USA). Primers were purchased from MWG.

Protocol:

PCR mix (50 µl total reaction volume)

- 24.5 µl MilliQ water
- 1 µl template DNA (rTri a 12 in pCR2.1)
- 5 µl Primer forward (rTri a 12 exp for)
- 5 µl Primer reverse (rTri a 12 exp rev)
- 4 µl $MgCl_2$
- 3 µl dNTP mix
- 5 µl Taq polymerase buffer
- 1.5 µl Taq polymerase

PCR program:

- 95℃ 3’
- 95℃ 1’
- 61℃ (T_A) 1’ } 35x
- 72℃ (T_X) 2’
- 72℃ 10’
- 4℃ ∞

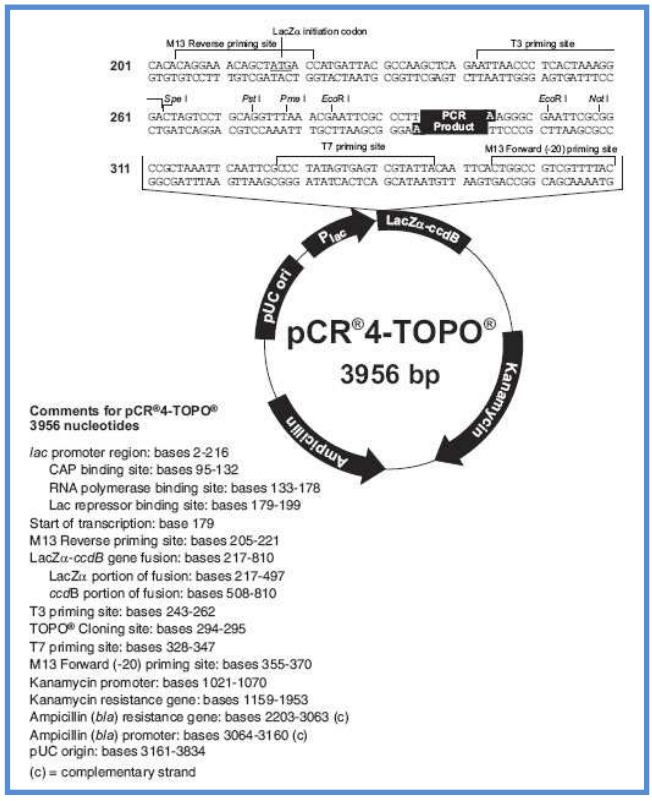


Fig.10: pCR4 TOPO vector (Copyright: Invitrogen, Carlsbad, CA, USA)

The resulting PCR product was ligated into the cloning vector pCR4-TOPO and competent Nova blue *E. coli* cells were transformed. After plasmid preparation,

a DNA sequencing experiment was performed. (For detailed information on ligation see chapter 1.10.1, for transformation see chapter 1.10.2, for plasmid DNA preparation chapter 1.12 and DNA sequencing see chapters 1.13 and 1.14).

2.3.2 Competent cells (*E. coli* BL21(DE3) & *E. coli* Nova Blue)

Two kinds of bacteria were used in this study:

- Nova blue *E. coli* (Novagen, Madison, WI, USA)
- BL21(DE3) *E. coli* (Invitrogen Corp., Carlsbad, CA, USA)

Nova blue *E. coli* cells were used as an amplification site for the cloning vector pCR4-TOPO and for the expression vector pET17b. BL21(DE3) *E. coli* cells are used for the expression of rTri a 12. Both strains were treated under chilling conditions with SEM buffer (contains divalent cations such as $CaCl_2$) to induce competence by weakening bacterial cells wall integrity.

Material:

- BL 21(DE3)/Nova blue overnight culture
- SOC medium
- Tetracycline (5 mg/ml; Nova blue cells only)
- SEM buffer
- DMSO (100%; Sigma)
- Liquid nitrogen

Protocol:

A BL21(DE3) (or Nova Blue) *E. coli* over night culture (1 ml) was diluted in 50 ml SOC (for Nova Blue cells: SOC + tetracycline) and grown in a 250 ml Erlenmeyer flask at 30° until an optical density (O D_{600nm}) of 0,6 was reached. After ten minutes on ice the culture was transferred into 50 ml Falcon tubes. Centrifugation was performed at 2,200 g at 4℃ for ten minutes. The resulting pellet was resuspended in 16 ml ice cold SEM buffer. After ten minutes on ice the suspension was centrifuged at 2,200 g at 4℃ fo r ten minutes. The resulting pellet was resuspended in 4 ml SEM buffer (on ice) and 280 µl DMSO were added. Following ten minute incubation on ice the now competent cells were frozen in liquid nitrogen.

After plasmid DNA preparation, sequencing experiments were performed (for detailed procedures on plasmid DNA preparation see chapter 1.12, for sequencing see chapters 1.13 and 1.14).

After obtaining a clone containing the proper restriction sites, the expression vector and the plasmid containing rTri a 12 (with *NdeI* and *EcoRI* restriction sites) were digested with *NdeI* and *EcoRI.*

2.3.3 Double Digest

For more detailed background information on restriction enzymes see chapter 1.14. Both, insert (rTri a 12) and vector (pET17b), were digested with *NdeI* (CA**|**TATG) and *EcoRI* (G**|**AATTC). Both enzymes produce sticky ends.

Material:

- pCR4 cloning vector containing insert (Invitrogen Corp., Carlsbad, CA, USA)
- pET17b expression vector (fig. 11&12; Novagen, Madison, WI, USA)
- 10x fast digest buffer
- Fast digest enzyme *NdeI*
- Fast digest enzyme *EcoRI*

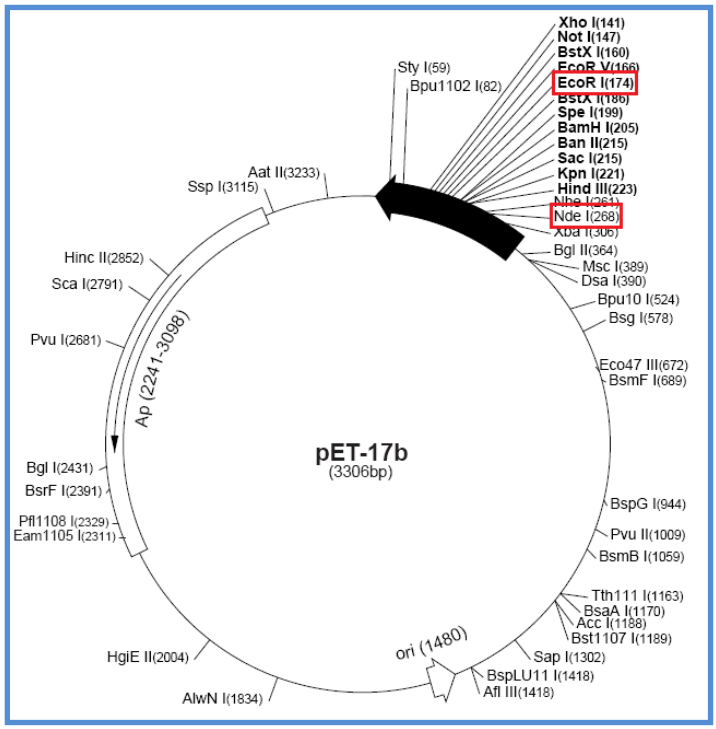


Fig.11: pET17b vector map (used restriction sites marked by red frames) ; (Copyright: Novagen, Madison, WI, USA)

Fast Digest restriction enzymes and 10x Fast Digest buffer were purchased from Fermentas (St. Leon, Germany). All reactions were carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech, High Point, NC, USA).

Protocol:

Fast digest (*NdeI*/*EcoRI)* of pCR4 (including wheat profilin insert) Reaction mix (20 µl total reaction

volume):

- 16 µl plasmid DNA
- 2 µl 10x fast digest buffer
- 1 µl fast digest enzyme (*NdeI*)
- 37℃ 30‘
- 65℃ 5‘
- 1 µl fast digest enzyme (*EcoRI*)
- 37℃ 30‘
- 65℃ 5‘

Fast digest (*NdeI*/*EcoRI)* of pET17b (expression vector)

Reaction mix (20 µl total reaction volume):

- 13.5 µl MilliQ water
- 2 µl 10x fast digest buffer
- 2.5 µl pET17b (0.4 µg/µl)
- 1 µl fast digest enzyme (*NdeI*)
- 37℃ 30‘
- 65℃ 5‘
- 1 µl fast digest enzyme (*EcoRI*)
- 37℃ 30‘
- 65℃ 5‘

Fig. 12: pET17b cloning site (used restriction sites in marked by red frame); (Copyright: Novagen, Madison, WI, USA)

2.3.4 Dephosphorylation

The digested vector was dephosphorylated with calf intestine alkaline phosphatase (removes 5’ and 3’ phosphates) to prevent re-ligation.

Material:

- Double digested pET17b

- 10x CIAP buffer
- CIAP (1U/µl)

CIAP and 10x CIAP buffer were purchased from Fermentas (Hilden, Germany) Reaction was carried out in a Primus 96^{plus} thermal cycler (MWG-Biotech, High Point, NC, USA).

Protocol:

Reaction mix (20 µl total reaction volume):

- 16 µl double digest of pET17b
- 2 µl 10x CIAP buffer
- 2 µl CIAP (1U/µl)
- 37℃ 30’
- 65℃ 15’

The total amount of digested pET17b and wheat profilin insert was loaded onto a 1.2% agarose gel. (for detailed procedures see Chapter 1.8).
After confirming the presence of vector and insert by agarose gel electrophoresis, gel extraction was performed (for detailed procedures see chapter 1.9).

2.3.5 Ligation

To combine the digested and dephosphorylated vector with the digested wheat profilin insert, a ligation reaction was performed.

Material:

- digested, dephosphorylated pET17b vector
- digested wheat profilin insert
- 10x fast link buffer
- 10mM ATP
- sterile MilliQ water
- Fast link DNA ligase (2 U/µl)

  Fast link ligase, 10 mM ATP and the 10x fast link buffer were purchased from Licor (Lincoln, NE, USA)

Protocol:

Reaction mix (20 µl total reaction volume):

- 6 µl insert
- 3 µl vector (0.4 µg/µl)
- 1.5 µl 10x fast link buffer
- 1.5 µl 10 mM ATP
- 2 µl sterile MilliQ water
- 1 µl ligase
- 22℃ 30’
- 70℃ 15’

To confirm the success of the ligation, agarose gel electrophoresis was performed (For detailed procedures see chapter 1.8). Competent Nova Blue *E. coli* cells were transformed with the ligation product (for detailed transformation procedures see chapter 1.10.2). Positive clones were selected on tetracycline and ampicillin containing LB-agar plates.

Several colonies were picked, analyzed by a PCR colony screen (For detailed procedures see chapters 1.7.2 + 1.7.3; for primers see chapter 2.3.1), and positive clones were sequenced (for detailed procedures see chapter 1.14).

Competent BL 21(DE3) *E. coli* cells were transformed with the correctly ligated plasmid (For detailed procedures see chapter 1.10.2).

2.3.6 Small Scale Expression Study

To assess the efficiency of rTri a 12 expression in BL21(DE3) *E. coli* cells, a small scale expression experiment was conducted.

Material:

- Transformed BL21(DE3) *E. coli* cells
- BL21(DE3) *E. coli* cells
- LB ampicillin medium
- IPTG

Protocol:

Five clones were picked and cultivated in ampicillin containing LB medium (LB-amp) over night at 30℃. 1.5 ml over night culture were diluted (in 30 ml LB-Amp medium) and grown to an optical density (OD_{600}) of 0.4. At this point, 0.4 mM IPTG was added to induce expression. Aliquots(0.5 ml) of the samples were taken at three consecutive time points (OD 0.4 being hour 0).
The aliquoted cultures were centrifuged at 13, 200 rpm for two minutes. The supernatant was discarded and the pellet resuspended in 100 µl 1x sample buffer.

2.3.7 Fermentation

A fermenter is used to cultivate bacteria on a larger scale. Here it is used as a means to produce rTri a 12 in sufficient quantity under controlled conditions (air supply, temperature regulator).

Material:

- 2L Fermenter (Ochs GmbH, Bovenden / Lenglern, Germany)
- LB-ampicillin medium
- overnight BL21(DE3) *E. coli* cell culture
- IPTG

Protocol:

A fermenter (maximum capacity: 2 litres) was used for the large scale expression of rTri a 12. 1.6 l of LB-ampicillin (0.1 mg/ml) medium were inoculated with 80 ml overnight *E. coli* culture (BL21 (DE3)) and grown at 30℃. After the bacterial culture reached an OD_{600nm} = 0.4, IPTG was added (0.4mM). The cultivation was continued for 5h. Then the bacteria were transferred into GS3 tubes and centrifuged at 7,000 g, at 4℃ for 30 minutes. Pellets were stored at -20℃.

3. Purification

3.1 Overview

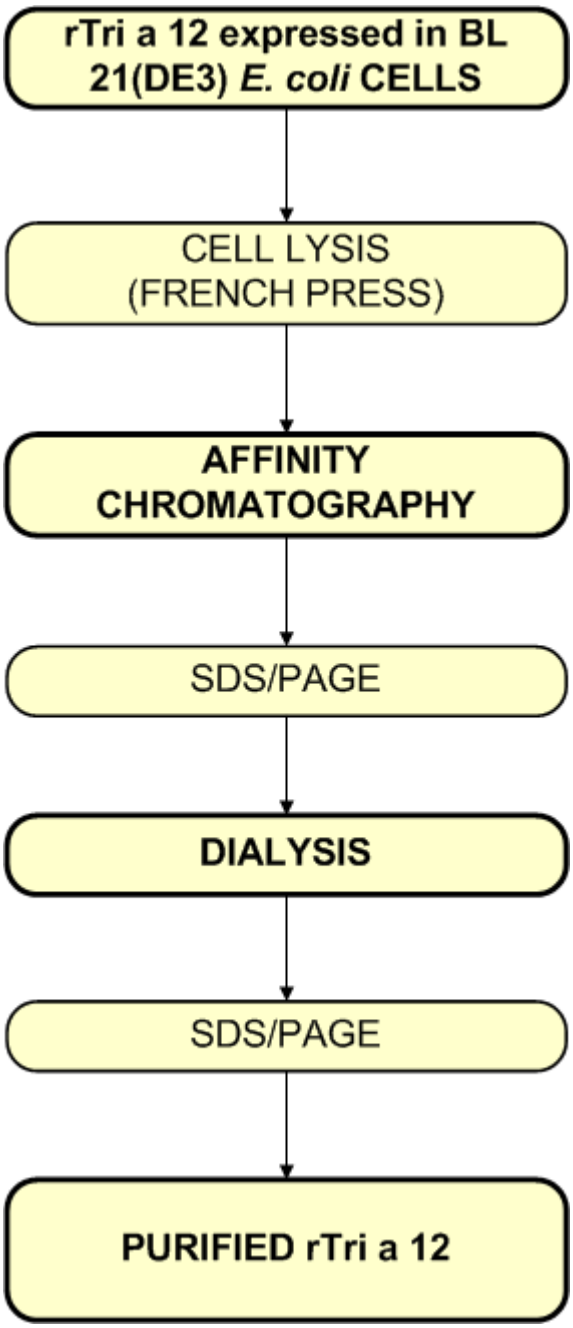


Fig.13: Purification overview

3.2 Buffers & Solutions

3.2.1 Column preparation buffers

Lysis buffer

50 mM NaH_2PO_4
300 mM NaCl
10 mM imidazol

Coupling buffer

0.2 M sodium borate, pH 8.5
10% isopropanol

Blocking buffer

1M ethanolamine, pH 8.0

Wash buffer 1

0.2 M sodium acetate, pH 4.0
10% isopropanol

Wash buffer 2

0.2 M sodium borate, pH 8.5

Wash buffer 3

0.2 M sodium borate, pH 8.5
0.5 M NaCl

3.2.2 Protein purification buffers

Start buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
1 mM DTT

Wash buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
2 M urea
1 mM DTT

Elution buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
6 M urea
1 mM DTT

Column regeneration buffer

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
8 M urea

3.2.3 Refolding buffers

Dialysis buffer 1

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
4 M urea
1 mM DTT

Dialysis buffer 2

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1 mM EDTA
2 M urea
1 mM DTT

Dialysis buffer 3

50 mM Tris-HCl, pH 7.4
150 mM NaCl

Dialysis buffer 4

5 mM Na_2HPO_4, pH 7.2

1 mM EDTA
1 mM DTT

3.3 French Press

After harvesting bacterial cells, disruption was performed using a French Press (fig.14). A French Press is a cell disruption method based on high pressure.

Material:

- French Press FA-031;(Spectronic Unicam, Cambridge, UK)
- Lysis buffer
- Cultivated BL21(DE3)
- Protease Inhibitor (Roche Diagnostics, Mannheim, Germany)

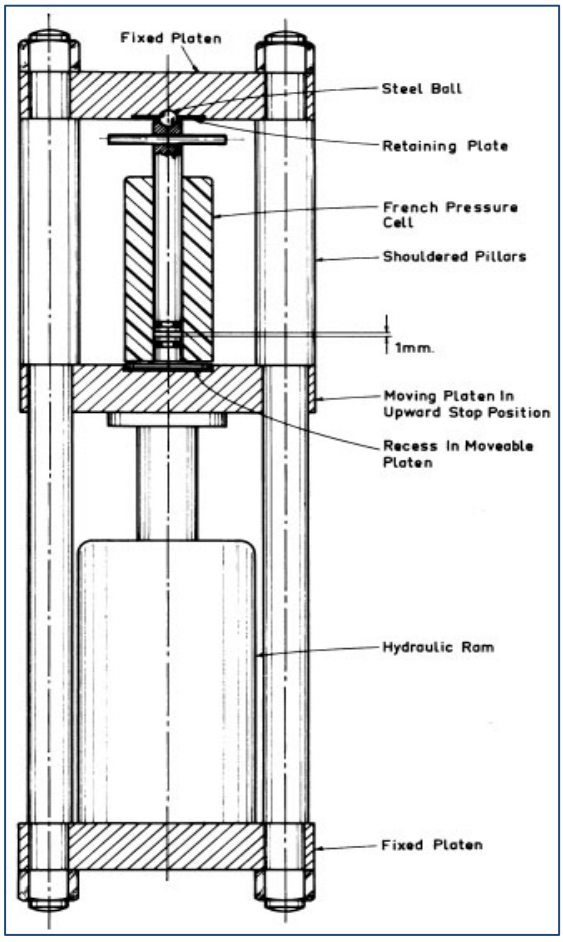


Fig.14: schematics of French press (Copyright:[74])

Protocol:

Cell pellets were resuspended in 30 ml lysis buffer on ice. To prevent degradation of rTri a 12, a protease inhibitor was added (one tablet Complete Protease Inhibitor). This suspension was filled in the French Pressure Cell, where it was disrupted three times (900-1, 000 psi). The resulting lysate was filled into SS34 tubes and centrifuged for 45 minutes at 14, 000 g. The supernatant and pellets were stored at 4℃. Samples of the (in)soluble fractions were prepared for 15% SDS-PAGE under denaturing condition (for detailed information on SDS-PAGE see chapter 4.3.1).

3.4 Affinity Chromatography

In the cell, profilin's ligands are actin, phosphoinositides, poly-L-proline (PLP) and proteins with proline-rich motifs [76]. For that reason poly-L-proline is used

as a hook to catch trapped profilin. Purification of rTri a 12 was performed by affinity chromatography. The underlying principle of this method is, that a matrix (CNBr-activated Sepharose) coupled ligand (poly-L-proline) will bind to profilin, discriminating all other proteins [75]. As urea is able to disrupt non-covalent bonds in proteins, it is used here (in buffer solutions) to elute purified profilin from the chromatography column.

Material:

Chromatography Column

- Chromatography column, diameter: 10 mm, length: 20cm (GE Healthcare, Uppsala, Sweden)
- CNBr-activated Sepharose 4B (Amersham Biosciences, Amersham, UK)
- Poly-L-proline, average MW 5000 (Sigma Aldrich, St. Louis, MO, USA)
- 1 mM HCl
- Coupling buffer
- Blocking buffer
- Wash buffer 1-3

Sample preparation

- BL21(DE3) *E. coli* lysate
- 0.45 µm filter + syringe

Chromatography run

- Start buffer
- Wash buffer
- Elution buffer
- Column regeneration buffer
- FPLC system ÄKTA (GE-Healthcare Biosciences, Amersham, UK)

All buffers were filtered and degassed.

Protocol:

Chromatography Column

First, 17.5 mg poly-L-proline were dissolved in 7 ml coupling buffer. A 100 µl aliquot was taken for photometric analysis of the efficiency of coupling (absorption at 214 nm). CNBr-activated Sepharose 4B (1 g) was transferred into a frit. A vacuum pump was used to filter 200 ml 1mM HCl through and thus let the Sepharose swell. The poly-L-proline solution and the swollen Sepharose were combined in a 50 ml Falcon tube and incubated under stirring at room temperature for 2 hours.

Afterwards the tube was centrifuged and the supernatant was analyzed photometrically, along with the aliquot from the poly-L-proline solution prior to coupling.

The gel was transferred to a chromatography column, where it was washed with wash buffer 1-3 and coupling buffer using a peristaltic pump.

Sample preparation

Before the *E. coli* lysate was applied to the column, it was dialysed (for detailed information on dialysis see chapter 3.5) against start buffer and also it was filtered by using a 0.45 µm sterile filter.

Chromatography run

The chromatography column was equilibrated with 18 ml (6-fold column volume) start buffer. Purification of rTri a 12 was achieved by using FPLC system ÄKTA. The chromatography column was connected to the ÄKTA FPLC system. Pump A (wash buffer), Pump B (elution buffer), sample pump and injection valve were washed with MilliQ water. Afterwards pump A was washed with wash buffer and pump B with elution buffer. The sample was applied to the FPLC/Chromatography system at a flow rate of 1 ml per minute. Fraction size was 9 ml (3-fold CV).

After completing the loading process a stepwise elution was performed. The first elution step was a buffer containing 1.5 M urea, the second step was performed with a buffer containing 4.5 M urea and the third step was done with buffer containing 6 M urea (100% elution buffer). All fractions were collected

and analyzed in a SDS PAGE (15%), followed by Coomassie staining. The column was washed with MilliQ-water, with column regeneration buffer and stored at 4℃.

3.5 Dialysis

To obtain folded protein and to get rid of small size contaminations, dialysis was performed. Also salt and urea do not provide a stable environment for long term storing of protein, therefore a sodium phosphate buffer solution was used as the final buffer during dialysis.

Material:

- Dialysis membrane (MWCO: 1,000 Da, 1 ml/cm);(Spectra/Por, Breda, NL)
- rTri a 12 solution
- Dialysis buffer 1-4

Protocol:

Total sample volume was 12 ml, therefore 18 cm of dialysis membrane were used. After carefully transferring the sample unto the membrane, it was closed on each side with clamps. Dialyzing was performed using three litres of respective buffer, overnight at 4℃ under constant stirring.The final dialysate was stored at -20℃.

4. Characterisation

4.1 Overview

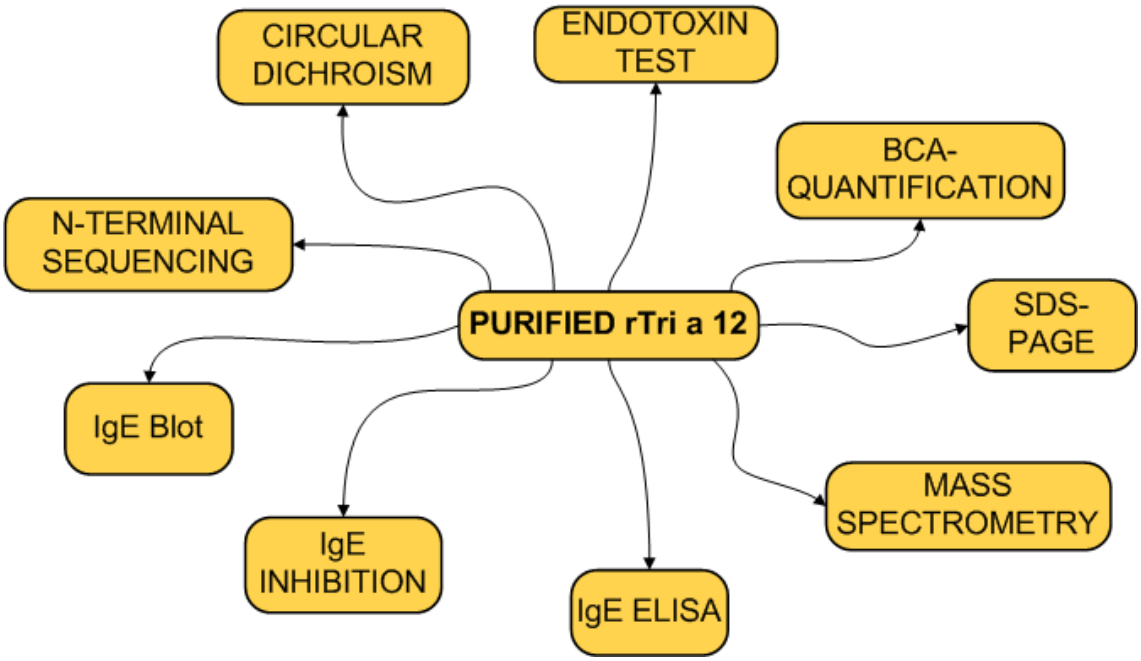


Fig.15: Characterisation overview

4.2 Buffers & Solutions

Reagent C

29.2% acryl amide
0.8% bisacryl amide
dissolved in distilled water and filtered

Lower buffer

1.5 M Tris-HCl, pH 8.8
0.4% SDS
dissolved in distilled water

Upper buffer

0.5 M Tris-HCl, pH 6.8
0.4% SDS
dissolved in distilled water

4x Sample buffer

200 mM Tris-HCl, pH 6.8
300 mM DTT
4% SDS
40% glycerine
0.04% bromophenol blue
dissolved in distilled water

10x Electrophoresis buffer

250 mM Tris pure
192 mM glycine
1% SDS
dissolved in distilled water

CBB

0.125% CBB R-250
50% Methanol
10% HAc
dissolved in distilled water

Destainer

20% Methanol
15% HAc
dissolved in distilled water

10x transfer buffer

250 mM Tris pure
192 mM Glycine
MilliQ water

1x transfer buffer

diluted 10x transfer buffer
20% Methanol

10x TBS

0.5 M Tris-HCl, pH 7.4
1.5 M NaCl
0.5% NaN_3

TBST

1x TBS
0.5% Tween 20
MilliQ water

1x AP buffer

100 mM Tris-HCl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$
MilliQ water

Gold buffer:

42 mM Na_2HPO_4 x 12 H_2O
8 mM NaH_2PO_4 x 2 H_2O
0.05% NaN_3
pH 7.5
0.5% Tween 20

4.3 Characterisation

SDS-PAGE was performed to separate the proteins obtained by affinity chromatography. Following the determination of the proteins' concentration by BCA [78], a LAL test was performed to determine the level of contamination by bacterial endotoxin. A circular dichroism procedure gave information about the structural integrity and the thermostability of rTri a 12. N-terminal sequencing revealed the first five amino acids of the protein, whereas mass spectrometry was performed to obtain the intact mass of rTri a 12. The following experiments were conducted to obtain information about rTri a 12 IgE binding properties: IgE blot, IgE inhibition blot and IgE ELISA.

4.3.1 SDS-PAGE

SDS-PAGE is a method used to separate proteins according to their molecular weight (electrophoresis). SDS is an anionic detergent, which denatures proteins. Its main functions are occupying polypeptide backbones of proteins,

blocking of hydrophobic interactions and disruption of hydrogen bonds. These abilities lead to linearization and negative charging of SDS treated proteins. With all proteins denatured and negatively charged, an electrophoretic separation is feasible.

DTT is added to break up disulfide bonds, which leads to loss of tertiary and quaternary structures.

The most frequently used buffer system for SDS-PAGE is the tris-glycine system described by Laemmli [77].

Material:

- Reagent C
- Upper buffer
- Lower buffer
- 4x sample buffer
- 10x electrophoresis buffer
- Sample
- rBet v 2 (1mg/ml; Biomay, Vienna)
- Protein marker (fig.16; Fermentas, Hilden, Germany)
- CBB
- Destainer

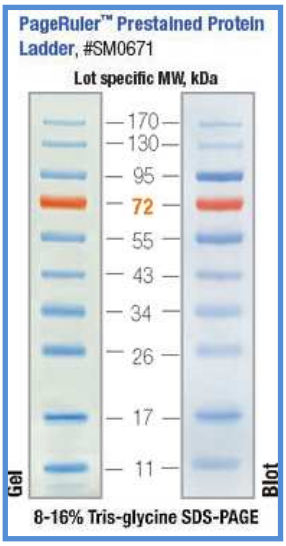


Fig.16: protein marker (Copyright: www.fermentas.com)

Chemicals	Resolving Gel	Stacking Gel
Reagent C	2.5 ml	300 µl
Lower buffer	1.25 ml	-
Upper buffer	-	500 µl
ddH_2O	1.25 ml	1.2 ml
TEMED	2.5 µl	1 µl
10% APS	25 µl	20 µl

Tab. 6: SDS-PAGE gel compositions

Protocol:

All samples were mixed with 4x SB and incubated at 95℃ for 10 minutes. After the samples were loaded into the gel pockets. Alongside a protein marker, rBet v 2 and a lysate from bacteria without pET17b + Tri a 12, the electrophoresis was performed for one hour (25 mA). Visualization was achieved by Coomassie Brilliant Blue R-250 staining for thirty minutes. Destaining was done overnight.

4.3.2 Bicinchoninic acid protein assay

To determine the concentration of rTri a 12 a BCA was performed. The BCA test is based on the reduction of copper ions ($Cu^{++} \rightarrow Cu^{+}$) by proteins (Biuret reaction) [78]. The resulting copper ion is then able to form a complex with two bicinchoninic acid molecules (chelate). Due to the violet colour of the chelate it is possible to measure absorption at 562 nm. More protein results in more Biuret reactions, which gives a stronger signal.

Material:

- BSA Stock solution: 2 mg/ml
- Sodium phosphate buffer, pH7.2.
- rTri a 12 in sodium phosphate buffer solution were diluted 1 : 5 and 1 : 10 in sodium phosphate buffer, pH7.2.
- Nunc MaxiSorp ELISA plate (96 well plate)
- Working reagents A+B (Pierce Protein Research Products, Rockford, IL, USA)
- ELISA READER (molecular devices, CA, USA)

Protocol:

Standards were made according to manufacturer's protocol:

Standard #	Buffer	BSA	concentration
Standard 1	105 µl	15 µl	250 µg/ml
Standard 2	108 µl	12 µl	200 µg/ml
Standard 3	111 µl	9 µl	150 µg/ml
Standard 4	114 µl	6 µl	100 µg/ml
Standard 5	117 µl	3 µl	50 µg/ml
Standard 6	60 µl	-	0 µg/ml

Tab. 7: BCA Standard preparation

After preparing the standards, rTri a 12 sodium phosphate buffer solution was diluted 1 : 5 and 1 : 10. Working reagent was produced by mixing reagent A with reagent B 50 : 1 (A : B). Twenty five micro litres of standards and sample were pipetted (in duplicates) into the wells of a 96-well plate. After the working reagent was added (200 µl) to every well, the plate was covered and incubated at 37℃ for 30 minutes. After five minutes of cooli ng, data were obtained from an ELISA reader at 562 nm.

4.3.3 Limulus amebocyte lysate test

A LAL test was performed to measure the contamination of purified rTri a 12 with bacterial endotoxin. This is a quantitative test for gram-negative bacterial endotoxin [79]. Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the Limulus Amebocyte Lysate (LAL). The activated enzyme then catalyses the splitting of p-nitroaniline (pNA) from colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released is measured photometrically at 405-410 nm after the reaction is stopped. The concentration of endotoxin in a sample is calculated from the absorbance values of a standard solution (tab.8).

Material:

- LAL reagent water
- LAL reagent
- Chromogenic substrate (Ac-Ile-Glu-Ala-Arg-pNA)
- Sample (rTri a 12)
- Stop solution (25% acetic acid diluted in LAL Reagent Water)
- 96 well microplate (Greiner bio-one, Frickenhausen, Germany)

LAL reagent, water and chromogenic substrate were purchased from Cambrex (Walkersville, MD, USA)

Protocol:

Standards were prepared according to manufacturer's protocol:

EU/ml	Endotoxin stock	Standards	LAL reagent water
1	0.1 ml		(x-1)/10 ml
0.5		0.5 ml	0.5 ml
0.25		0.5 ml	1.5 ml
0.1		0.1 ml	0.9 ml

Tab. 8: LAL standards

Escherichia coli endotoxin was reconstituted in 1 ml LAL reagent water and mixed vigorously for at least 15 min. The LAL reagent was dissolved in 1.4 ml LAL reagent water. Samples were diluted in LAL reagent water in optional dilutions.

To measure the endotoxin concentration of purified rTri a 12, a 96 well microtiter plate was heated to 37℃ before it was c oated with 50 µl sample, standards, and blank (LAL reagent water), in double. Ten minutes after addition of 50µl LAL to every well, 100 µl substrate solution (37℃) were added. Six

minutes later 100 µl stop solution were added. Optical density was measured at 405 nm.

4.3.4 Circular Dichroism

Circular dichroism (CD) spectroscopy is a type of absorption spectroscopy that provides information on the structures of biological macromolecules. CD is the difference between the absorption of left and right handed circularly-polarised light and is measured as a function of wavelength. Chiral or asymmetric molecules produce a CD spectrum because they absorb left and right handed polarised light to different extents. Proteins are composed of optically active elements and due to their adoption of different types of three-dimensional structures each type of molecule produces a distinct CD spectrum (fig.17). Ultraviolet or vacuum ultraviolet (190 nm – 300 nm) light is best suited for examining the structures of proteins. These are the regions of the electronic transitions of the peptide backbone and side chains in. Peptide bonds dominate between 170-250 nm (fig.17), whereas aromatic amino acids do so between 250-300 nm. Disulphide bonds are displayed at 250 nm. α-helices produce well defined negative bands at 222 nm, whereas β-sheets display less-defined bands at 217 nm. CD spectroscopy requires only very small amounts of material (< 100 µg) [80].

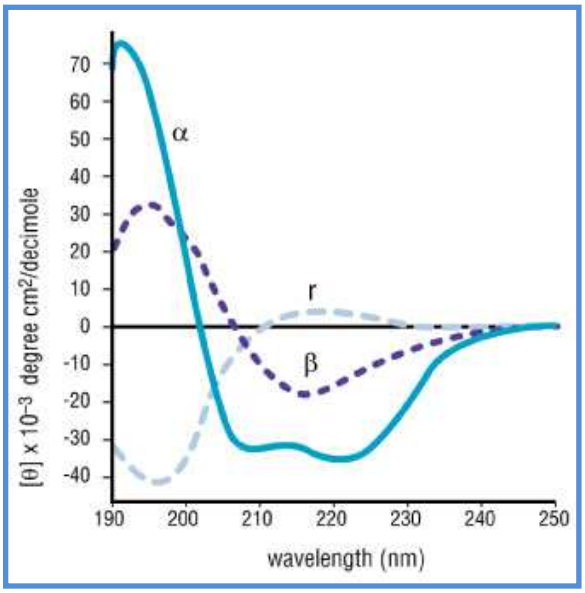


Fig. 17: Example for CD spectrum; structural units marked by α-helix, β-strand and loop (r) (Copyright: New Science Press)

Material:

- purified rTri a 12 a
- JASCO J-810 spectropolarimeter (Jasco, Essex, UK)
- Neslab RTE-11-M temperature control system (Thermo Neslab Inc., Newington, NH, USA)
- quartz cuvette of 0.1 cm path length

Protocol:

Spectra of purified rTri a 12 were recorded in aqueous solutions with a temperature control system equipped spectropolarimeter. Measurements were performed with a quartz cuvette. Far UV CD spectra were recorded at 25℃, with a band width of 1 nm, a response time of 1 second, and a data pitch of 1 nm. Measurement range was between 190 nm and 260 nm and obtained spectra represent an average of 3 consecutive scans. Final spectra were obtained after subtraction of the baseline recorded under identical conditions. All measurements were performed using 5 mM sodium phosphate pH 7.2 and protein concentration of 0.1 µg/µl. Thermal denaturation was monitored in the temperature range of 25℃ to 95℃ at a fixed wavele ngth of 210 nm. The temperature slope was set at 1℃/min and full CD sp ectra were recorded from 25℃ to 95℃ every 10 degrees using the parameters listed above. The reversibility of the unfolding process was determined by monitoring a full CD spectrum after cooling the denatured protein to 25°C.

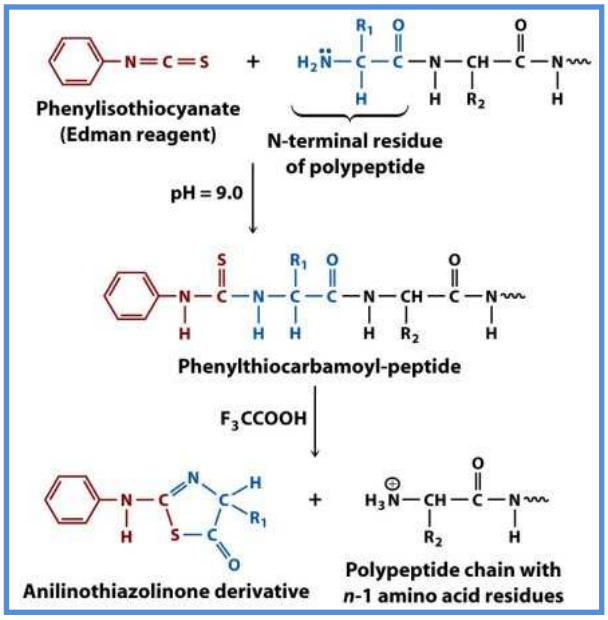


Fig. 18: Edman chemistry (Copyright: University of Montana, division of biological sciences)

4.3.5 N-terminal sequencing

Based on the Pehr Victor Edman degradation, the sequence of the N-terminus of proteins can be deciphered [81]. The amino acid sequence is obtained by labelling and (later) cleaving the amino-terminal residue without disrupting the peptide bonds between the following amino acid residues (fig.18).

The uncharged terminal amino group reacts with phenylisothiocyanate (PITC, Edman reagent) to form a phenylthiocarbamoyl derivative. This derivative is cleaved under mildly acidic conditions. The resulting molecule is called phenylthiohydantoin (PTH)-amino acid, which can be identified by chromatography. This procedure is repeated as often as is required to obtain the N-terminal amino acid sequence. A graphic description of

the Edman degradation is shown in figure X. The coupling of N-terminal amino groups of a protein with PITC (top) forming an open chain thiourea derivative (middle), the N-terminal amino acid is split off as anilinothiazolinone (ATZ) derivative (bottom) with trifluoroacetic acid (F_3CCOOH). This unstable ATZ-derivative is converted into a more stable PTH derivative, which is separated by reversed phase HPLC and identified by comparison with a standard.

Material:

- 50 pmol rTri a 12
- Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, USA)
- Prosorb Cartridge

Protocol:

Fifty pico mol of rTri a 12 were adsorbed on a Prosorb cartridge and analyzed with the amino acid sequencer overnight. The resulting sequence was compared to the database sequence of Tri a 12 by using the BLAST software.

4.3.6 Mass Spectrometry

Experiment was performed by Dr. Peter Briza (University of Salzburg, Department of Molecular Biology).

Material:

- Sample
- Global Ultima Q-Tof instrument (Waters, Manchester, UK)
- Horse heart myoglobin (Sigma, St. Louis, USA)
- Proteoextract trypsin digestion kit (Calbiochem, San Diego, USA)
- RP-HPLC (Waters, Milford, USA)
- [Glu]-fibrinopeptide B (Sigma, St. Louis, USA)
- Jupiter 5µm, C4, 300Å column, 4.6 x 250mm (Phenomenex, Aschaffenburg, Germany)
- guard cartridge, 3 x 4 mm, wide-pore C4, “Security Guard” (Phenomenex, Aschaffenburg, Germany)

Protocol:

For mass determination of intact rTri a 12, the protein was dissolved in 25 % (v/v) acetonitrile in 0.1% (v/v) aqueous formic at a concentration of 1 pmol/µl and directly infused into a Global Ultima Q-Tof instrument with electrospray ionization. The infusion rate was 1 µl/min, using the Waters Nanoflow spray head with nitrogen as desolvation gas and a capillary voltage of 3.4 kV. The instrument was calibrated with horse heart myoglobin. Spectra of multiple charged ions were recorded for 3 minutes in a mass/charge range from 400 to 1900. Spectra were then combined and deconvoluted using the MaxEnt1 software supplied with the instrument. For sequence analysis, 5 µg of rTri a 12 were digested with the Proteoextract trypsin digestion kit. The resulting peptides were separated by capillary RP-HPLC directly coupled to the mass spectrometer (pre-column Waters Nanoease Symmetry300 trap column, separating column Waters Nanoease Atlantis dC18, connected via a 10 port stream select valve). The flow rate was adjusted to 300 nl/min by T-splitting. Peptides were eluted with an acetonitrile gradient (solvent A: 0.1% (v/v) formic acid, 5% (v/v) acetonitrile; solvent B: 0.1% (v/v) formic acid, 95% (v/v) acetonitrile; 5-45% B in 90 min). For sequence analysis, the instrument was calibrated with the fragment ions of [Glu]-fibrinopeptide B. Data were acquired in the Data Directed Analysis (DDA) mode. Survey and fragment spectra were analyzed using the software PLGS version 2.2.5 with automatic and manual data verification. For sequence identification a mini database comprising the trypsin and rTri a 12 sequences and a combined SwissProt/Trembl database were used.

Recombinant Tri a 12 was subjected to RP-HPLC with on-line electrospray-ionization mass spectrometry analysis using a modification of the method of Moreno *et al*. [82]. Recombinant Tri a 12 was dissolved in water and applied to a Jupiter 5µm, C4, 300Å column (4.6 x 250mm) equipped with a guard cartridge. Peptides were eluted with an acetonitrile gradient (solvent A: 0.1% (v/v) trifluoroacetic acid; solvent B: 0.085% (v/v) trifluoroacetic acid, 90% (v/v) acetonitrile, 0.9%-90% solvent B in 15 min). Recombinant Tri a 12 was analysed by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) after in-gel trypsin digestion at the University of Salzburg by Dr. Peter Briza (Department of Molecular Biology).

4.3.7 IgE blot

Blotting: Blotting is a standard technique for transferring proteins (or nucleic acids) from poly acrylamide gels onto a membrane (e.g. nitrocellulose). This is achieved by applying an electric current to the gel-membrane blot sandwich (fig.19).
The negatively charged proteins will migrate onto the nitrocellulose membrane, as the anode attracts negatively charged molecules.

Fig.19: Blotting sandwich

Material:

- 10x transfer buffer
- 1x transfer buffer
- 10x TBS
- TBST
- 1x AP buffer
- NBT (50 mg/ml in 70% DMF/MilliQ water)
- BCIP (25 mg/ml in MilliQ water)
- Whatman paper
- rTri a 12
- nitrocellulose membrane
- IgE antibodies (BD-Biosciences Pharmingen, San Diego, USA)
- profilin allergic person's serum and NHS

Protocol:

rTri a 12 was separated by 15% SDS-PAGE under reducing conditions (for detailed procedure see chapter 4.1). The gel was blotted onto a nitrocellulose membrane: gel, nitrocellulose membrane and whatman paper were soaked with

1x transfer buffer. The blot sandwich was assembled according to figure 19. The blotting procedure was performed for fifty minutes in a Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, USA) at 4℃ and 25 mA. After blotting was accomplished the membrane was dried and cut into strips. After blocking the strips with TBST + 3% milk powder at RT for 30 minutes (followed by washing with TBST), strips were incubated with patients' sera (1:5 diluted in TBST + 0.5% BSA) at 4℃ over n ight. After removing the sera, the strips were washed with TBST for forty minutes (buffer change all 10 minutes), bound IgE was detected by incubation with alkaline phosphatase conjugated anti-human IgE antibodies (1:1000 dilution with TBST + 0.5% BSA) at RT for two hours. After washing with TBST for thirty minutes the membrane was equilibrated with AP buffer. To visualise bound IgE, BCIP (60 µl) and NBT (60µl) were diluted in 10 ml AP buffer. The membrane strips were incubated in this solution for five minutes at room temperature in the dark. The reaction was stopped with deionized water.

4.3.8 IgE Inhibition

In this experiment the IgE binding capacity of purified rTri a 12 and of natural wheat profilin was compared. Natural wheat profilin was extracted from wheat seeds.

Material:

- wheat seed extract
- profilin allergic patients serum, normal human serum (NHS)
- rTri a 12
- rBet v 2 (Biomay, Vienna, Austria)
- Gold buffer
- Milk powder, BSA
- IgE ^{125}I antibody (MedPro, Vienna, Austria)
- Blotting material (see IgE Blot chapter 4.7)

Protocol:

Diluted patient's sera (1:5 in Gold buffer + 0.5% BSA) were incubated with either rTri a 12 (100µg/ml) or rBet v 2 (100 µg/ml) for two hours. Buffer- and NHS served as negative controls.

An extract from ground wheat corn (*T. aestivum cv. Edison*) was separated via SDS PAGE (80 µl wheat seed extract) and blotted onto a nitrocellulose membrane (see chapter 4.3.7). Afterwards the membrane was cut in 4 mm broad strips. The strips were blocked for forty minutes with gold buffer/3% milk powder. After washing with gold buffer for ten minutes, the strips were incubated with diluted serum (see above) overnight at 4℃. Bound IgE was detected with I^{125} labelled anti-human IgE (1:40; diluted in 50 mmol/L sodium phosphate buffer/0.5%Tween 20/1% BSA pH 7.5) and visualised by autoradiography. NHS and buffer controls were included.

4.3.9 IgE ELISA

ELISA is a widely used technique in immunological research. Usually, the assay employs two antibodies. The first antibody recognizes the allergen. The second antibody binds to the first antibody. The second antibody is coupled to an enzyme (e.g. alkaline phosphatase), which catalyzes a colourless, chromogenic substrate to a quantifiable dyed product.

Material:

- MaxiSorp 96 well plate (Nunc; Roskilde, Denmark)
- 25 mM $NaHCO_3$, pH 9.5 (coating buffer)
- TBST, milk powder, BSA
- Wheat allergic patients' sera, Baker asthmatics' sera, and NHS
- Purified rTri a 12
- Monoclonal anti human IgE Antibody (BD-Biosciences Pharmingen, San Diego, USA), alkaline phosphatase labelled
- p-nitrophenyl phosphate tablets, to prepare 5 ml (Sigma, St. Louis, USA)
- ELISA reader (Spectra Max plus 384; Molecular Devices GmbH, Munich, Germany)

Protocol:

Purified rTri a 12 was diluted in 25 mM $NaHCO_3$ (pH 9.6) to a concentration of 10 µg/ml. The 96 well plate was coated with 1 µg protein per well and incubated at 4℃ over night. Following the disposition of the coating solution, the plate was washed three times with TBST. After two hours of blocking with 200 µl TBST+3% milk powder (at room temperature), the plate was washed again with TBST(3x). Sera were diluted in 1:5 TBST+0.5% BSA. The 96-well plate was incubated with sera from wheat allergic patients overnight at 4℃. After removing the sera and washing the plate three times with TBST, alkaline phosphatase-conjugated mouse anti-human IgE antibody (1:1000; TBST+0.5% BSA) was added. After incubating the plate for two hours in the dark (RT) the wells were washed again three times with TBST. The assay was developed with the p-nitrophenyl phosphate substrate. Optical density values from normal human serum samples were obtained and standard deviation calculated. All OD values from serum samples above mean value plus 3x standard deviations were considered positive.

The experiment was repeated with 25 sera from wheat allergic- and baker asthmatic patients (provided by the Floridsdofer Allergiezentrum, Vienna, Austria). An IgE ELISA was also performed with Tri a 19 instead of rTri a 12. Conditions were the same as for the rTri a 12 ELISA (including the sera).

4.3.10 Sera

Sera of wheat allergic patients were selected according to convincing case history, based on their clinical symptoms after ingestion of wheat and subsequent positive skin prick test (SPT) and CAP/RAST. Sera were provided by EUROPREVALL serum bank (n = 8; sera B207-B214) and by Dr. Wolfgang Hemmer from the Floridsdofer Allergiezentrum (n = 25; Vienna, Austria). For detailed information on patient's case history, age and diagnosis results see table 9.

As negative control, normal human sera (NHS) from non-responding, non-allergic individuals were used.

Patient	Age	History	SPT	RAST (class)	CAP (kUA/l)
1594	19	Rhinitis	Wheat Rye Oat	w 2.2 r 2.0	100
1826	42	Rhinitis respiratory symptom	Wheat Rye	w 4.5 r 4.8	700
3115	22	respiratory symptom	neg	w 2.8 r 2.9	50
3238	56	Wheat allergy	Wheat	w 3.4	30
3243	24	respiratory symptom	Wheat Oat	w 2.4	190
3261	76	angiooedema	Wheat Rye	w 2.8	30
3306	43	Baker respiratory symptom	Wheat	w 3.6 r 2.2	1000
3322	18	Confectioner Dermatitis	neg	w 2.1 r 2.0 b 2.0	60
3383	50	Wheat allergy	neg	w 3.3 o 2.0	360
3400	31	Confectioner Rhinitis	nt	w 2.5 r 2.6	260
3407	45	Wheat allergy	neg	w 2.5	290
3422	37	Pizza cook Rhinitis	nt	w 2.0	2000
3486	49	Respiratory symptom	nt	w 2.4 r 2.3	930
3523	22	Wheat allergy	neg	w 2.4	170
3699	24	Confectioner Rhinitis	nt	w 2.3	30
3869	41	Wheat allergy	neg	w 2.2	100
3889	20	Baker Rhinitis	Wheat	w 2.2 r 2.3	600
3901	70	baker	nt	w 2.2 r 2.1	20
3906	26	Wheat allergy	Wheat	w 2.3	240
3956	23	Wheat allergy	neg	w 4.0	80
3962	33	Wheat allergy Exantheme	Wheat	w 2.5	330
3971	52	Wheat allergy	Wheat	w 2.7	470
3980	40	Wheat allergy	Wheat	w 3.0	40
4083	22	Baker	neg	w 2.0 r 3.4	760
4220	33	Wheat allergy	Wheat	w 2.0	140

Tab. 9: patients index; nt = not tested; neg = negative; w = wheat; r = rye; o = oat; b = barley

III. RESULTS

1. Cloning

Total RNA was isolated from 2 days old wheat sprouts. RNA extract was investigated via a photometric scan and agarose gel electrophoresis. Complementary DNA first strand synthesis was performed using total RNA extract and a T25NN primer. This was followed by PCR with gene specific primers. The PCR product was cloned into the cloning vector pCR2.1. Following

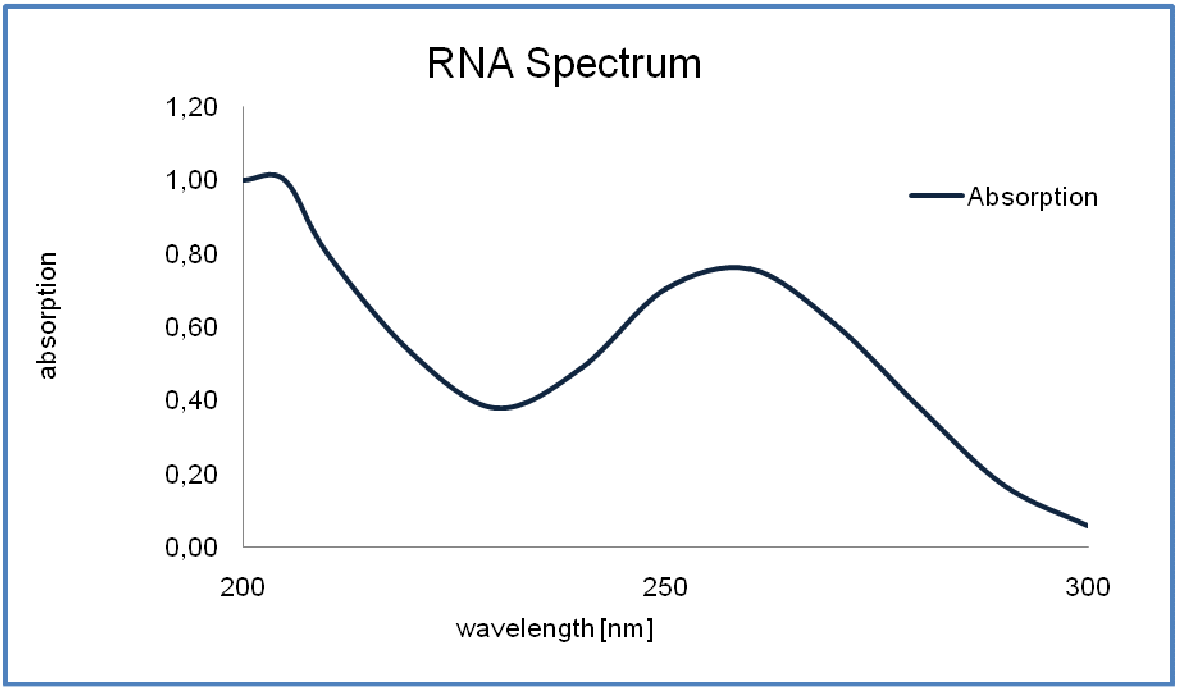


Fig.20: photometric wavelength scan of total RNA extract

transformation of TOP10 *E. coli* cells, single clones were isolated and the respective inserts sequenced (ClustalW, Transseq).

The RNA spectrophotometric scan (Fig.20) and the RNA gel electrophoresis (Fig.21) demonstrated the successful isolation of RNA from two days old wheat sprouts (severed from seed). The spectrum showed the expected peak at 260 nm and the 28S and 18S RNA signals are clearly visible on the RNA agarose gel (fig.21;lane 2).

To assess the quality of the extracted RNA the optical density OD_{260nm}/OD_{280nm}) ratio was determined.

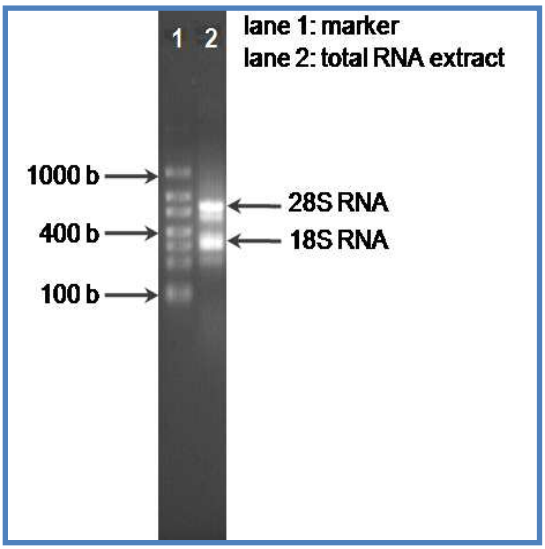


Fig.21: agarose gel electrophoresis of total RNA extract

OD_{260nm} = 0.759

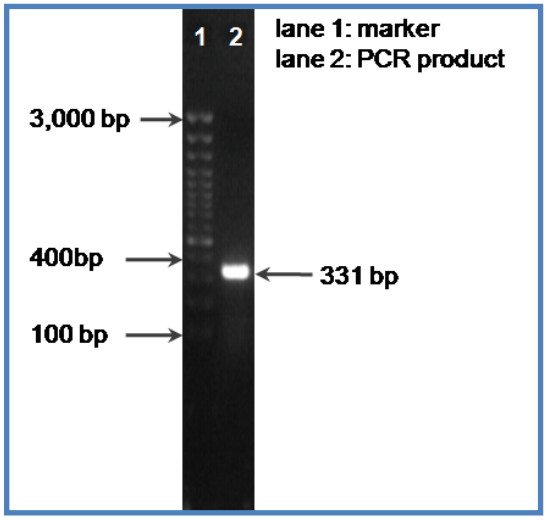
OD_{280nm} = 0.381

OD_{260nm}/OD_{280nm} = **1.986**

To calculate the RNA concentration the following formula was used:

$$RNA\ concentration = OD260nm \times 20\ (dilution\ factor) \times 40(RNA)[69]$$

RNA-concentration = 607.2 µg/mL



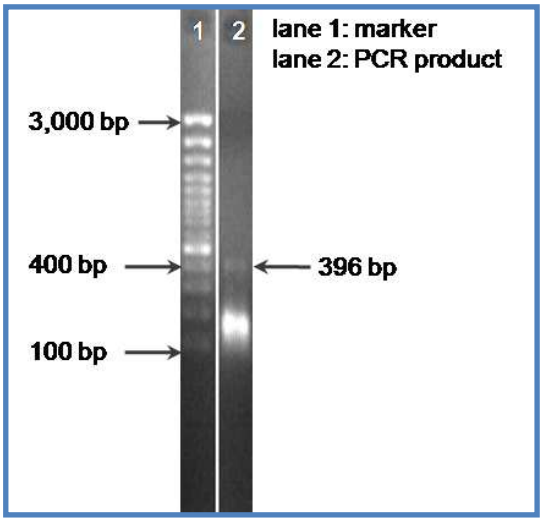


Fig.22: agarose gel electrophoresis of rubisco ophoresis of profilin PCR-product

Complementary DNA synthesis was performed using a T25NN primer.

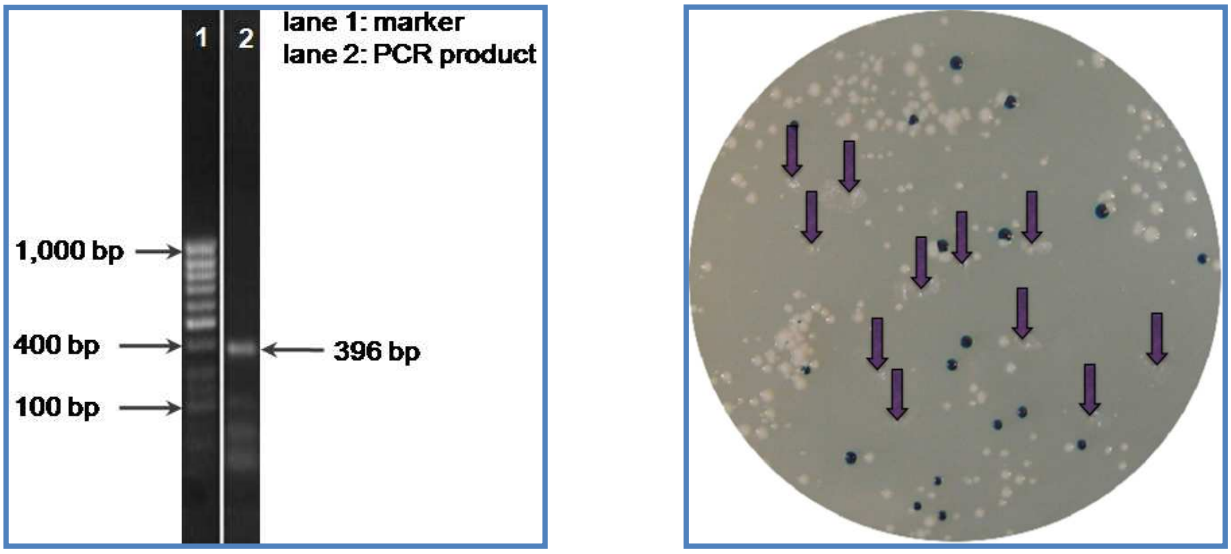


Fig.24: agarose gel electrophoresis of gelexPCR ed TOP10 *E. coli* on

The rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) housekeeping gene served as a control for the integrity of the obtained cDNA. PCR was performed successfully for a rubisco gene fragment of 331 bp (fig.22; lane 2).

PCR was performed to amplify profilin encoding sequences (from cDNA). Primers were designed according to available wheat profilin sequences. With an annealing temperature of 60℃ a weak signal at 396 bp (fig.23; lane 2) was visible in agarose gel electrophoresis.

To obtain a stronger PCR signal the first PCR product was extracted from the agarose gel and then used as a template for another PCR. The annealing temperature was raised by one degree to 61℃ to min imize unspecific amplification of PCR product, resulting in a stronger signal (Fig. 24, lane 2) at 396 bp.

After ligation of the PCR product into the pCR2.1 vector, competent *E. coli* TOP 10 cells were transformed. Single colonies of the transformed *E. coli* were cultivated and later plated onto LB-amp-x-gal medium petri dishes. Following transformation picked colonies (fig.25) were analysed further by plasmid DNA preparation and restriction analysis. Subsequently, DNA sequencing was performed.

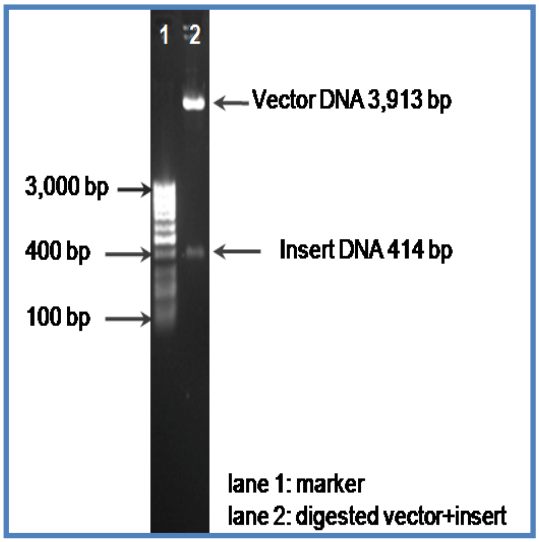


Fig.26: agarose gel electrophoresis of digested cloning vector plus insert

The *EcoRI* digest of the pCR2.1 Vector (including the insert) showed signals of the plasmid (fig.26, lane 2, top) and the insert (fig.26, lane 2, bottom) in the expected range.

1.1 Sequence analysis

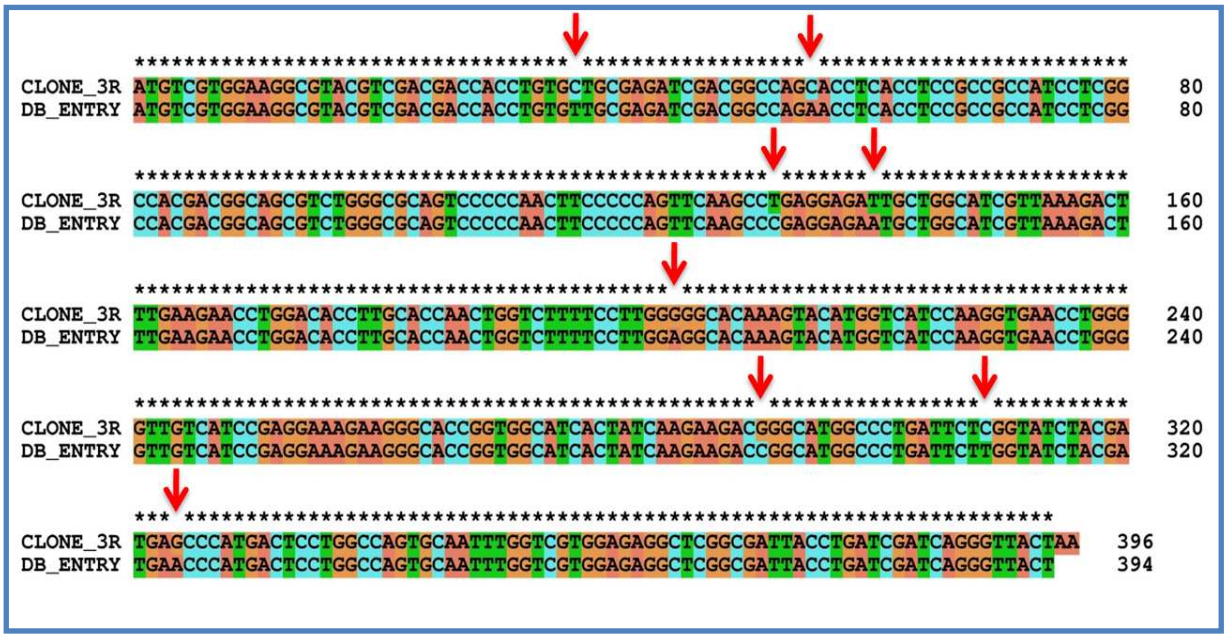


Fig.27: DNA sequence alignment between the obtained sequence (CLONE_3R) and the published wheat profilin sequence (AccNr.: X89827). The sequence of CLONE_3R showed a length of 396 bp, compared to 394 bp of the database sequence. Differences between the two sequences are marked by red arrows.

None of the sequenced clones matched exactly the database entry (homology: 98%, fig.27).There were differences in the DNA sequence at eight positions (fig.27, red arrows), which resulted in two different amino acid residues (fig.28).

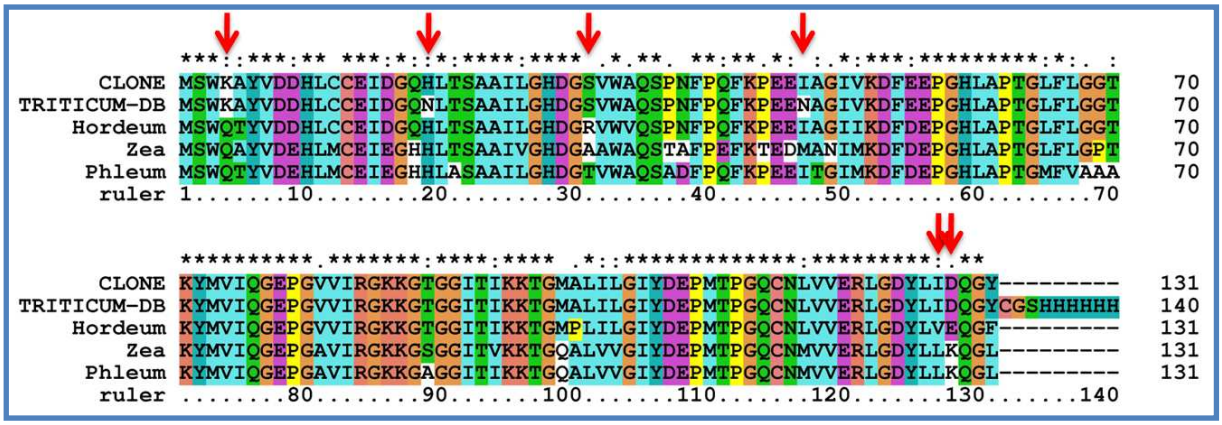


Fig.28: amino acid sequence alignment between the obtained sequence and the published sequences from wheat (Triticum-DB; AccNr.:P49234), barley (Hordeum;AccNr.:AAA92503), maize (zea; AccNr.: NP001105450), and timothy grass (phleum; AccNr.: ABG81290). Differences of interest are marked by arrows.

Compared to the protein database entry of the wheat (*Triticum aestivum*) profilin isoform 3 (there are two differences in the amino acid sequence. At position 20 (fig.28) the clone shows a histidine residue, whereas the database profilin shows an asparagine residue. At position 48 (fig.28) an isoleucin replaces the asparagine of the database sequence.

Profilin amino acid sequences of other, closely related plants, show homology with the clone at positions 20 and 48 (fig.28).

Other differences in the amino acid sequence occurred at position 4, 31, 129 & 130 (fig.28). At position 4 the clone sequence and the sequence of wheat profilin isoform three matched (both lysine), whereas all other sequences showed a glutamine residue. At positions 31, 129 and 130 the clone sequence and the database entry of wheat profilin matched, all other sequences differed from that at these positions.
A theoretical mass of 14,032 kDa and a theoretical pI of 5.04 were determined by Protparam.

2. Expression

PCR was performed with primers including *NdeI* and *EcoRI* restriction sites. After a double digest of profilin insert and pET17b with *NdeI* and *EcoRI,* vector and insert were ligated. After transformation of BL21(DE3) *E. coli* cells, several colonies were picked, sequenced and cultivated. One clone was chosen for a small scale expression experiment.

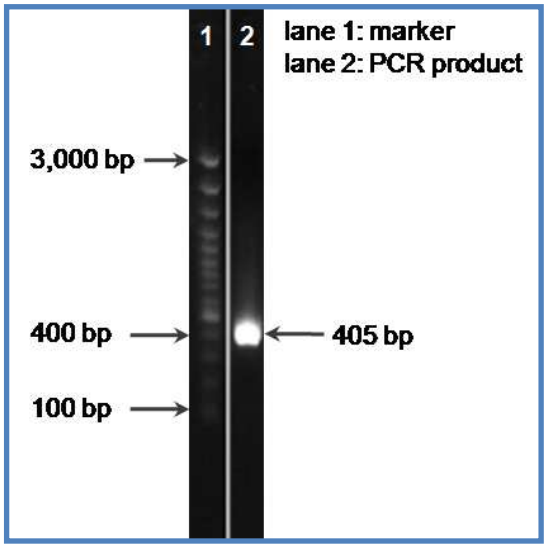


Fig.29: agarose gel electrophoresis of wheat profilin PCR-product (with added restriction sites)

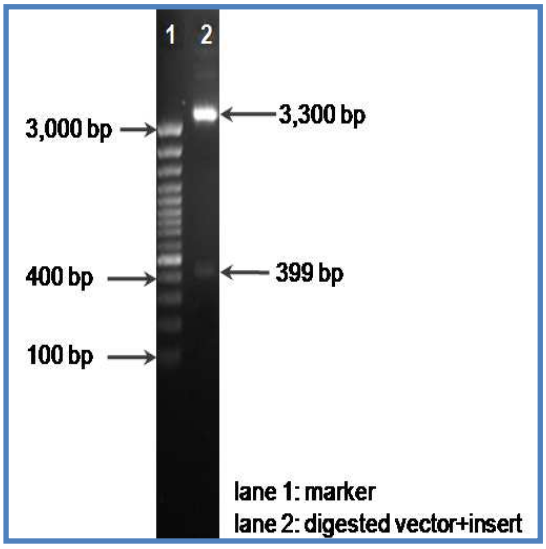


Fig.30: agarose gel electrophoresis of *NdeI/EcoRI* digested expression vector containing insert

NdeI and EcoRI restriction sites were added via PCR to the wheat profilin sequence.The resulting product showed a length of 405 bp (fig.29, lane 2). Digested expression vector/profilin-insert construct showed two bands in agarose gel electrophoresis. One band was visible at 3,300 bp, which represents the digested pET17b vector (fig.30, lane 2, top). The other signal

was visible at 399 bp (fig.30; lane 2, bottom), which represents the digested insert.

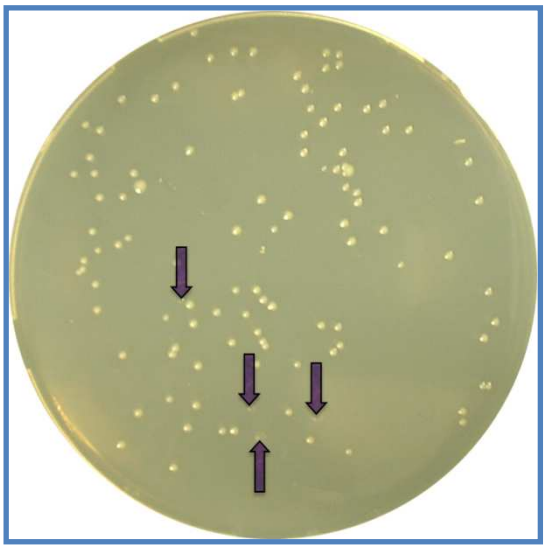


Fig.31: colonies of transformed BL21(DE3) *E. coli* cells on LB-amp plate (picked colonies are marked by arrows)

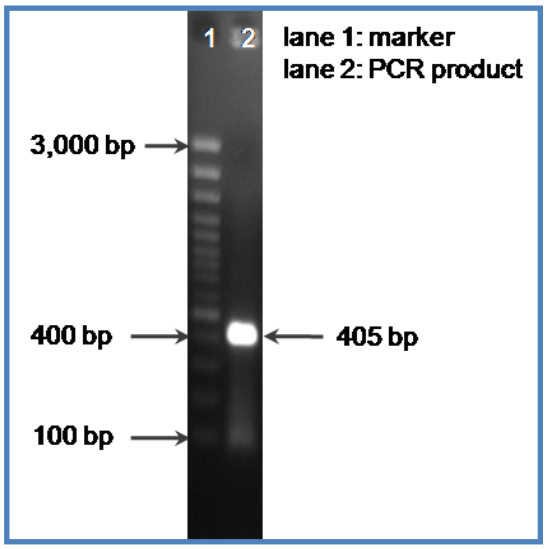


Fig.32: agarose gel electrophoresis of a PCR colony screen, which confirmed the presence of wheat profilin

After transformation of BL21(DE3) *E. coli* several colonies (fig.31; marked by arrows) were picked for further analysis. Agarose gel electrophoresis of PCR colony screens confirmed the presence of wheat profilin (405 bp band) in the genetic makeup of the bacteria of that colony (fig.32).

After the PCR colony screen, a plasmid DNA preparation was performed, followed by the DNA sequencing thereof. Sequence analysis showed that all clones contained the profilin DNA sequence (fig.33).

One clone with the correct sequence is shown in figure 33. The profilin sequence is flanked by the correct restriction sites (fig.33: *NdeI* at the 5' end, and *EcoRI* at the 3' end; in orange) and the pET17b DNA (fig.33; in red).

5'-TTTAAGAAGGAGATATACATATGTCGTGGAAGGCGTACGTCGACGACCACCTGTGCT
GCGAGATCGACGGCCAGCACCTCACCTCCGCCGCCATCCTCGGCCACGACGGCAGCGT
CTGGGCGCAGTCCCCCAACTTCCCCCAGTTCAAGCCTGAGGAGATTGCTGGCATCGTTA
AAGACTTTGAAGAACCTGGACACCTTGCACCAACTGGTCTTTTCCTTGGGGGCACAAAGT
ACATGGTCATCCAAGGTGAACCTGGGGTTGTCATCCGAGGAAAGAAGGGCACCGGTGG
CATCACTATCAAGAAGACGGGCATGGCCCTGATTCTCGGTATCTACGATGAGCCCATGA
CTCCTGGCCAGTGCAATTTGGTCGTGGAGAGGCTCGGCGATTACCTGATCGATCAGGGT
TACTAAGAATTCTGCAGATATCCATCACA - 3'

Fig.33: DNA sequence of clone containing the flanking regions of pET17b in red, *NdeI* and *EcoRI* restriction sites in orange, profilin sequence in black.

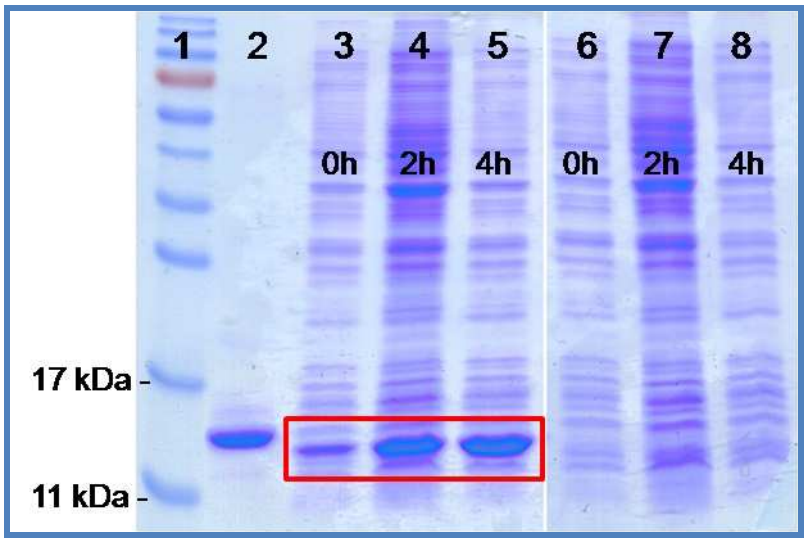


Fig.34: SDS-PAGE (15%) of BL21(DE3) *E. coli* protein extract containing rTri a 12 (lanes 3-5) compared to BL21(DE3) *E. coli* protein extract without rTri a 12 (lanes 6-8). rBet v 2 served as positive control (lane 2)

In SDS-PAGE (15%) a strong band at 14 kDa was detectable in the BL21(DE3) batches, which corresponds to expressed rTri a 12 (lanes 3-5; 0-4h). Even without IPTG a moderate level of expression was achieved (fig.34, lane 3, 0h). After the addition of IPTG, strong bands were visible at 14 kDa after two and four hours (fig.34, lanes 4&5, 2h&4h) post induction. In contrast, bacteria without rTri a 12 showed no comparable signals at 14 kDa (fig.34, lanes 6-8). Birch pollen profilin served as appositive control (fig.34, lane 2).

3. Purification

Following *E. coli* harvest, purification of rTri a12 from the soluble fraction was achieved by affinity chromatography. After dialysis to a suitable storage buffer (5 mM Na_2HPO_4, pH 7.2), SDS-PAGE was performed to check the success of the purification (dialysis).

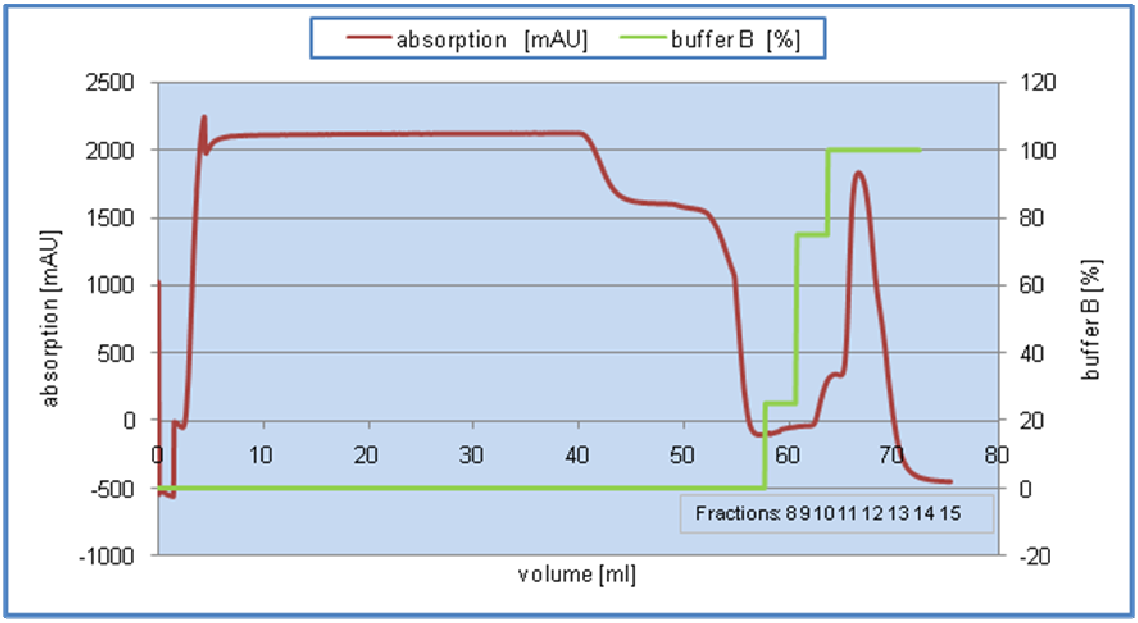


Fig.35: chromatogram of BL21(DE3) *E. coli* expressing rTri a 12, loaded onto a poly-L-proline chromatography column

Elution of rTri a 12 was achieved by a stepwise increase in the urea concentration of the elution buffer (fig.35, buffer B). The highest concentration of urea (buffer B) showed the best results, regarding rTri a 12 elution. Collected fractions (fig. 35, fractions 8-15) were analyzed further by SDS-PAGE (fig.36)

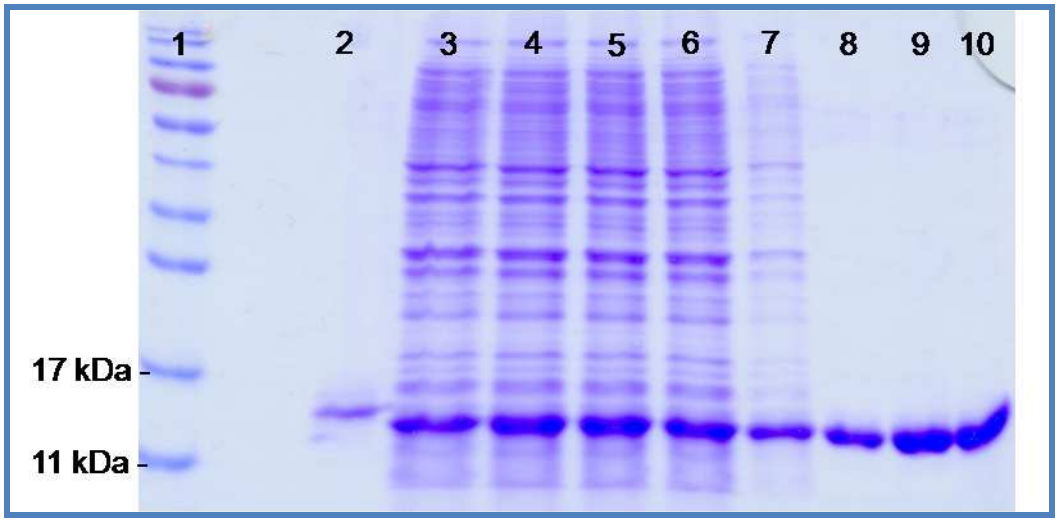


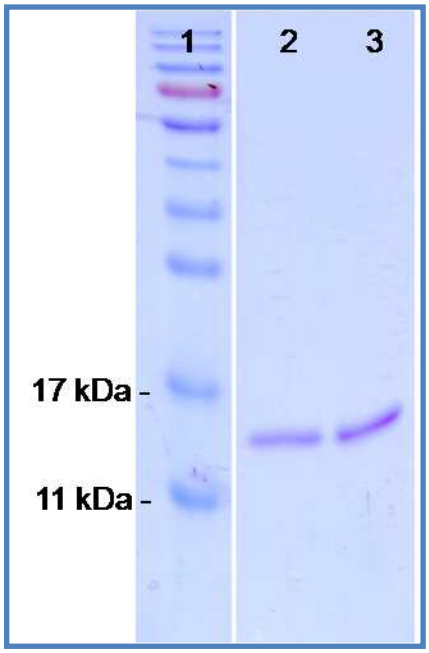
Fig.36: SDS-PAGE (15%) analysis of rTri a12 purification: E.coli lysate was purified by poly-L-proline affinity chromatography.
lane 1: protein marker; lane 2: purified rBet v 2; lane 3-7: flow through fractions; lanes 8-10: elution fractions 11-13.

Fractions 11, 12 and 13 contained a high amount of purified rTri a 12 (fig.36, lanes 8-10). Even the flow through fractions contained rTri a 12 in high quantity, although highly contaminated by bacterial protein (fig.36, lanes 3-7).

Recombinant Bet v 2 (fig.36, lane 2) was used as an indicator for the approximate position of rTri a 12.

4. Characterisation

Protein concentration was determined by BCA. An endotoxin test (LAL) was performed to determine the level of bacterial endotoxin contamination. N-terminal sequencing was performed to confirm the correct sequence of the first five amino acids. Mass spectrometry confirmed the anticipated molecular weight and circular dichroism showed that the protein was folded correctly. To characterize the IgE binding properties of rTri a 12 IgE blot, IgE inhibition (with natural wheat seed extract) and IgE ELISA (rTria 12 & Tri a 19) were performed.



After dialysis of purified rTri a 12, quantification was determined by BCA (for detailed procedure see chapter 4.1). As shown in figure 37 (lanes 2&3), 1.3 and 2.6 µg of purified rTri a 12 (final batch) were analysed by SDS-PAGE (15%). In both lanes a band is visible at 14 kDa.

Fig.37: SDS-PAGE (15%) of defined amounts of rTri a 12 post dialysis. Lane 1: protein marker; lane 2: 1.3 µg rTri a 12; lane 3: 2.6 µg rTri a 12

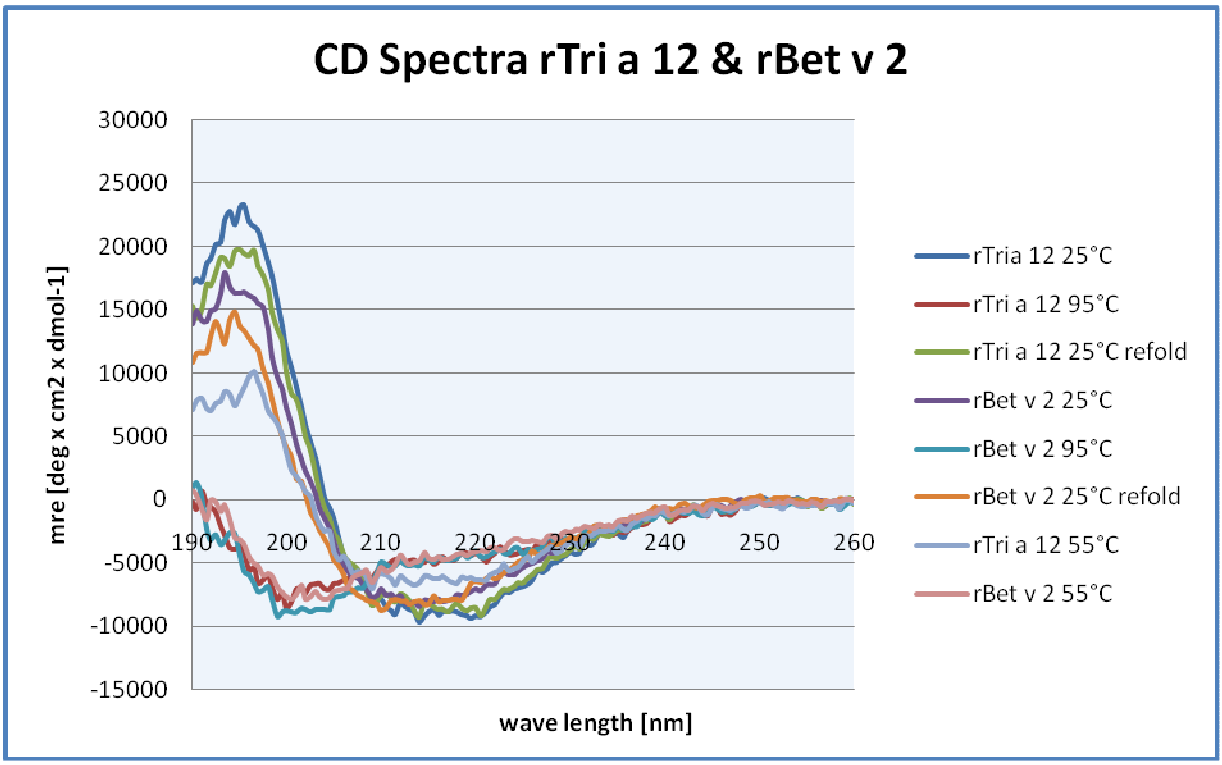


Fig.38: CD spectra of rBet v 2 and rTri a 12 at temperatures ranging from 25℃ to 95℃

Structural integrity was investigated using circular dichroism. rTri a 12 showed intact secondary structures, as did rBet v 2 (birch pollen profilin) which served as a control protein. In contrast to birch pollen profilin, wheat profilin showed a higher degree of stability when exposed to higher temperatures. rBet v 2 already lost its structural cohesion at 55℃, where as rTri a 12 retained some of its structure at the same temperature (fig.38).

N-terminal sequencing revealed the sequence of the first five amino acids (initial methionine cleaved off): (M)**SWKAY**. This sequence matched exactly the published profilin amino acid sequence (fig.28, row 2).

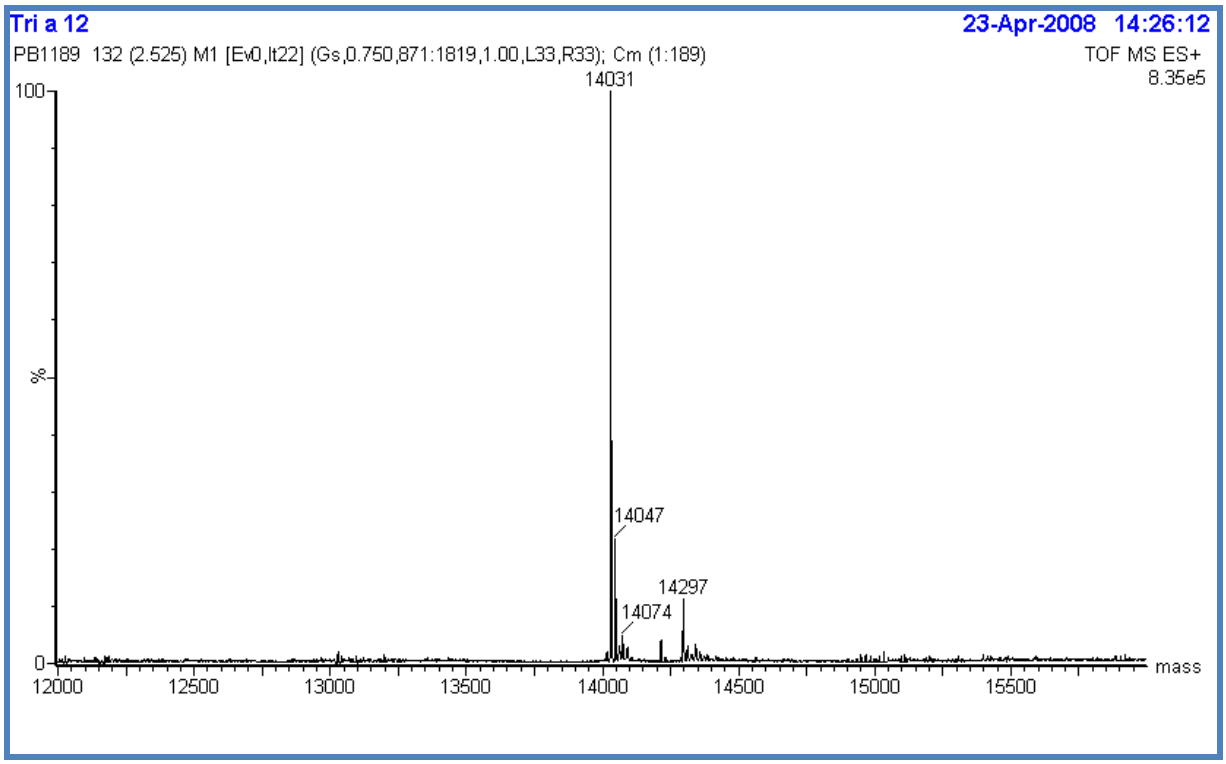


Fig.39: mass spectrogram of rTri a 12

Intact mass was determined by mass spectrometry. Compared to the theoretical mass of 14032 Da the intact mass was determined at 14031 Da (fig.39).
Tryptic digestion was able to confirm 43% of the profilin sequence (fig.40).

MSWKAYVDDHLCCEIDGQHLTSAAILGHDGSVWAQSPNFPQFKPEEIAGIVKDFEE
PGHLAPTGLFLGGTKYMVIQGEPGVVIRGKKGTGGITIKKTGMALILGIYDEPMTPGQ
CNLVVERLGDYLIDQGY

Fig.40: Mass analysis of rTri a 12 after tryptic digestion confirmed the amino acid residues shown with green background.

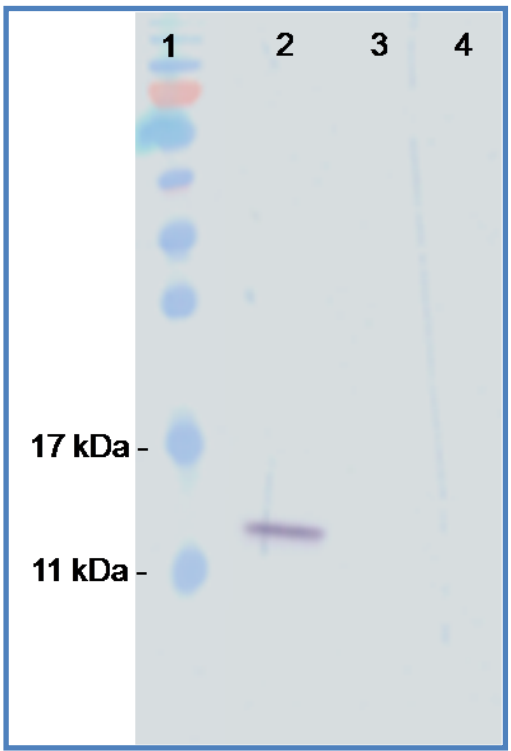


Fig.41: IgE blot of rTri a 12; lane 1: protein maker; rTri a 12 was recognised by specific IgE antibodies of a person allergic to profilin (lane 2). Normal human serum and buffer served as negative controls (lanes 3&4)

An immunoblotting (IgE blot) experiment demonstrated the successful recognition of rTri a 12 by specific IgE from the patient's

serum (specific IgE antibodies) allergic to profilin. NHS and buffer served as negative controls (fig.41).

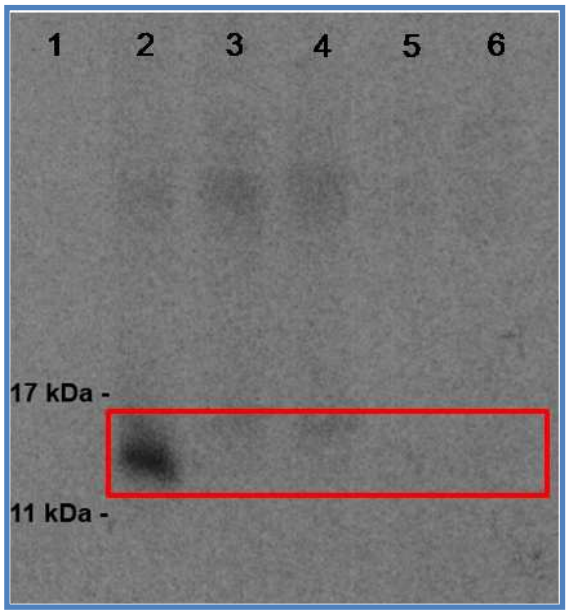


Fig.42: IgE inhibition blot with natural wheat seed extract; Untreated serum (specific IgE) recognised natural wheat profilin (lane 2). Treatment with rTri a 12 successfully inhibited the recognition of natural profilin from wheat seed extract (lane 3). Also, rBet v 2 inhibited the recognition of natural profilin (lane 4). Normal human serum and buffer served as negative controls (lanes 5&6).

IgE inhibition with a natural wheat seed extract was performed to compare the IgE binding capability of recombinant and natural wheat profilin. Recombinant Bet v 2 served as positive control. NHS and buffer served as negative controls. Untreated (by rTri a 12 or rBet v 2) serum was able to recognize natural wheat profilin from the extract (fig.42; lane 2). Preincubating the serum with either rTri a 12 or rBet v 2 resulted in a strong inhibition (fig. 42; lanes 3&4), with rBet v 2 showing a slighty less effective inhibition.

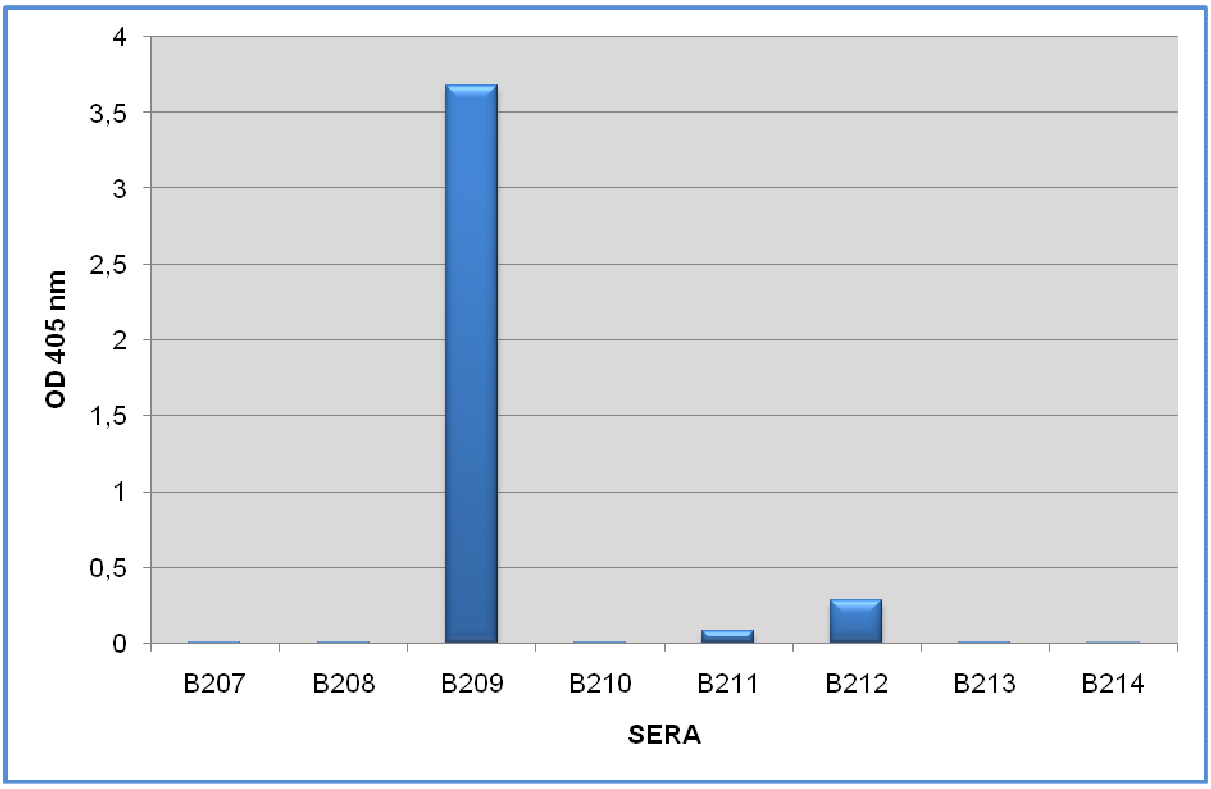


Fig.43: rTri a 12-IgE ELISA with wheat allergic patient's sera

An ELISA was performed to assess the IgE binding capability of recombinant wheat profilin. Eight sera from wheat allergic patients were tested, of which three reacted positively (fig.43; B209, B211, B212; 38% of sera recognised rTri a 12).

To evaluate the role of wheat profilin in bakers' asthma another ELISA was performed with sera from 25 wheat allergic patients (table 9). Eleven of these sera were obtained from persons with allergic reactions against wheat flour. 14 sera where obtained from persons with a known allergic reaction after ingesting wheat containing products.
13 patients were positively tested with SPT, 7 sera reacted negatively, and 5 sera were not tested at all. CAP/RAST was performed for all patients (table 9).

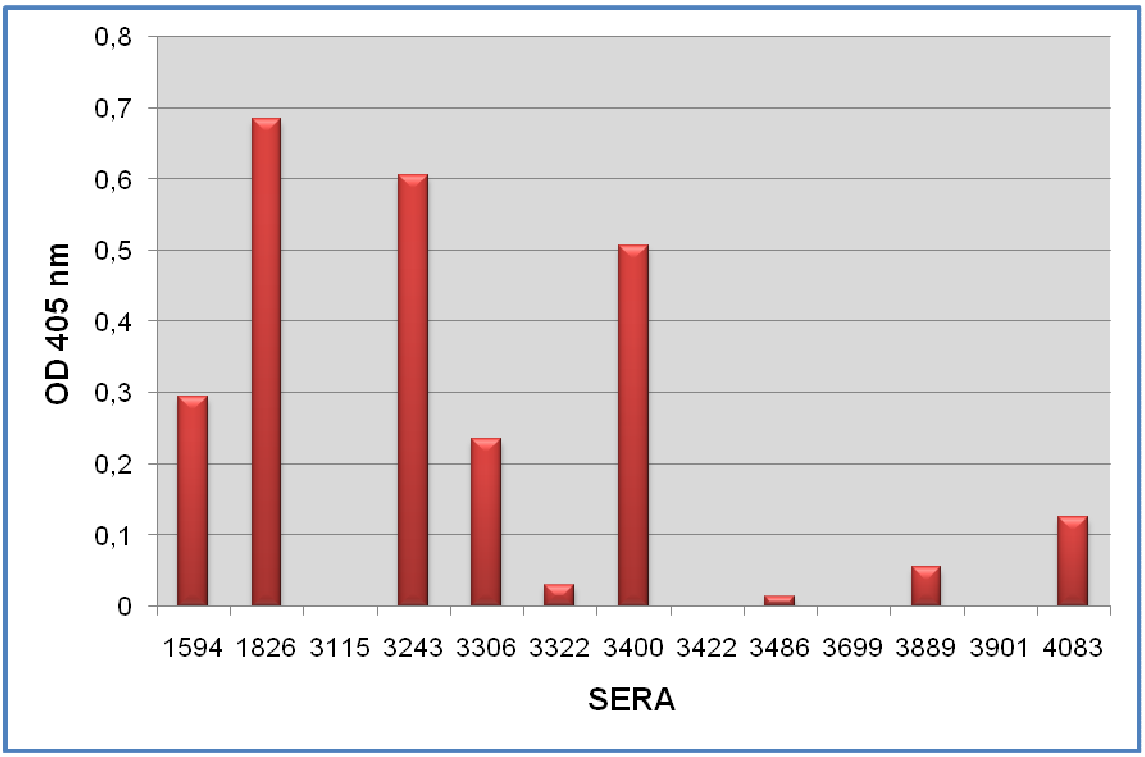


Fig.44: rTri a 12-IgE ELISA with 13 sera of patients suffering from bakers' asthma

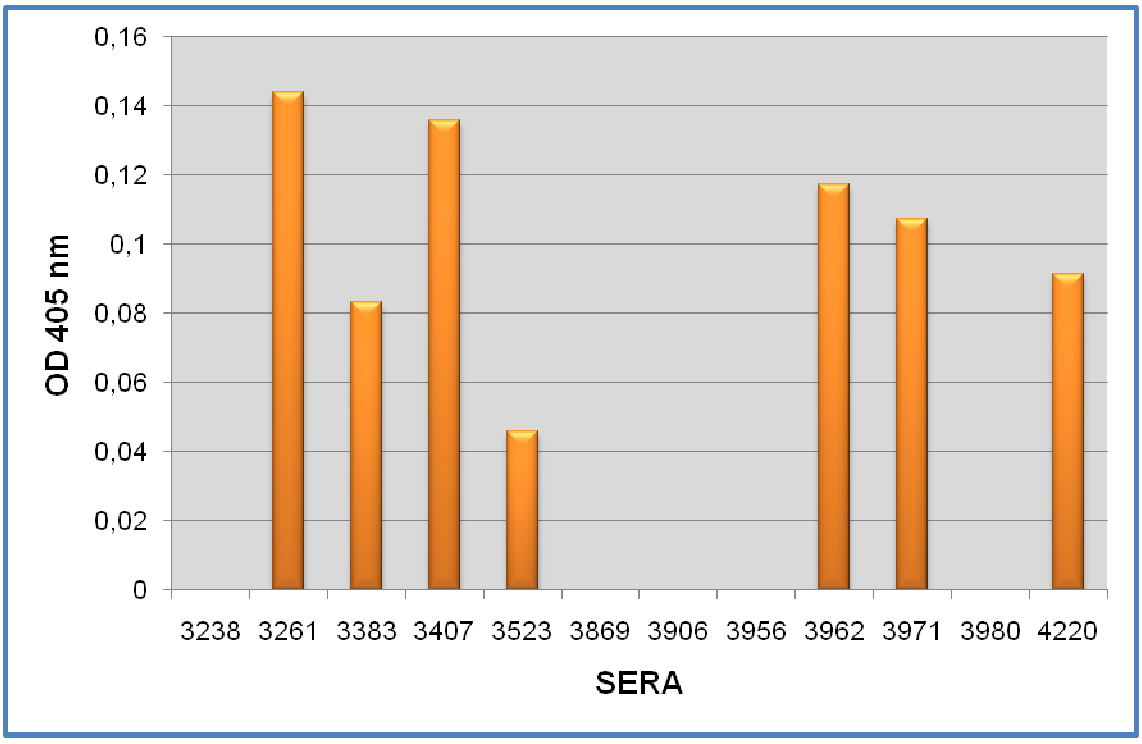


Fig.45: rTri a 12-IgE ELISA with 12 sera of patients suffering from wheat allergy

Sixteen out of 25 sera from persons with wheat related allergic reactions showed wheat profilin specific IgE (64% of patients recognised rTri a 12; fig. 44&45). Of the 25 patients, 13 suffered from bakers' asthma. Nine sera out of 13 showed rTri a 12 specific IgE (fig. 44; 69.23%).12 patients suffered from wheat food allergy. Seven sera out of 12 showed wheat profilin specific IgE (fig.45; 58.3%).

The 25 sera were further investigated by IgE ELISA to screen for patients with an IgE reactivity towards Tri a 19, a major allergen in WDEIA. Only two individuals, both suffering from bakers' asthma, showed specific IgE towards Tri a 19 (fig.46; 3115, 3243).

Patients suffering from bakers' asthma reacted more readily with rTri a 12 than patients suffering from wheat food allergy (69.23% vs. 58.3%). Also, patients suffering from bakers' asthma showed a more powerful IgE reaction compared to wheat food allergics (average OD 405nm for bakers' asthmatics: 0.199; average OD 405nm for wheat food allergics: 0.060).

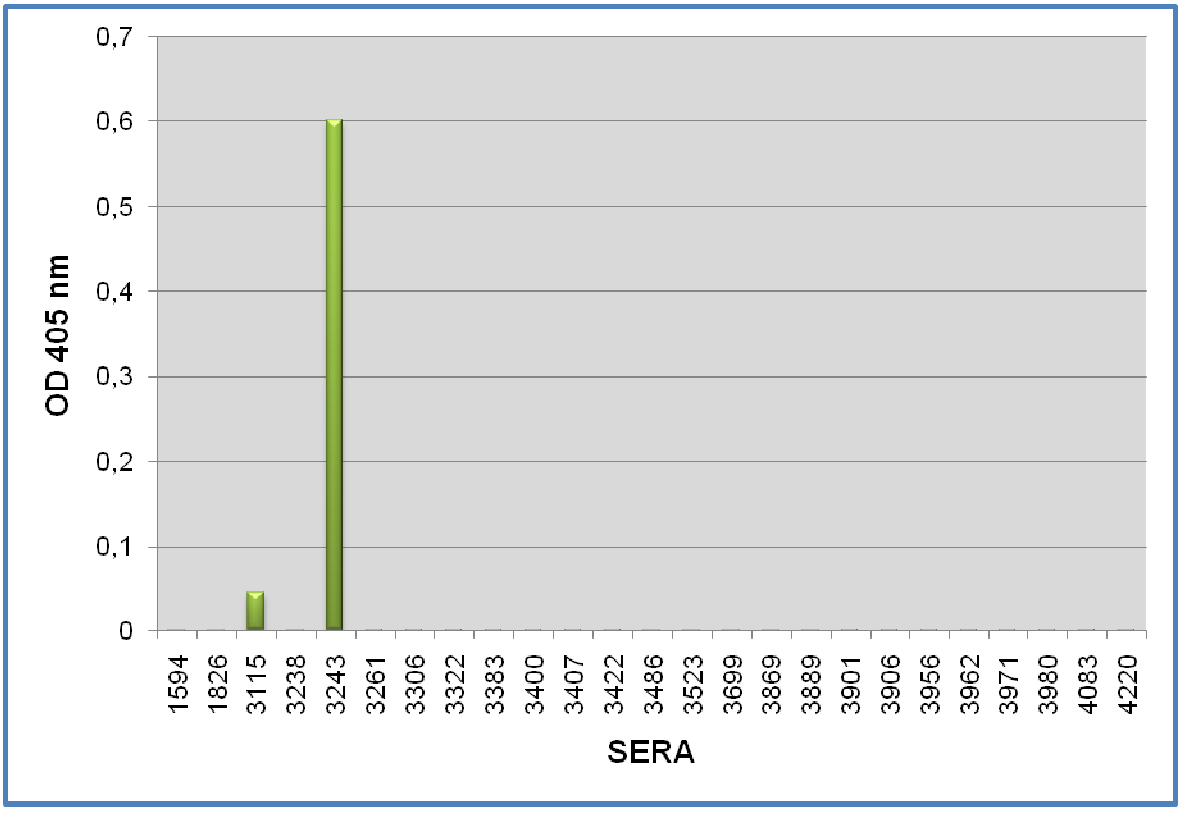


Fig.46: Tri a 19-IgE ELISA with 25 sera of patients suffering from either wheat allergy or bakers' asthma; only 2 patients (3115&3243) suffering from bakers' asthma showed specific IgE reactivity.

IV. DISCUSSION

Allergy diagnosis and specific immunotherapy (SIT) are performed with crude extracts from the offending food. These extracts are often difficult to prepare, possess varying protein content, and during the extraction procedure allergens can be degraded. Therefore results, be it in diagnosis or in therapy, obtained with this extracts are of questionable quality [66]. Patients with strong reactions towards allergens are also at risk when tested with food extracts.

Recombinant allergens are a way to circumvent this multitude of problems. Purified and well defined, they provide the necessary properties to diagnose allergies correctly and in a comparable fashion.

Profilin is an ubiquitous protein, to which 10-20% of allergic patients are sensitized [83]. To what extent these sensitizations result in clinical symptoms is unclear [84]. Profilin is also the main agent of cross sensitization in pollen related food allergies [85, 21].

The aim of the study was to obtain enough recombinant wheat profilin, its characterisation on the molecular level, and to assess its IgE binding capability.

A further goal was to obtain information on the sensitization patterns towards wheat profilin in patients suffering from wheat food allergy and bakers' asthma.

RNA was extracted successfully (fig.20&21). Following cloning and sequencing, the cDNA sequence of Tri a 12 was identified with a coding sequence of 396 bp using PCR with gene specific primers (fig.23&24). The total length of the deduced amino acid sequence consists of 131 amino acid residues (fig.28), a theoretical mass of 14,032 Da, and an isoelectric point of 5.04. Protein alignments of Tri a 12 using ClustalW revealed high identities with profilins of barley, timothy grass, and maize (fig.28). The DNA sequence of the obtained profilin showed deviations from the published sequence at eight positions (fig.27). At the amino acid level two different amino acid residues were observed (fig.28).

Tri a 12 was cloned into the pET17b expression vector (fig.29-33) and expressed as a non fusion protein in BL21(DE3) *E. coli* cells. After lysating the cells using a french press, profilin was purified by affinity chromatography (fig.35). SDS-PAGE followed by Coomassie staining showed a band at the expected height of 14 kDa (fig.36). Recombinant Tri a 12 (rTri a 12) was identified by N-terminal sequencing of five amino acid residues (M)SWKAY (initial methionine was cleaved off). Mass spectrometry revealed an intact mass of 14,031 Da (without the starting methionine;

fig.39). A tryptic digest resulted in a 43% sequence identity (fig.40). Circular dichroism was performed to get information about the structural integrity of Tri a 12. At 55℃ it starts to loose its structural cohesion (fig.38), at 95℃ Tri a 12 is completely denatured. After a cool down to 25℃ recombinant wh eat profilin was able to regain its structural integrity.

To assess the IgE binding qualities of rTri a 12, IgE immunoblotting was performed. Recombinant wheat profilin was recognised by IgE antibodies of a profilin allergic person (fig.41). IgE inhibition with a natural wheat seed extract showed that rTri a 12 possesses a comparable IgE binding capability to natural wheat profilin. Birch pollen profilin was tested alongside rTri a 12 as a positive control (fig.42).

Recombinant wheat profilin's role in wheat food allergy and bakers' asthma was determined by an IgE ELISA with eight sera from the EUROPREVALL serum bank. Three out of eight sera showed a positive signal (37.5%; fig.43). This demonstrated the proposed minor role of profilin in wheat food allergy.

Another series of Ige ELISA experiments with 25 sera provided by the Floridsdorfer Allergiezentrum (tab.9). 13 Sera were from patients suffering from bakers' asthma and 12 sera from patients suffering from wheat food allergy were tested (fig.44+45; 16 out of 25 (64%) showed specific IgE directed against rTri a 12. If the test results are divided into wheat allergics (fig.45) and bakers' asthmatics (fig.44) a clear difference is visible. People suffering from bakers' asthma showed a higher rate of positve signals (9 out of 13; 69.23%) as wheat allergics (7 out of 12;58.3%). Also the reaction was stronger in persons suffering from bakers' asthma, the strongest signal from a person suffering from bakers' asthma reached an OD_{405nm} = of 0.7 (fig.44; serum nr. 1826), whereas the strongest signal visible in wheat allergics was OD_{405nm} = 0.14 (fig.45; serum nr. 3261).

These results indicate a more prominent role of wheat profilin in bakers' asthma, which is a disease caused by inhalant allergens, compared to wheat food allergy, which is caused by ingesting the culprit food.

Tri a 19, a major allergen in wheat dependant excercise induced anaphylaxis (WDEIA), was also tested in an IgE ELISA experiment (fig.46). Only 2 out of 25 sera tested, showed a positive result (fig.46; sera nr. 3115&3243).

In summary it could be demonstrated that rTri a 12 has comparable IgE binding qualities as natural wheat profilin. It was also demonstrated, that wheat profilin is eagerly recognised by a clear majority (fig.44; 72,72%) of patients suffering from

bakers' asthma, whereas a poor reactivity of wheat food allergics was observed (fig.45).

To evaluate rTri a 12 role as a clinically relevant allergen, data regarding pollen sensitisation should be obtained and SPT should be performed to get in vivo test results.

Recombinant wheat profilin (rTri a 12) will be subjected to the allergen library established in the EC-project

EUROPREVALL ("The Prevalence, Cost and Basis of Food Allergy across Europe"). By the use of well-defined individual allergens, component-resolved diagnostics will be established and sera from a large number of food allergic patients (total = 2,000) from numerous clinical centres across Europe will be screened. Thus, comparable data on the prevalence of food allergy in Europe will be feasible in the near future.

V. ABBREVIATIONS

In alphabetical order:

AP	Alkaline phosphatase
APS	Ammonium persulfate solution
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CD	Circular dichroism
cDNA	Complementary deoxyribonucleic acid
CIAP	Calf intestine alkaline phosphatase
DBPCFC	Double blind placebo controlled food challenge
DEPC	Diethylpyrocarbonat
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Di-thio-threitol
EAST	Enzymeallergosorbent test
EDTA	Ethylenediamine tetraacetic acid
ECF-A	Eosinophil chemotactic factor of anaphylaxis
ELISA	Enzyme linked immuno sorbent assay
FA	Formaldehyde agarose
FPLC	Fast protein liquid chromatography
GSP	Gene specific primers
HMW	High molecular weight
IUIS	International Union of Immunological Societies
LB	Lysogeny broth
LTP	Lipid transfer protein
M-MuLV	Moloney murine leukemia virus
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	Messenger ribonucleic acid
NBT	Nitro blue tetrazolium chloride
NHS	Normal human serum
OD	Optical density

PAF	Platelet activation factor
PAGE	Poly acryl amide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SB	Sample buffer
SDS	Sodium dodecyl sulfate
SOC	SOB-Medium + glucose
SPT	Skin prick test
TAQ	*Thermus aquaticus*
TBE	Tris borate EDTA
TBS	Tris buffered saline
WDEIA	Wheat dependant exercise induced anaphylaxis
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

VI. REFERENCES

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt warden, ersuche ich um Meldung bei mir.

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VII. ZUSAMMENFASSUNG

Krankheiten die nach dem Verzehr von Weizenprodukten bzw. nach der Inhalation von Weizenmehl auftreten sind Zöliakie, Weizenallergie und Bäckerasthma.
Eine weitere, jedoch selten auftretende Krankheit, ist die durch den Verzehr von Weizen mit nachfolgender körperlicher Betätigung ausgelöste Anaphylaxie (WDEIA).
Diese vier verschiedenen Krankheitsbilder unterscheiden sich in ihren Symptomen und in ihrer Reaktion auf unterschiedliche Allergene. Ein potentieller Auslöser für Weizenallergie, WDEIA und Bäckerasthma ist Profilin aus Weizen.
Ziel dieser Arbeit war es, Profilin (Tri a 12), ein Protein aus Weizen (*Triticum aestivum*) zu klonieren, als rekombinantes nicht-Fusionsprotein in *E. coli* zu exprimieren, zu reinigen und seine Eigenschaften zu untersuchen.
Gesamt-RNA wurde aus Weizen (Cultivar *Edison*) isoliert und mittels PCR konnte die Tri a 12 cDNA kloniert und sequenziert werden.
Die kodierende Sequenz besteht aus 396 Basenpaaren (inklusive Start/Stoppcodon) und wurde in den Expressionsvektor pET17b kloniert. Für die Expression bei 30℃ wurden *E. coli* BL21(DE3) Zellen verwendet.
Rekombinantes Tri a 12 wurde aus der löslichen Zellfraktion isoliert und mittels Affinitätschromatographie gereinigt. Tri a 12 besteht aus 131 Aminosäuren (inklusive Start-Methionin) und hat eine intakte molekulare Masse von 14,031 Da und einen isoelektrischen Punkt von 5,04.
N-terminales Sequenzieren der ersten fünf Aminosäuren und Massenspektroskopie verifizierten die Sequenz von Weizenprofilin. Zirkuläre Dichroismus Spektroskopie zeigte, dass rTri a 12 nach Reinigung als gefaltetes Protein vorliegt und eine gemischte α/β-Sekundärstruktur besitzt. IgE Bindung wurde mittels IgE Immunoblot und IgE ELISA untersucht. Hierfür wurden Seren von Patienten mit diagnostizierten allergischen Symptomen nach dem Verzehr von Weizenprodukten bzw. Weizenmehlinhalation verwendet.
Tri a 12, ein Mitglied der Profilin-Familie, wurde identifiziert und charakterisiert. Es stellt nun ein weiteres Allergen dar, welches für allergen-spezifische Diagnose und zur Bestimmung der Kreuzreaktivität zwischen verschiedenen Profilinen verwendet werden kann.

VIII. ABSTRACT

There are three major diseases caused by either wheat consumption or wheat flour inhalation: celiac disease, wheat allergy and bakers' asthma.
A fourth disease is the very rare wheat-dependant exercise induced anaphylaxis (WDEIA). These four diseases display different symptoms and are caused by different allergens. One potential causative agent is wheat profilin.

Total RNA from wheat (cultivar *Edison*) was isolated and PCR with gene specific primers was performed. cDNA encoding for Tri a 12 was cloned and sequenced. The coding sequence consisting of 396 bp (including start- and stopcodon) was cloned into the expression-vector pET17b. *E.coli* BL21(DE3) was used as host strain for expression at 30℃. Recombinant Tri a 12 was isolat ed from the soluble cell fraction and purified by affinity chromatography. Tri a 12 has a deduced amino acid sequence of 131 amino acid residues (including initial methionine) and an intact molecular mass of 14, 031 kDa and an isoelectric point of 5.04.
N-terminal sequencing of the first five amino acid residues and mass spectroscopy confirmed the expected wheat profilin sequence.
Circular dichroism spectroscopy showed that rTri a 12 was expressed and purified as a folded protein since it displayed a mixed α/β-secondary structure. IgE binding properties were investigated by IgE immunoblotting and IgE ELISA.
Sera from diagnosed wheat allergic patients were used for the investigation of IgE binding properties.
Tri a 12, a member of the profilin protein family, was identified and characterised. It represents another molecule suitable for component-resolved diagnosis and for defining cross reactivity among profilins.

IX. CURRICULUM VITAE

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