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Examination of allergenic properties of recombinant banana glucanase (*Musa acuminata*)

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Ispitivanje alergenih osobina rekombinantne glukanaze banane (*Musa acuminata*)

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Abstract

Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food. Currently, food allergy is a major health problem, with an estimated prevalence of about 5% in young children and 3-4% in adults, and the prevalence is increasing. Food allergies present a challenge in the field of diagnostics, since the currently employed allergen extracts frequently give rise to poor specificity and sensitivity of tests. Recombinant DNA technology developed in the 1980s has provided the means for producing allergens that are equivalent to their natural counterparts. The proteins are produced as biochemically defined molecules with consistent structural and immunologic properties. Several hundred allergens have been cloned and expressed as recombinant proteins, and they can provide the means for making a very detailed diagnosis of a patient's sensitization profile. The production of recombinant proteins in a well-characterized form has become an important issue in the pharmaceutical industry.

Banana *(Musa acuminata*) is a very popular fruit worldwide. It is available year round and is very present in human nutrition. However, in 1991 symptoms of allergic reaction to banana were described for the first time. The molecular basis of allergic reactions to banana has been ascribed to five proteins, designated Mus a 1 to 5. The Mus a 5 allergen is a β-1, 3-glucanase belonging to the PR-2 family of proteins and is involved not only in plant defense, but also in diverse physiological and developmental processes.

In this thesis banana β-1,3-glucanase was produced as a heterologous protein in *Escherichia coli*. Its gene (GenBank GQ268963) was cloned into a pGEX-4T expression vector as a fusion protein with glutathione-*S*-transferase (GST). BL21 cells transformed with the GST-Mus a 5 construct were employed for production of the protein. The conditions for protein expression were optimized by varying the temperature (25, 30 and 37

°C) and duration of protein expression (3, 6 and 12 h). The level of protein production was analyzed by densitometry of the sodium dodecyl sulfate–polyacrylamide gel (SDS–PAG) after electrophoretic resolution of the respective cell lysates. The optimal protein expression for downstream processing was obtained after 12 h of cell growth at 25 °C upon addition of IPTG. In addition, since a large amount of rGST-Mus a 5 was detected in inclusion bodies we designed an enzyme assay for monitoring expression and solubility of GST fusion proteins targeted to inclusion bodies. The activity of rGST was assayed under denaturing conditions using a colorimetric assay and it was shown that rGST preserves 50% of activity in 4M urea. This assay was further successfully used to monitor rGST-Mus a 5 expression in inclusion bodies.

Recombinant GST-Mus a 5 purified by glutathione affinity chromatography revealed a molecular mass of about 60 kDa. The IgE and IgG reactivity of the rGST-Mus a 5 was confirmed by dot blot and 2D blot analysis with sera of individual patients from subjects with banana allergy and polyclonal rabbit antibodies against banana extract, respectively. These results indicate that purified recombinant glucanase is a potential candidate for banana allergy diagnosis.

Key words: Food allergy, recombinant allergens, banana, β-1, 3- glucanase, GST

Scientific field: Life sciences

Scientific discipline: Biochemistry

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Naslov: Ispitivanje alergenih osobina rekombinantne glukanaze banane (*Musa acuminata*)

Izvod

Alergije na hranu se definišu kao nepoželjne imunološke reakcije koje se javljaju svaki put prilikom konzumiranja određene hranljive namirnice. Danas alergije na hranu predstavljaju značajan zdravstveni problem, i smatra se da oko 5% dece i 3-4% odraslih ljudi pati od ovog poremećaja, a uočena je i tendencija rasta broja alergičnih pacijenata. Istovremeno, čak i samo dijagnostifikovanje alergije na hranu predstavlja izazov, s obzirom da se u tu svrhu trenutno upotrebljavaju alergeni ekstrakti, koji su uzrok slabe specifičnosti i osetljivosti dijagnostičkih testova. Rekombinantna DNK tehnologija je razvijena tokom 80-tih godina prošlog veka i pružila je mogućnost proizvodnje rekombinantnih proteina koji bi bili ekvivalentni prirodnim alergenima. Ovi proteini se proizvode kao biohemijski definisani molekuli sa ujednačenim strukturnim i imunološkim osobinama. Do danas je nekoliko stotina različitih alergena klonirano i eksprimirano u obliku rekombinantnih proteina. Ovi rekombinantni alergeni su omogućili znatno detaljniju dijagnostiku senzitizacionih profila alergičnih pacijenata. Iz ovog razloga je proizvodnja dobro okarakterisanih rekombinantnih proteina postala važno pitanje u farmaceutskoj industriji.

Banana (*Musa acuminata*) je veoma popularno voće širom sveta i prisutno je tokom cele godine. Međutim, 1991. godine su prvi put opisani simptomi alergijske reakcije na ovo voće. Do danas je identifikovano 5 proteina koji čine molekulsku osnovu alergije na banany. Ovi proteini su označeni kao Mus a 1 do 5. Alergen Mus a 5 predstavlja β-1, 3-glukanazu iz PR-2 porodice proteina i učestvuje kako u odbrani biljke, tako i u mnogim drugim raznolikim fiziološkim i razvojnim procesima.

Tokom izrade ove disertacije, β-1, 3-glukanaza je proizvedena kao heterologi protein u bakteriji *Escherichia coli.* Gen glukanaze iz banane (GenBank GQ268963) je ukloniran u ekspresioni vektor pGEX-4T sa glutation-S-transferazom (GST). BL21 ćelije transformisane sa GST-Mus a 5 konstruktom su korišćene za proizvodnju proteina. Uslovi

za ekspresiju proteina su optimizovani variranjem temperature (25, 30 i 37°C) i dužine trajanja proteinske sinteze (3, 6 i 12 h). Nivo proizvodnje proteina je analiziran denzitometrijom SDS–PA gela nakon elektroforetskog razdvajanja ćelijskih lizata. Optimalna proizvodnja proteina za njegovo dalje procesovanje je dobijena gajenjem ćelija nakon dodatka IPTG na 25 °C tokom 12 h. S obzirom da je znatna količina rGST-Mus a 5 detektovana u inkluzionim telima, tokom izrade ove disertacije osmišljen je enzimski esej za proveru ekspresije i solubilnosti GST fuzionih proteina u inkluzionim telima. Aktivnost rGST-a je merena pod denaturišućim uslovima i pokazano je da rGST zadržava 50% svoje aktivnosti u 4M urei. Ovaj esej je zatim uspešno korišćen i za detekciju ekspresije rGST-Mus a 5 u obliku inkluzionih tela.

GST-Mus a 5 prečišćen je afinitetnom hromatografijom sa glutationom i određena je molekulska masa od 60 kDa. IgE i IgG reaktivnost izolovane glukanaze potvrđena je u “dot blot-u” i 2D imunoblotu sa pojedinačnim serumima osoba alergičnih na bananu, i sa poliklonskim zečijim antitelima na ekstrakt banane. Dobijeni rezultati ukazuju da je prečišćena rekombinantna glukanaza dobar potencijalan kandidat za dijagnozu alergije na bananu.

Ključne reči: Alergije na hranu, rekombinantni alergeni, banana, β-1, 3-glukanaza, GST

Naučna oblast: Prirodno-matematičke nauke

Uža naučna oblast: Biohemija

UDK broj: 577.112

Abbreviations

ACN - acetonitrile

TFA - trifluoroacetic acid

GST - glutathion-S-transferase

GSH - glutathione

IPTG - isopropylthio-β-galactoside

cDNA - complementary DNA

tRNA - total RNA

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

RPC - reversed phase chromatography

TEMED - N, N, N′, N′- Tetramethylethylenediamine

APS - ammonium persulfate

IEF - isoelectric focusing

2D-PAGE - two-dimensional gel electrophoresis

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

NBT - nitro blue tetrazolium chloride

BSA - bovine serum albumin

PVDF - polyvinylidene fluoride

CDNB - 1-Chloro-2, 4-dinitrobenzene

ELISA - Enzyme-Linked Immunosorbent Assay

IgE - immunoglobulin E

GST - glutathione-*S*-transferase

SPT – skin prick test

FcεRI - high-affinity IgE receptor

MHC - major histocompatibility complex

Contents

1. Introduction

1.1. Immune system and allergy

The immune system must distinguish between innocuous and pathological antigens to prevent unnecessary and self-destructive immune responses (Akdis, 2004). When the immune system exerts an inappropriate response that induces tissue damage, a condition is marked as hypersensitivity (Roith, 2006). Typically, hypersensitivities are classified into four different types (Marc, 2009). Nowadays, the most common form of hypersensitivity is allergic response, which is termed Type 1 or immediate hypersensitivity (Roith, 2006).

Allergic reactions are symptomatic responses to a normally innocuous environmental antigen (Jacquet, 2009). The interaction of environmental and genetic factors can lead to the development of atopic disorders in some individuals, but not in others, following allergen exposure (Taylor, 2005). During the last 30 years, there has been a considerable increase in the incidence of atopic diseases in industrialized societies and it has been estimated that at least 20% of the population worldwide is susceptible to an atopic disease (Miescher, 2002).

The generation of the allergen-specific $CD4^+$ T helper (Th) cells provides the initial event responsible for the development of an allergic disease. The current view is that naïve T cells, under the influence of IL-4 differentiate into Th2 cells when they are activated by antigen-presenting cells (APCs) (Taylor, 2005). The secretion of IL-4, IL-5, IL-9 and IL-13 and probably some other recently identified cytokines, such as IL-25, IL-31, and IL-33 by Th2 cells mediate the allergen-specific Th2 response characterized by the production of allergen-specific IgE antibodies (Jacquet, 2009). IL-4 and IL-13 induce immunoglobulin

(Ig) class switching in B cells, leading to excessive IgE production with subsequent mast cell activation and mediator release. IL-5 contributes to the development of eosinophilic inflammation and enhances mucus production of the airway epithelia (Gerhold, 2007).
Diagnostic tests for food allergy frequently have poor specificity and sensitivity (Palacin et al., 2008). Therefore replacement of allergen extracts with a panel of IgE reactive molecules from particular allergen source is a promising strategy for the improvement of allergy diagnosis.

The molecular basis of banana allergy has been related to five IUIS (International Union of Immunological Societies, www.allergen.org) nominated allergens: profilin (Mus a 1), class I chitinase (Mus a 2), non-specific lipid transfer protein (Mus a 3), thaumatin-like protein (Mus a 4) and beta-1,3-glucanase (Mus a 5). Beta-1,3-glucanase belongs to the PR-2 family of proteins and is involved in various physiological and developmental processes (Peumans et al., 2000).

Unlimited availability of recombinant proteins have advantage over natural molecules regarding their unique feature such as purity and homogeneity, providing batch-to-batch consistency not achievable with natural molecule. The aim of this work was molecular characterization of recombinantly produced Mus a 5, in terms of primary structure, IgG and IgE reactivitivity for employment in component-resolved allergy diagnosis.

1.1.1. Mechanisms of immune tolerance to allergens

There has been a long-term belief, as well as experimental evidence, that the immune system has inducible peripheral mechanisms of immune tolerance to allergens (Akdis, 2004). Dendritic cells (DCs) are cornerstones in the activation of the innate and adaptive immune system, as well as in the maintenance of peripheral tolerance (Soyer, 2013).

Many studies report that dendritic cells, the most powerful antigen presenting cells (APCs), orchestrate the induction of Th1, Th2, Th9, Th17, Th22 or Treg responses (Jacquet, 2009; Soyer, 2013). Many factors are decisive in the process of Th polarization including the type

of antigen, the presence of microbial compounds, the route of exposure and the genetic background of the host. All these factors will act at the level of DC to induce signals triggering Th subset cell differentiation (Jacquet, 2009). During a healthy immune response, monocytes differentiate into tolerogenic DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL18, and prime T regulatory cells (Treg) to induce tolerance (Soyer, 2013).

Indeed, Treg cells have been clearly implicated as potent inducers of a non-responsive state in several immune-mediated pathologies like autoimmunity and allergy. It has been shown, in allergy, that Treg cells can be transferred conferring specific tolerance to subsequent challenges with an allergen. In addition, depletion of the regulatory T cells can have a detrimental effect in allergic airway hyperreactivity (Agua-Doce, 2012).The differentiation of T-cell clones to Th1, Th2, Th9, Th17, Th22 and Treg cells is a complex event (Figure 1).

Differentiation to the Th1 phenotype is induced by IL-12 and IFN-γ, which are secreted by DCs, and lead to the activation of signal transducers and activators of transcription (STAT) 1 and STAT4. STAT1 stimulates expression of T-box transcription factor (T-bet), the main transcriptional factor of Th1 differentiation. Th2 cell development is promoted by IL-4, which signals through activation of STAT6 after engagement of IL-4R with GATA-3. GATA-3 is the main transcriptional factor of Th2 cells, and is important for Th2 cytokine production (Soyer, 2013).

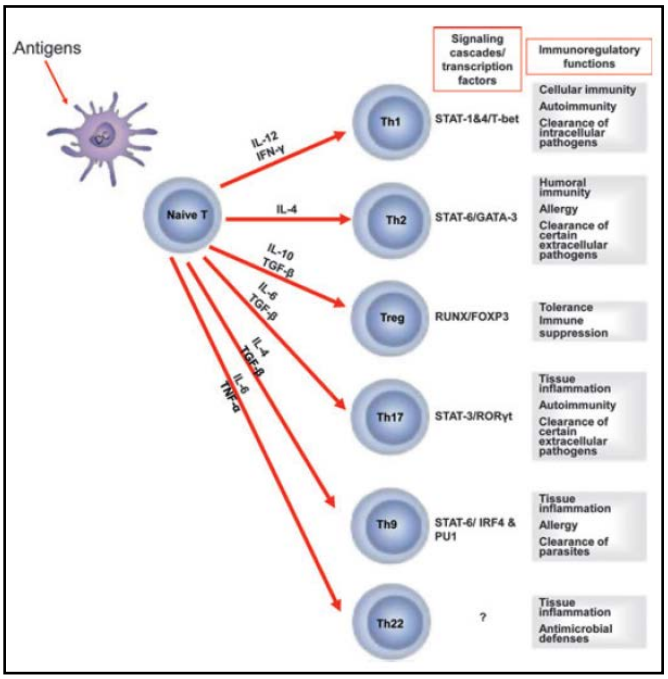

Figure 1. Differentiation of T cells into effector CD4$^+$ T cell lineages, Th1, Th2, Th17, and Treg cells is stimulated by the presentation of antigen by dendritic cells (DCs) to naïve T cells. Certain cytokines and other cofactors released from DCs and other cells induce the differentiation of naïve T cells, which involves the activation of distinct signaling cascades and transcription factors. Each effectors T-cell subset has unique functions in immune regulation (Soyer, 2013).

Transcription factors are key molecules involved in the determination of Th1/Th2 balance. The shift toward Th2 determines allergic response, while Th1 cytokines are supposed to suppress these reactions. STAT6 plays an important role in signal transduction pathway used by IL-4 and IL-13, as well as in class switching to IgE and Th2 cytokine production (Kumar, 2012).

T regulatory cells are thought to mediate the phenomenon of antigen-specific tolerance (Gerhold, 2007). Treg cells can be divided into three major subsets (Kumar, 2012). Natural Tregs (nTregs) develop in the thymus, express constitutively CD25 (IL-2Rα) and the transcription factor fork head box protein 3 (Foxp3), and act in an antigen-independent manner immunosuppresively (Gerhold, 2007; Chinen, 2004). In the periphery, a group of

adaptive antigen-specific Tregs (aTregs) develop from still unknown ($CD25^-$) precursor cells in response to foreign antigens. Tregs of this type become $CD25^+$ during their development; but only some of them express Foxp3 (Gerhold, 2007).

Type 1 of regulatory cells (Tr1), also known as inducible Treg cells, are defined by their ability to produce high levels of IL-10 and transforming growth factor-β (TGF-β)(Agua-Doce, 2012). Tr1 cells are induced by high and increasing doses of allergens. Allergen-specific Th1 and Th2 responses are down-regulated by these Tr1 cells. Th3 cells, similar to Tr1 cells, are inducible upon activation with an appropriate antigen or anti-CD3 antibody and produce high levels of TGF-β, with variable amounts of IL-4 and IL-10 (Taylor, 2005; Braga, 2012).

In healthy individuals, the immune response to allergens can be regarded as specific unresponsiveness of T cells or active peripheral tolerance induction by subsets of Treg cells (Soyer, 2013). There are several major mechanisms, by which Treg cells contribute to the control of allergen-specific immune responses (Figure 2) (Braga, 2012). IL-10 down-regulates the expression of MHCII and other co-stimulatory molecules on monocytes/macrophages and dendritic cells. That way, Treg cells suppress antigen-presenting cells that support the generation of effectors Th2 and Th1 cells (Braga, 2012). IL-10 is also a suppressor cytokine of allergen-induced proliferation of Th1 and Th2 cells (Taylor, 2005).

Some studies showed that TGF-β, produced by Treg cells, can directly inhibit GATA3 expression, thus impairing Th2 differentiation. Since the Th2 response is impaired, the production of IL-4 is diminished, and this has a direct impact on B-cell class switch, preventing IgE and favoring IgA production (Agua-Doce, 2012). Treg cells also have regulatory function on B cells by suppression of allergen-specific IgE and induction of IgG4, IgA or both. IL-10 has two major effects on B cells: it decreases ε-transcript expression and therefore IgE production, and enhances g4 transcript expression and IL-4-induced IgG4 production (Braga, 2012). Furthermore, Treg cells also prevent allergic

inflammation through action on mast cells, basophiles and eosinophiles. Treg cells suppress FcεRI-dependent mast cell degranulation through cell-cell contact (Soyer, 2013).

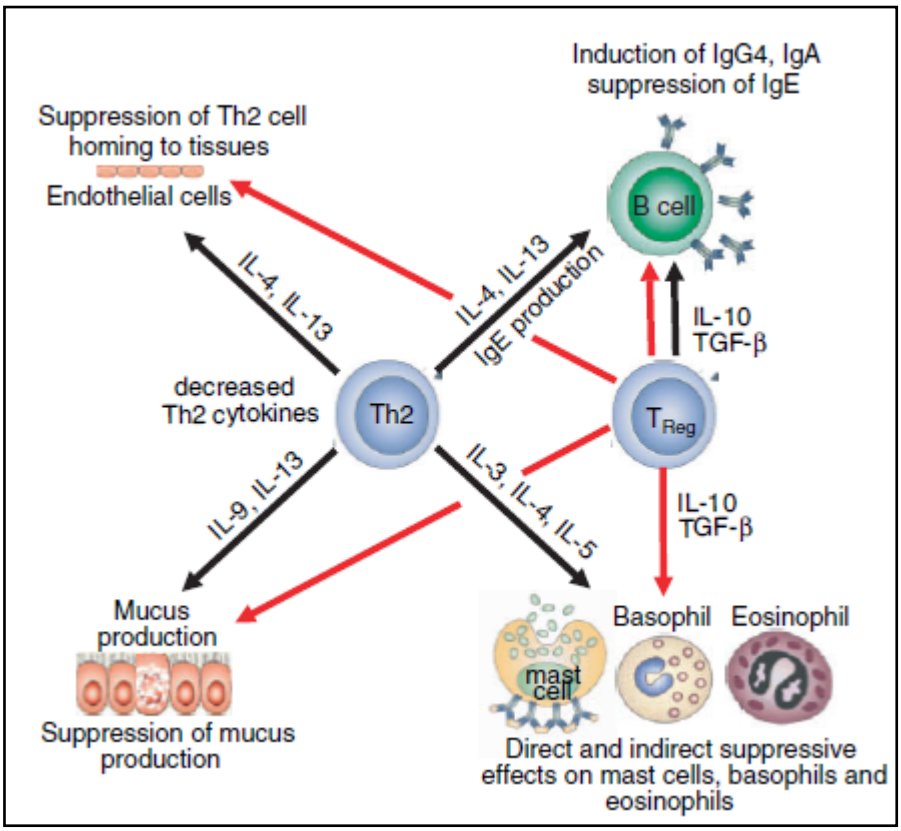

Figure 2. Suppression of Th2 cell-mediated features of allergic inflammation by Treg cells. Treg cells utilize multiple suppressor factors to regulate undesired activity of effector Th2 cells. IL-10 and TGF-β suppress IgE production and induce the non-inflammatory immunoglobulin isotypes IgG4 and IgA, respectively. Furthermore, these two cytokines directly suppress allergic inflammation induced by effector cells such as mast cells, basophils and eosinophils. In addition, Th2 cells are suppressed by Treg cells and can therefore no longer provide cytokines such as IL-3, IL-4, IL-5, IL-9 and IL-13. These cytokines are required for the differentiation, survival and activity of mast cells, basophils, eosinophils and mucus-producing cells, as well as for the tissue homing of Th2 cells (red line indicates suppression, black line indicates stimulation) (Taylor, 2005).

1.1.2. Pathogenesis and mechanisms underlying the allergic responses

The defining hallmark of an atopic disease is the production of specific IgE antibodies against allergens (Miescher, 2002). Allergen sensitization and the production of IgE is the first step in the development of an allergic disease (Bochner, 2004). Cytokines produced by allergen-specific Th2 play a central role in the regulation of the IgE response. IL-4 and IL-13 are essential for the production of IgE antibodies (Miescher, 2002).

Upon primary exposure, food allergens are captured by antigen presenting cells, especially dendritic cells (DCs). The allergens are internalized by DCs due to receptor-mediated endocytosis process, macropinocytosis, or phagocytosis. The allergens are detected by ubiquitin, and these ubiquitinized allergens move to the proteosomal complex and ultimately get degraded to peptide fragments. The degraded peptide fragments are presented by major histocompatibility complex class II (MHC II) and recognized by naïve $CD4^+$ T cells. These $CD4^+$ T cells differentiate into Th2 cells, especially in the presence of adequate amount of IL-4 (Kumar, 2012; Mills, 2003).

Activation of the allergen-specific Th2 cells leads to expression of IL-4 and IL-13, which induce class switching to IgE (Stone, 2010). Isotype switching to IgE requires 2 signals. The first signal is provided by IL-4 and IL-13 and their receptors on B cells. Binding of IL-4 and IL-13 to their receptors initiates a signaling cascade that results in the translocation to the nucleus of signal transducer and activator of transcription 6 molecules (STAT6) which becomes an active dimer after phosphorylation (Miescher,2002). STAT6 activates transcription at the IgE isotype-specific, Sε switch region (Stone, 2010).

In addition to the switch signal provided by IL-4 and IL-13 a second signal for efficient activation of B cell is required. The second signal is an interaction of CD40 on B cells and CD40-ligand on Th2 cells (Miescher, 2002). After activation by allergens, the subset of $CD4^+$ T lymphocytes produces a spectrum of cytokines like IL-4, IL-5, IL-6, IL-9, Il-10 and IL-13 that cause the increase of serum IgE levels and induction of eosinophiles, which,

in turn, mediate the clinical symptoms. The IgE immunoglobulin attaches to the FcεRI of mast cells or basophils (Kumar, 2012). The FcεRI is a multimeric cell surface receptor composed of α-, β- and two γ-chains and exists in two isoforms. The α- and β- subunits are found to be involved in attachment of IgE, while γ- subunits are involved in phosphorylation process (Miescher, 2002).

Upon secondary exposure to the allergen, IgE activates the allergic cascade effectively via the high-affinity receptor, FcεRI, on blood- and tissue cells (Kumar, 2012). The cross-linking of FcεRI molecules initiates the association of the receptor with lipid rafts containing Lyn, a membrane anchored member of the Src family of protein tyrosine kinases. Lyn kinase trans-phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of FcεRI through the Src homology 2 (SH2) domain. This ITAM phosphorylation induces fully activation of Syk, which initiates a series of events such as generation of inositol triphosphate (IP_3) (Saito, 2013). IP_3 causes Ca^{2+} release from intracellular repository, including endoplasmic reticulum, which ultimately leads to mast cell degranulation (Kumar, 2012).

Cross-linking of IgE-FcεRI complexes on the mast-cell surface by allergens leads, within minutes, to the so-called ``early phase`` of the allergic reaction, which involves mast-cell degranulation and the synthesis of lipid mediators (Gould, 2008). Prostaglandins, cytokines, leukotrienes, histamine, heparin, platelet-activation factor (PAF), eosinophil chemotactic factor of anaphylaxis, proteolytic enzymes and other mediators are secreted by degranulated mast cells or basophils. These mediators may cause smooth muscle dilatation, capillary disruption, local swelling and other symptoms. In some individuals, those reactions may occur very vigorously leading to anaphylaxis or sometimes even death (Kumar, 2012). A brief mechanistic pathway of allergies is given in Figure 3.

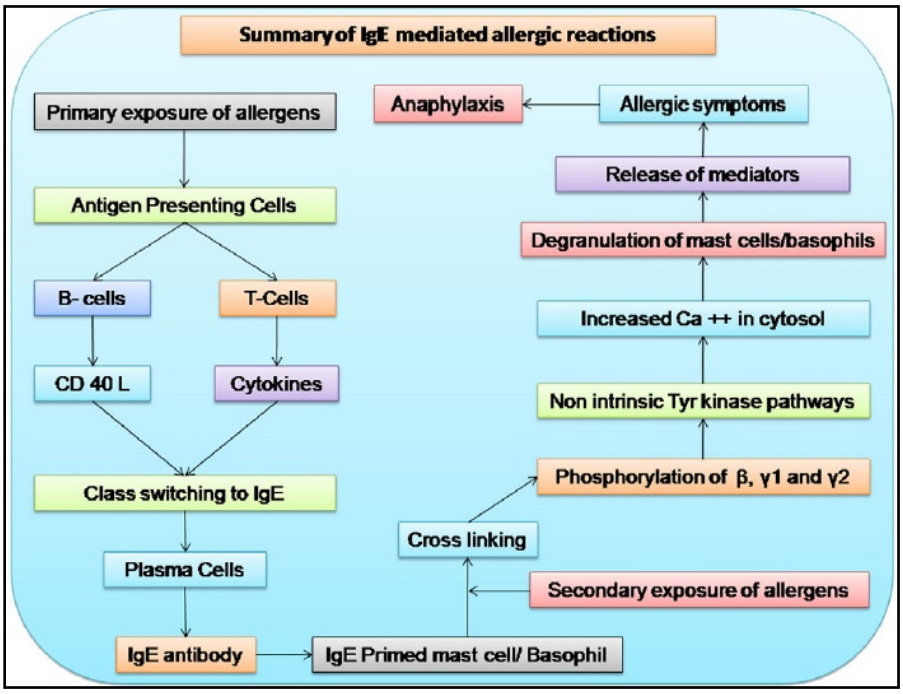

Figure 3. A brief outline of mechanistic pathway that occurs during allergen induced mast cell degranulation (Kumar, 2012).

1.1.3. Molecular genetics of allergies

It has been widely accepted that allergic diseases develop in atopic individuals and that they have a genetic basis. More than one gene is likely involved in the expression of atopy (Miescher, 2002). Atopy manifests itself as an IgE response to allergens. Traditionally, finding the gene responsible for the susceptibility to certain disease required the discovery of a biochemical or physiological abnormality leading to the isolation of an aberrant protein that was partially sequenced. This amino acid sequence was then used to produce an oligonucleotide to screen for the expressed gene, which could then be fully sequenced. However, in many diseases there is no known abnormal protein (Anderson, 1998).

In order to identify genes that are relevant to atopic diseases two approaches have been followed. Firstly, a positional cloning, which links the inheritance of specific chromosomal

regions with the inheritance of the disease (Miescher, 2002; Cookson, 2000). The second method, the study of candidate genes, is based on the identification of polymorphisms in a gene, which is plausible candidate for being responsible for the disease (Miescher, 2002; Anderson, 1998).

Such studies have identified several genes or loci that may be involved in atopy. On chromosome 11q13, in the region of gene encoding the β-subunit of the high affinity IgE receptor (FcεRI), two polymorphisms (ile/leu181 and val/leu183) have been reported (Anderson, 1998; Cookson, 2000). Linkage for atopy was also found with polymorphisms in gene cluster region on chromosome 5. The 5q23-31 region contains genes coding for IL-3, IL-4, IL-5, IL-9 and IL-13 (Anderson, 1998).

The MHC region on chromosome 6 has shown consistent linkage to asthma-associated phenotypes in several studies and may be considered to be a major locus influencing allergic diseases (Cookson, 2000). The major control of the allergen-specific IgE response appears to be associated with particular HLA-D types. For example, more than 90% of the IgE responders to the ragweed Amb V (Ra5) are HLA-Dw2 (Anderson, 1998).

Determining the inheritance of a set of polymorphisms in a subject may allow the prediction of those at risk of developing allergic diseases and may allow disease prevention strategies to be more accurately directed. Similarly, the inheritance of a set of polymorphisms may indicate the clinical course, its severity, and its response to treatment (Anderson, 1998).

1.2. Allergens as allergy elicitors

The inquiry into which features make a protein allergenic is of a high interest to allergologists worldwide. It has been argued by some that any immunogenic protein presented in the right context and in sufficient quantity to the immune system of an atopic

individual can become an allergen. Other authors attribute allergenicity to certain architectural and structural features of the proteins. For this discussion, the comparison of allergenic and non-allergenic members in various protein superfamilies could provide further insights (Breitender, 2008). Even though a definite answer to the question: ``what makes an antigen an allergen? `` cannot be fully answered yet, the term allergen is used to describe two distinct molecular properties: the property to sensitize (i.e. induce the immune system to produce high-affinity antibodies, particularly of the IgE class) and the property to elicit an allergic reaction (i.e. to trigger allergic symptoms in a sensitized subject). Complete allergens have all these properties (Aalberse, 2000).

Aspects of protein structure likely to be relevant for allergenicity are solubility, stability, size, and the compactness of the overall fold. One of the structural features clearly related to stability is the presence of disulfide bonds. Both inter- and intrachain disulfide bridges constrain the three-dimensional folds such that perturbation of the structure by heat or chemicals is limited and frequently reversible (Breitender, 2005). These aspects reflect dependency of allergenicity on transport over mucosal barriers and susceptibility to proteases. Posttranslational modifications may affect allergenicity in different ways. It may induce new epitopes and it may affect solubility, stability, size and susceptibility toward proteases. However, it is likely that features other than structure are more relevant for allergenicity. It is not realistic to assume that the requirements for sensitization to food allergies in early childhood are very similar to those for late-onset sensitization to airborne occupational allergens (Aalberse, 2000).

1.2.1. Nomenclature of allergens

The biochemistry of allergens is underpinned by a Linnean system of nomenclature that is maintained by the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee. The systematic nomenclature was adopted by WHO/IUIS and published in the Bulletin of the WHO in 1986 and in revised form in 1994. Allergens are named using the first 3 letters of the genus,

followed by a single letter for the species and a number indicating the chronologic order of allergen purification (Chapman, 2007).

The massive accumulation of sequence data of, particularly, plant proteins in recent years has made the classification of allergens into protein families possible. By comparing sequences and structures, related proteins can be grouped together into families (if they have residue identities of 30% or greater, or if they have lower sequence identities, but their functions and structures are very similar), and related families can be grouped together into superfamilies (Radauer, 2007).

1.3. Food Allergy

1.3.1. Prevalence and risk factors

Adverse reactions to food can be classified as either IgE-mediated or non-IgE mediated. Non allergic adverse reactions are commonly caused by host factors, such as enzyme deficiencies, or by inherent toxic properties of food, as is the case with food poisoning (Sicherer, 2006). Food allergy, on the other hand, is defined as an ‘adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food’ (Boyce, 2010). Currently, food allergy is a major health problem, with an estimated prevalence of about 5% in young children and 3-4% in adults and the prevalence is increasing (Sicherer, 2010; Burks 2012). The major food allergens are milk, egg, peanuts, shellfish, fish, wheat, and soy (Sicherer, 2010). In most cases exposure to these food allergens causes only mild allergic reactions. However, in severe cases anaphylaxis can be induced, mainly in people allergic to peanut and other tree nuts (Koplin, 2011).

Allergies are the result of a complex interplay between genetic predisposition and environmental factors. Specific genetic loci that may modulate individual risk of food allergy remain to be identified (Hong, 2009). Some environmental factors, such as delayed

introduction of solid foods in infants, effects of breastfeeding, and early colonization of the intestine by appropriate microbiotica have been shown to be important for the development of a healthy immune system. In addition, the acid content of the stomach also plays a role in allergic sensitization, since an increased pH is associated with increased allergic sensitization (Cochrane, 2009; Untersmayr, 2003). Nevertheless, it is not yet fully understood how all these factors play a role in the development of food allergy.

1.3.2. Immune system of the gastrointestinal tract

The gastrointestinal mucosa is an extensive structure responsible for digestion and absorption of nutrients as well as protection from pathogenic organisms (Sicherer, 2006). The highly elaborate architecture of the intestinal mucosa increases the total intestinal surface to about 300–400 m^2, with a total length of the small and large intestine of approximately 8 m. The intestinal mucosa is colonized with numerous (up to 10^{14}) luminal bacteria derived from more than 500 different species (Schenk, 2008). The intestinal barrier, formed by a monolayer of epithelial cells, displays a number of specialized protective adaptations: the cells form tight junctions that act as a physical barrier, goblet cells secrete thick mucus which forms a viscose layer, and paneth cells secrete microbicidal a-defensins, lysozyme and cathelicidins (Frey, 1996; Boman, 2000). Approximately 70% of all the lymphocytes of the human body are concentrated in the intestinal intraepithelial and subepithelial layers, and the largest pool of tissue macrophages is located in the intestinal wall (Macpherson, 2004). These features, coupled with the presence of proteolytic enzymes, bile salts, and extreme pH needed for digestive processes results in a highly functional defense system. In addition, components of the innate immune system (NK cells, polymorphonuclear leukocytes, macrophages) and adaptive immune system (intraepithelial lymphocytes, Peyer's patches, IgA, cytokines) form an active barrier towards foreign proteins (Chehade, 2005; Mowat, 2003).

1.3.3. Oral tolerance

Despite the complex interplay of the mucosal system, approximately 2% of intact food proteins are absorbed through the mature GI tract and reach the lymphatic and portal circulation. (Sicherer, 2006) After ingestion, most food proteins are digested by gastric acids and enzymes in the stomach and intestines.

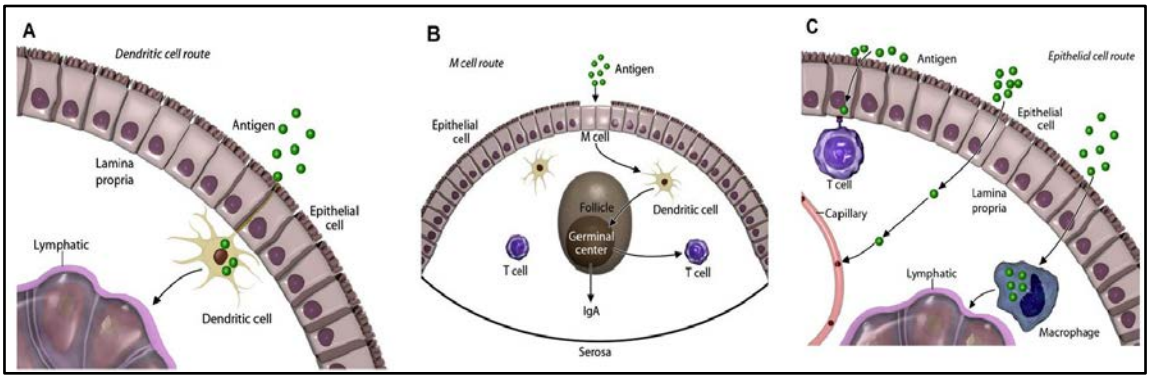

Figure 4. Antigen sampling in the gut. A) Dendritic cells extend processes through the epithelium and into the lumen. B) M cells overlying Peyer's patches take up particulate antigens and deliver them to subepithelial dendritic cells. C) Soluble antigens possibly cross the epithelium through transcellular or paracellular routes to encounter T cells or macrophages in the lamina propria (Chehade, 2005).

The remaining food proteins and peptides are subsequently transferred from the lumen to the mucosa via gut epithelial cells, either by specialized M cells present in Peyer's patches, through epithelial cells, or by direct sampling of mucosal dendritic cells (Figure 4) (Mowat, 2003; Burks, 2008). In the mucosa, dendritic cells process these proteins and peptides, move to T cell areas and present them on MHCII where they can interact with naïve T cells. The presence of costimulatory molecules is important in determining the subsequent immune response: interaction of CD28 present on T cells with CD80 and CD86 present on dendritic cells induces T cell activation, whereas interaction of CD80 and CD86 present on

dendritic cells with CTLA-4 present on T cells down-regulates T cell activation (Krummel, 1995; Vandenborre, 1999).

In normal individuals, presentation of processed proteins and peptides by dendritic cells to naïve T cells will lead to the induction of oral tolerance, a state of active inhibition of immune responses to antigens encountered in the intestine. Oral tolerance allows individuals to encounter immense quantities of dietary protein and commensal bacteria without inciting a dynamic immune response (Chehade, 2006). However, in certain individuals, caused by factors mentioned before, presentation of the food protein on MHCII results in the induction and activation of Th2 cells which then stimulate B cells to produce allergen specific IgE leading to sensitization and a subsequent allergic reaction upon repeated exposure. Food hypersensitivity likely results from either a failure in establishing oral tolerance or a breakdown in existing tolerance. Several factors, including antigen properties, route of exposure, and genetics and age of the host, contribute to the failure of oral tolerance (Burks, 2008).

1.3.4. Food allergens

Aspects of protein structure that are likely relevant for allergenicity are solubility, stability, size, and compactness of the overall fold. In fact, most allergens are relatively small, hydrophilic, and stable proteins, apparently lacking bacterial homologues (Emanuelsson, 2007). These aspects reflect the dependency of allergenicity on transport over mucosal barriers and susceptibility to proteases (Hauser, 2008).

Despite the wide range of foods that humans consume, surprisingly few foods account for the vast majority of food allergies. Milk, egg, peanut, wheat, and soy are responsible for most food-induced reactions (Sampson, 2004). Two forms of IgE-mediated food allergy have been proposed. Class 1 food allergy results from sensitization through the GI tract. The class 1 food allergens are generally 10 to 70 kDa in size and highly stable when subjected to heat, acid, or proteases. Examples of class 1 food allergens include milk

(caseins), peanut (vicillins), egg (ovomucoid), and nonspecific lipid transfer proteins (Sicherer, 2006). On the other hand, sensitization can be facilitated if the gastrointestinal tract is surpassed and the antigen is presented via an alternative route. An example of this is the oral allergy syndrome (OAS), also known as the food-pollen syndrome. Oral tolerance is not induced since sensitization occurs through the respiratory tract. The patient is sensitized with pollen via the airways and exhibits an allergic reaction to a food antigen with a structural similarity to the pollen protein (Fernandez-Rivas, 2006; Bohle, 2007). Class 2 food allergy results from sensitization to inhalant allergens that are partially homologous to proteins in certain fruits and vegetables, and principally occurs in adolescents and adults. Class 2 allergens are heat labile and susceptible to digestive processes. Consequently, symptoms occur when the food is ingested in the raw form, but not when cooked. Pollen allergic individuals are at risk due to the homology between specific pollen proteins and proteins in certain foods (Sampson, 2006). Of patients with allergic rhinitis, it has been estimated that 23 to 76% of patients experience oral symptoms to at least one food (Sicherer, 2001). The cooked version of the foods is typically tolerated due to disruption of the protein conformation or tertiary structure (Sampson, 2006).

1.3.5. Plant-derived food allergens

So far the allergen list of the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee comprises 130 plant-derived food allergens. Based on sequence homology these allergens can be classified into only 27 out of 9,000 known protein families. These families comprise the prolamin and cupin superfamilies, pathogenesis related proteins, profilins, thaumatin-like proteins, oleosins, expansins, a number of enzymes and protease inhibitors among others (Hauser, 2008). Many of the known plant food allergens are homologous to pathogenesis-related proteins (PRs), proteins that are induced by pathogens, wounding, or certain environmental stresses. PRs have been classified into 14 families. Allergens other than PR homologs can be allotted to other well-known protein families. In fruits and vegetables with high water content, such as cherries, papayas, bananas, or avocados, that are prone to fungal attack, the chitinases, proteases, and

antifungal proteins are the predominant allergens. The allergens in hard and dry seeds such as wheat berries, rice, mustard seeds, or soybeans fall into two classes: the seed storage proteins and the enzyme inhibitors. They prevent the endosperm from being digested by insects or fungi (Breiteneder, 2000).

In general, any innocuous environmental antigen that is able to trigger a Th2 or an IgE response is defined as allergen. Depending on the percentage of allergic individuals reacting to a protein of a given allergenic source, major (> 50%) and minor (< 50%) allergens can be distinguished. Both major and minor allergens can be found in most plant food protein families. The seed storage proteins for example are considered as major allergens of the prolamin superfamily that are likely to act as potent class I food allergens. In contrast, profilins and PR-proteins are regarded as class II food allergens due to their instability to heat and gastric digestion. For instance, profilin has been identified as a major allergen in patients suffering from pollen-food syndrome caused by melon. Digestibility analysis of melon profilin revealed its stability in human saliva but not in simulated gastric fluid, which is typical for class II food allergens (Rodriguez-Perez, 2003).

1.3.6. Diagnosis of food allergy

The diagnostic approach to allergic food reactions includes assessing the patient's medical history, a physical examination, and identification of the suspected food. Elimination diets are used both in diagnosis and in therapy of food allergies (Sampson, 2004) Identification of the suspected food also includes skin tests and *in vitro* assays, which can confirm a sensitization to the food. However, for the conclusive diagnosis of a food allergy it is necessary to demonstrate, by an oral challenge, that the food to which a sensitization has been found is responsible for the patient's symptoms (Fernandez- Rivas, 2004).

Lewis and Grant described skin prick testing (SPT) for the first time in 1924 and it was modified by Pepys in 1970. This is the easiest, fastest, and less expensive method to identify specifc IgE antibodies (Demoly, 2003). Drops of different glycerinated allergen

extracts, placed on the volar surface of the forearm are passed by hypodermic needle at a low angle with the bevel facing up into the epidermal surface. The needle tip is then gently lifted upward to elevate a small portion of the epidermis not inducing bleeding. The immediate reaction (wheal and erythema) is measured after 15 to 20 minutes. A positive test is considered when diameter wheal is greater than 3 mm (Hamilton, 2003). Several factors contribute to variability in the prick test results. Bleeding and dermographism can lead to a false-positive wheal and erythema. False-negative tests are consequences of antihistamine use, low potency of extracts and poor technique (Demoly, 2003). Nowadays, the skin prick test is usually done with fresh foods. In this test the lancet is plunged several times into the food immediately before pricking the patient's skin with it. The inconveniences of the prick–prick test are the impossibility of standardization and the dependence on the availability of the fresh food (Ortolani, 1989; Fernandez- Rivas, 2004).

Allergen-specific IgE antibody (sIgE) *in vitro* assays have developed significantly over the past 40 years and continue to evolve in the areas of allergen characterization, antibody detection, and instrumentation. Originally described in the late 1960s, the radioallergosorbent test (RAST) was the first routine technique used for determining sIgE in serum (Hamilton, 2003). This 'first generation' assay employed cyanogen bromide-activated paper discs as the solid phase (allegrosorbent) and used a birch pollen specific IgE calibration curve from which the levels of sIgE could be interpolated. Results were semi-quantitative and organized into classes of reactivity related to skin prick test positivity. Second generation methods, introduced in the early 1990s, offered marked improvements in assay convenience and performance. These included: developments in solid phase allergosorbent materials or use of a liquid-allergen matrix resulting in enhanced antigen–antibody kinetics and assay sensitivity, the use of nonisotopic detection methods (spectrophotometry, fluorimetry, enzyme enhanced chemiluminescence), reporting of quantitative sIgE results (kU/L) standardized to a WHO International Reference preparation for IgE. In addition, systems became semiautomated, resulting in faster turn-around times for results. Further refinements have been introduced with 'third generation' assays, most notably in the extension of the lower limit of detection to 0.1 kU/L, and in the availability

of random access automated analytical platforms (Crameri, 2006; Hamilton, 2004; Ahlstedt, 2006; Ebo, 2006).

Skin prick tests and *in vitro* IgE assays are useful methods to demonstrate the presence of food specific IgE antibodies, but they do not establish the diagnosis of clinical food allergy. The oral challenge is the diagnostic test which provides conclusive evidence of a food allergy (Fernandez-Rivas, 2004; Bock, 1988) Oral food challenges may be performed openly (patient and physician are aware of the food ingested), single-blind (only the physician knows the content of the challenge) or double-blind (neither the patient nor the physician is aware of the content of the challenge). The blind challenges may be placebo-controlled. The double-blind placebo controlled food challenge (DBPCFC) is considered the *gold standard* for the diagnosis of food allergy (Sampson, 1999). Food challenges are performed in the hospital where emergency care is immediately available. The food is administered to the patient in fasting conditions, starting with a dose unlikely to provoke symptoms, according to the eliciting dose reported in the medical history or in the last positive provocation test. Incremental amounts of food are given at time intervals slightly longer than expected to produce symptoms, until a positive reaction appears or the patient eats a normal amount of the food (Fernandez- Rivas, 2004).

1.3.7. Therapy of food allergies

Currently, the primary recommendation for food allergy patients is strict avoidance of the allergenic substance. Antihistamines and corticosteroids are used to treat acute allergic symptoms and in anaphylaxis epinephrine is used as the first line of treatment. However, these drugs only ameliorate symptoms and do not stop or cure progression of the disease (Sicherer, 2006; Burks, 2012). In addition strict avoidance of the allergenic food is also difficult to achieve. Patients have inadvertent cross contact with allergens or foods that have hidden allergens (Sicherer, 2006). One study illustrated that for 156 reactions to peanut and tree nuts, 50% of accidental ingestions in food establishments were due to

“hidden ingredients. This illustrates the difficulties that food-allergic patients face with daily experiences, such as purchasing prepared foods or eating in restaurants (Furlong, 2001).

A potentially curative treatment for (food) allergy is allergen-specific immunotherapy (SIT). SIT is already used for more than a hundred years as a desensitizing therapy and is presently successfully used to treat allergic rhinitis, asthma and wasp and bee venom hypersensitivity (Fitzhugh, 2011). The aim of SIT is to induce peripheral T cell tolerance and to increase the thresholds for IgE mediated mast cell and basophile activation. The high success rate of SIT (varying from 80-90 %, depending on the antigen used) is promising. However, disadvantages include the long duration of the treatment (3-5 years), the amount of allergen injections needed (about 80) and allergic side effects. Key mechanisms of successful SIT are the induction of regulatory T cells and an increase of IgG4 that may capture the allergen before reaching effector cell-bound IgE (Kunding, 2010). Unfortunately conventional immunotherapy is not as effective in treating food allergies. Recently, both sublingual immunotherapy (SLIT), where the allergen is held under the tongue, and oral immunotherapy (OIT), where the allergen is simply swallowed, have shown promise as potential treatments (Burks, 2008). The implementation of recombinant proteins with an altered IgE-binding epitope may be a form of immunotherapy that holds future promise. The altered IgE-binding epitopes could prevent binding of the patient’s IgE to the engineered protein within the vaccine (Sampson, 2004).

1.4. Recombinant DNA technology

Technology of recombinant DNA or genetic engineering is a new field of biotechnology that developed in the last three decades. Techniques of genetic engineering allow the possibility of introduction and understanding of complex biological processes such as mechanism of eukaryotic gene expression during development, immunological response,

cell division etc. These techniques have found a broad application not only in scientific research but also in many other fields of life (Allison 2007).

Employement of recombinant DNA technology allows production of any protein-encoding gene . The target gene is derived from the original chromosome using an endonuclease enzyme and then integrated in a vector as a plasmid or phage. After that, the vector is transformed into a prokaryotic or eukaryotic expression system. The target protein is produced in desired amounts by culturing these microorganisms. Cloning of DNA from any organism entails five general procedures:

1. Cutting the DNA of interest at precise locations by sequence-specific endonucleases (restriction endonucleases)
2. Selection of cloning vector (a delivery agent), typically plasmids or viral DNAs
3. Joining two DNA fragments covalently by using DNA ligase
4. Moving recombinant DNA from the test tube to a host cell that provides the machinery needed for DNA replication
5. Selection and identification of host cells that contain recombinant DNA (Allison, 2007).

The bacterium *Escherichia coli* (*E. coli*) is the first organism used for recombinant DNA work and still the most common host cell. *E. coli* has many advantages: its DNA metabolism (like many other of its biochemical processes) is well understood, many naturally occurring cloning vectors associated with *E. coli*, such as plasmids and bacteriophages, are well characterized; and techniques are available for moving DNA from one bacterial cell to another. *E. coli* can also be used for amplification of DNA in large quantities so that it can be introduced into the desired host cells, such as mammalian, yeast or special bacterial cells. The host cells will then synthesize the foreign protein from the recombinant DNA. After the cells are grown in vast quantities the foreign recombinant protein can be isolated and purified in large amounts. Recombinant DNA technology is not only an important tool in scientific research, but it has also affected the diagnosis and treatment of diseases and genetic disorders in many areas of medicine (Davies 1981).

However, high levels of recombinant protein expression in *E. coli* can lead to the formation of insoluble inclusion bodies, which represent a set of structurally complex aggregates often perceived to occur as a stress response when the recombinant protein is expressed at high rates (Sorensen and Mortensen, 2005).

1.4.1. Recombinant fusion proteins

The purification of recombinant proteins can be simplified by incorporating a tag of known sequence into the protein. It is also a marker for expression and facilitates detection of the recombinant protein, which may simplify downstream purification protocol. The two most commonly used tags are glutathione-S-transferase (GST) and 6 × histidine residues $(His)_6$.

1.4.2. GST fusion proteins

Yields of active, correctly folded recombinant proteins can be improved by using a cloning strategy in which the target protein is linked to a fusion partner, leading to high yield expression of the protein in soluble form (Hannig and Makrides, 1998). Glutathione *S*-transferase (GST) is among the most frequently used fusion partners. The crystal structure of recombinant *Schistosoma japonicum* GST has been determined and matches that of the native protein. Recombinant fusion proteins containing the complete amino acid sequence of GST exhibit a functional GST enzymatic activity. In addition to improving solubility, GST allows detection of the expressed recombinant protein using enzyme assays and GST-specific antibodies, and also facilitates recombinant protein purification (McTigue et al., 1995). Although most GST fusion proteins are expected to be soluble, many of them, even relatively small ones (40-50 kDa), are partially or completely insoluble following lysis of prokaryotic cells (Frangioni and Neel, 1993; Hansen and Eriksen, 2007). Therefore GST fusion proteins accumulated in inclusion bodies are considered to be undetectable by GST-tag assays, designed for quantitative measurement of GST activity under native conditions.

1.4.3. Production of reagents for allergy diagnosis by recombinant DNA technology

When the allergen nomenclature system was adopted in 1986, allergens were mainly identified based on their electrophoretic and chromatographic behavior and by reactivity to antisera. This was not only unsatisfactory as far as standardization is concerned, but the processes of allergic sensitization and immunotherapy could not be studied in the framework of antigen processing and B- and T-cell epitopes. Recombinant technologies developed in the 1980s permitted the cloning of allergens, beginning with the major house dust mite allergen Der p 1 and hornet allergen Dol m 5 (Fang et al., 1988; Thomas et al., 1988). After this, a wave of cloning with IgE immune screening resulted in the cloning of Der p 2, Der p 5, Bet v 1, Bet v 2, and Dac g 2 along with Fel d 1 cloned (Fritsch et al., 1998; Lynch et al., 1997; Seppälä et al., 2005). Recombinant allergens have been used to define the important allergens for a wide range of allergies and to develop new types of immunotherapy, some of which have shown efficacy in human trials. The pure recombinant allergens have been used to solve the tertiary structures of these proteins. Proprietary recombinant allergens are now being used in improved diagnostic tests (Asturias et al., 1997; Gessler and Patocchi, 2007; Niederberger et al., 2001).

Recombinant DNA technology provides the means for producing allergens that are equivalent to their natural counterparts and also genetically engineered variants with reduced IgE-binding activity. The proteins are produced as biochemically defined molecules with consistent structural and immunologic properties. Several hundred allergens have been cloned and expressed as recombinant proteins, and they can provide the means for making a very detailed diagnosis of a patient's sensitization profile. Clinical development programs are now in progress to assess the suitability of recombinant allergens for both subcutaneous and sublingual immunotherapy. Recombinant hypoallergenic variants, which are developed with the aim of increasing the doses that can be administered while at the same time reducing the risks for therapy-associated side effects, are also in clinical trials for subcutaneous immunotherapy. Grass and birch pollen preparations have been shown to be clinically effective, and studies with various other

allergens are in progress. Personalized or patient-tailored immunotherapy is still a very distant prospect, but the first recombinant products based on single allergens or defined mixtures could reach the market within the next couple of years (Barber et al., 2009; Heiss et al., 1999).

A great variety of recombinant plant, mite, mold, mammal, and insect allergens have been expressed in heterologous hosts (e.g., Escherichia coli). The number of biologically active recombinant allergens available for experimental, diagnostic, and therapeutic purposes is increasing tremendously. Recombinant allergens have proven to be valuable tools to investigate T-cell and B-cell recognition of allergens as well as to study mechanisms of specific IgE regulation. The immunologic equivalence of many relevant recombinant allergens with their natural counterparts has been demonstrated, and the three-dimensional structures of several recombinant allergens have been described recently (Kaiser et al., 2003; Markovic-Housley et al., 2003). Recombinant allergens have been used for successful *in vitro*, as well as *in vivo*, allergy diagnosis, and work is in progress to produce recombinant allergen derivatives with reduced anaphylactic potential to improve current forms of immunotherapy (Asturias, 2009; Gafvelin et al., 2007; Holm et al., 2004; Li et al., 2003; Sampson et al., 2003).

By using recombinant DNA technology, defined and safe allergy vaccines can be produced that allow us to overcome many, if not all, of the problems associated with the use of natural allergen extracts, such as insufficient quality, allergenic activity, and poor immunogenicity. Some of these vaccines have undergone successful clinical evaluation up to phase III studies. Furthermore, they introduce a strategy for allergen-specific immunotherapy based on recombinant fusion proteins consisting of viral carrier proteins and allergen-derived peptides without allergenic activity, which holds the promise of being free of side effects and eventually being useful for prophylactic vaccination (Bousquet et al., 1998).

A large percentage of allergenic proteins are of plant origin. Hence, plant-based expression systems are considered ideal for the recombinant production of certain allergens. First

attempts to establish production of plant-derived allergens in plants focused on transient expression in *Nicotiana benthamiana* infected with recombinant viral vectors (Schmidt et al., 2008). Accordingly, allergens from birch and mugwort pollen, as well as from apple have been expressed in plants. Production of house dust mite allergens has been achieved by *Agrobacterium*-mediated transformation of tobacco plants. Beside the use of plants as production systems, other approaches have focused on the development of edible vaccines expressing allergens or epitopes thereof, which bypasses the need of allergen purification. The potential of this approach has been convincingly demonstrated for transgenic rice seeds expressing seven dominant human T cell epitopes derived from Japanese cedar pollen allergens. Parallel to efforts in developing recombinant-based diagnostic and therapeutic reagents, different gene-silencing approaches have been used to decrease the expression of allergenic proteins in allergen sources. In this way hypoallergenic ryegrass, soybean, rice, apple, and tomato were developed (Holm et al., 2004).

1.5. Component resolved allergy diagnosis

Employing allergen extracts for diagnosis and treatment is widespread and supported by a long and successful clinical tradition. However, based on the knowledge that has been gathered during the last 20 years of *in vitro* allergy diagnostics, extract-based approaches for diagnosis and treatment are controversially disputed. Above all, biological extracts are heterogeneous mixtures of allergenic and non-allergenic components. The actual allergenic molecules may only account for a minor fraction of the total content of the extract. Most allergen sources (e.g., birch pollen, animal dander, storage mites, etc.) have at least several major and minor allergen molecules. Standardized mixtures containing all the relevant allergens in the appropriate (biologically and diagnostically relevant) concentrations are difficult or even impossible to produce and the performance of commercially available allergen extracts from different manufacturers can therefore vary significantly. This is due to several reasons. Firstly, some allergens might resist even sophisticated extraction procedures and may therefore be absent or under-represented in the resulting extract. The

extractability of an individual allergen is a major prerequisite for test quality as it directly affects the assay sensitivity. Secondly, variable allergen stability leading to the partial or complete degradation of allergens during the extraction procedure could potentially interfere with the production of high quality extracts. Thirdly, biological materials are intrinsically heterogeneous regarding their allergen content. For example, when collecting tree pollen or fruit, the expression of certain allergens is affected by environmental factors, such macro- or micro-climate, pollution, or the developmental stage of the collected pollen. When considering food allergens, many different breeds exist for the common species and their allergen content is known to be different. Also protein content varies greatly with the ripening state so extracts can show a significant difference in composition and/or allergenicity (Gavrovic-Jankulovic et al. 2005). In addition, biological raw materials may be contaminated by compounds of extrinsic origin. For example, animal hair and dander can contain mites and even a fraction of these alien allergens may eventually lead to false-positive results when the corresponding extracts are used for testing in certain allergic patients (van der Veen et al. 1996). Preparations of fruit or vegetable extracts can be contaminated with moulds or herbicides which can also induce false-positive results. Finally, many biological sources contain allergens with a strong potential for cross-reactivity. Cross-reactivity originates from IgE antibodies binding to structurally similar epitopes from homologous proteins contained in different species. The use of heterogeneous extracts containing such structures bears the risk of producing false-positive or clinically irrelevant results.

During the previous decade, a greater understanding of the problems mentioned above has led to numerous research activities focused on improving the standardization of the allergens being used for testing as well as for treatments (van Ree et al., 2004).

Panels of recombinant allergens are available for many of the most important allergen sources (Barber et al., 2009; Bublin et al., 2010; Heiss et al., 1999; Pittner et al., 2004). These panels are designed to assemble the epitope complexity of their biological counterparts. Testing with these purified recombinant (or natural) allergenic components allows the majority of allergic patients to be diagnosed correctly and has several other

benefits. First and most importantly, assay standardization using well-characterized single molecules is superior to testing even with well standardized extracts and it also has positive effects on assay sensitivity and specificity. Moreover, the IgE-reactivities of an individual patient can be resolved at the level of individual disease-eliciting components. Hence, the term Component-Resolved Diagnosis (CRD) was introduced for this particular diagnostic approach. In certain cases the resulting reactivity pattern of serum IgEs can be used to improve the treatment for an individual patient significantly. For example, in the house dust mite (HDM), *Dermatophagoides pteronyssinus*, more than 20 allergens have been identified so far (Pittner et al., 2004). Patients who are sensitized against HDM could be tested at least for the major allergens Der p 1 and Der p 2 as well as for Der p 10, a minor allergen with a high potential for cross-reactivity. Since HDM extracts are generally standardized only for their content of Der p 1 and Der p 2, a patient who does not have specific IgE against either of the two proteins will probably not benefit from specific immunotherapy with HDM extracts. On the other hand, if the patient sample reacts with Der p 10 they are most probably sensitized against other mite species as well (e.g. *D. farinae*), and should not be treated with HDM extracts (Pittner et al., 2004). A similar constellation can be found in the European White Birch, *Betula verrucosa*. Extracts of this species produced for specific immunotherapy (SIT) are usually standardized against the major allergen Bet v 1 to which more than 90% of all patients have specific IgE. Patients who are mono-sensitized against this particular allergen can therefore be considered highly suitable for receiving SIT. On the contrary, an exclusive sensitization towards the minor birch allergen Bet v 2 makes SIT not recommendable for two reasons. Firstly, the allergen might not be present to a sufficient extent in the extract used for therapy and secondly, Bet v 2 belongs to the protein family of highly cross-reactive profilins that can be found in a wide range of biological sources (http://www.allergen.org/viewallergen.php?aid=130). Therefore, cross-reactivity towards an allergen of a different biological source has to be considered as the primary cause of the reactivity against birch pollen extract (Mobs et al., 2010).

In other cases, CRD may help to reveal the route of sensitization of a particular allergen and this information can be used to prevent further allergen exposure more effectively. For example, in natural rubber latex (*Hevea brasiliensis*) reactivity against the major allergens Hev b 1 and Hev b 3 correlates with subcutaneous sensitization that has been acquired via previous surgery. Patients with Spina bifida (SB) who typically have several surgical treatments in the first few years of life often react with these two allergens. On the other hand, the allergens Hev b 2, Hev b 4 and Hev b 6 are more frequently the cause of occupational allergy (e.g. in health care workers) who come into contact with latex products via skin exposure (Levy and Leynadier, 2001). Moreover, a significant percentage of latex-extract positive patients may have been sensitized against a cross-reactive allergen, such as a profilin (*e.g.* Bet v 2). Clinically non-relevant cross-reactions against so-called cross-reactive carbohydrate determinants (CCD) have been described as the cause of false-positive *in vitro* results when testing for latex (Coutinho *et al*., 2008).

Determination of the precise allergy-eliciting molecule may help patients to take preventive life-saving measures which are particularly important in patients who are prone to systemic reactions when they come into contact with a particular allergen. For example, allergens of the lipid transfer protein (LTP) family are frequently found in tree nuts (e.g. peanut and hazelnut) and fruits (e.g. peach and cherry) and these allergens are strong elicitors of life-threatening systemic reactions (Schocker *et al*., 2004). Other allergens of the same biological sources (e.g. Cor a 1, Ara h 1) have not been found to induce severe reactions in the affected patients. Therefore, using CRD with the corresponding allergenic molecules can be used to provide identification of patients who are prone to systemic reactions and equip them with an appropriate emergency set.

Despite these promising results, CRD procedures are not yet established in routine diagnostic settings. Basically, there are two reasons for this: firstly, even if a comprehensive IgE reactivity profile could be obtained with a panel of recombinant allergens therapies based on the same molecules are still in a proof-of-concept stage of development. Secondly, only very few tests based on recombinant allergen panels are commercially available and these panels still lack important allergens. Allergy diagnosis on

the basis of single molecules would require a large number of individual allergens contained in the test format. For example, a comprehensive CRD-based test for allergy against grass pollen (*e.g*., timothy grass), mites (*e.g*., *Dermatophagoides pteronyssinus*) and latex (*Hevea brasiliensis*) would have to employ at least 30 individual protein components. Still this test could lack components of some other important species (*e.g*. other grass pollen and mites) that might be of relevance. Also, some naturally occurring allergen sources might contain allergens that have not been identified yet. As a consequence, a small percentage of patients would go undiagnosed if not all the available allergens are employed in the assay. Moreover, for many natural allergens a large number of isoforms has been identified and each of these isoforms might exhibit a (slightly) different IgE binding capacity *in vitro* (Corti *et al*., 2005; Sowka *et al*., 1999). It has also been shown that some recombinant allergens bind IgE with high affinity and resemble their natural counterpart quite well, whereas other artificial molecules only bind a minor fraction of IgE in contrast to the purified natural allergen. These differences mainly originate from certain post-translational modifications which are not an integral part of the gene expression machinery of the heterologous host where the allergens are expressed (*e.g*., *E.coli*) or from incorrect 3D folding of the artificial molecule (Pauli and Malling, 2010).

Growing efforts in the molecular characterization of allergens and the development of innovative assay designs will permit medical experts to use recombinant allergen-based approaches for the diagnosis and treatment of allergic diseases in the future. In combination with established standards, the interpretation of these novel test formats should be guided by diagnostic decision trees and guidelines that could be proposed by international committees of allergy experts. Large-scale studies will reveal reactivity patterns that may help to improve the predictive value of allergy diagnosis by using component diagnostic signatures for *in vitro* testing, disease prevention in early life and monitoring of specific immunotherapies. Combined efforts in allergy research and clinical diagnosis will lead to a better understanding of the effectiveness of single allergen molecule-based approaches and will help to improve the treatment of allergic diseases significantly.

1.6. Allergy to banana and banana allergens

Banana (*Musa acuminata*) is a very popular fruit worldwide. It is available year round and is very present in human nutrition. However, in 1991 symptoms of angioedema and anaphylactic shock caused by banana ingestion were described for the first time (M'Raihi *et al*., 1991). Allergic reactions to banana manifested in edema formation, collapse, urticaria, swelling of the hands, oral pruritus, rhinitis, cough, asthma and anaphylaxis.

It was noticed that people allergic to bananas quite often had the same reaction after contact with pollen of other plants (Reindl *et al*., 2002; Grob *et al*., 2002) or latex products, and by using sera of allergic patients it was demonstrated that this is caused by cross-reactive IgE antibodies (Sanchez *et al*., 1999). In 1994 the term "latex-fruit syndrome" was established, which is based on the fact that 52% of patients allergic to latex are also sensitized to fruits, like avocado (36%), chestnut (36%), bananas (28%), kiwi (20%) and papaya (12%). (Lavaud *et al*., 1992; Anibarro *et al*., 1993; Blanco *et al*., 1994; Makinen-Kiljunen, 1994).

Although many people think they are allergic to bananas, they actually have another form of intolerance to this fruit. Banana contains certain amines that cause digestive problems in people with a weak or sensitive digestive system. The enzyme diamine oxidase which participates in the digestion of these amines is not synthesized by some people and they are hence not able to break down the proteins that are found in bananas and experience digestive problems, symptoms that are often missinterpreted as a true allergic reaction to banana (Palacin *et al*., 2001).

1.6.1. Banana allergens

Banana belongs to the group of fruits with high water content, along with cherries, papaya, and avocado. It is therefore susceptible to fungal attack, so it is of no surprise that proteases, chitinases, and antifungal proteins are among the most dominant proteins found in this fruit. The mentioned proteins are actually associated with pathogenesis related

proteins (PR proteins) in plants whose expression is induced by pathogens, mechanical wounds or enviromental stress (Breiteneder, 2000).

Familiar banana allergens are designated Mus a 1 to 5. Mus a 1 is a 14 kDa profilin showing an IgE-binding prevalence of 44% in 16 sera with suspected banana allergy and a high cross reactivity with its homologous allergens from other plant foods, latex and pollens. Mus a 2 corresponds to a 33 kDa class I chitinase harboring a hevein-like N-terminal domain, and represents a major banana allergen in patients suffering latex-fruit syndrome. It displays very similar structural (*i.e*. amino acid sequence) and immunological properties to Pers a 1, its homologous class I chitinase and allergen from avocado fruit. Currently, three additional banana allergens have been added to the list: Mus a 3.0101, a non-specific lipid transfer protein (LTP), and Mus a 4.0101 and Mus a 5, corresponding to a thaumatin-like protein (TLP) and β-1,3-glucanase, respectively (Palacin *et al*., 2011).

Banana endo-β-1,3-glucanase and thaumatin-like protein are pathogenesis-related (PR) proteins (Barre *et al*., 2009). The glucanase is a 33 kDa protein belonging to the PR-2 plant protein family (Barre *et al*., 2009), whose putative role in the latex-fruit syndrome has been suggested, but not clearly demonstrated (Wagner *et al*., 2005). The PR-2 family also comprises well-known allergens from rubber latex, Hev b 2 (Heucas *et al*., 2001), and olive pollen, Ole e 9 (Yagami *et al*., 2002). Both are N-glycosylated glucanases (Yagami, 2002; Yagami *et al*., 1998), which have been proposed to be involved in latex- and/or pollen-plant food cross-reactivity (Yagami, 1998; Palomeras *et al*., 2005; Barber *et al*., 2008), and behave commonly as minor allergens, although sensitization to Ole e 9 significantly increases in geographical areas where olive pollen exposure is high (Leone *et al*., 2006). The 21 kDa banana TLP is the second PR protein (PR-5 family) targeted as a potential allergen (Breiteneder, 2004). Homologous allergenic proteins have been described in plant foods and pollens (Gavrović-Jankulović *et al*., 2002). Thus, Act d 2 from kiwifruit (Palacin *et al*., 2008), a food related to latex and banana allergy, has been recently identified as a major allergen in Spanish adults with kiwifruit allergy (Oberhuber *et al*., 2008).

Interestingly, the apple thaumatin-like allergen Mal d 2 is glycoprotein with complex asparagine-linked glycans (Palacin *et al*., 2008; Oberhuber *et al*., 2008; Breiteneder, 2000).

1.6.2. β-1, 3-glucanase

β- 1, 3-glucanase was designated as Mus a 5. In s study where sera from 51 banana allergic patients were examined, purified natural Mus a 5 reacted with serum IgE from 84% of sera (Grob *et al*., 2002).

The biological role of this protein is the hydrolyses of 1,3-β-D-glycosidic bonds in 1,3-β-D-glucans, such as laminarin and paramilon, and the degradation of glucans involved in the composition of hyphae filamentous fungi, and thus it has a protective role, and belongs to PR-2 proteins. The amount of β-1,3-glucanase in banana increases significantly during ripening (Breiteneder, 2000).

The E.C. number of β-1,3-glucanase is 3.2.1.39. This enzyme is a homodimer that is stable at temperatures up to 70°C . The molecular weight of one subunit is 33.451 kDa (Heucas *et al*., 2001). Two Mus a 5 isoforms were determined by 2D immunoblot technique (pI 7.7 and 8), and both isoforms have an identical N-terminal amino acid sequence of the first 10 amino acids (Aleksic *et al*., 2012). Banana glucanase is made up of 312 amino acid residues and the amino acid sequence of this protein (without the signal sequence) is given in Figure 5.

IGVCYGMLGNNLPPPSEVVSLYKSNNIARMRLYDPNQAALQALRNSNIQVLLDV
PRSDVQSLASNPSAAGDWIRRNVVAYWPSVSFRYIAVGNELIPGSDLAQYILPAM
RNIYNALSSAGLQNQIKVSTAVDTGVLGTSYPPSAGAFSSAAQAYLSPIVQFLASN
GAPLLVNVYPYFSYTGNPGQISLPYALFTASGVVVQDGRFSYQNLFDAIVDAVFA
ALERVGGANVAVVVSESGWPSAGGGAEASTSNARTYNQNLIRHVGGGTPRRPG
KEIEAYIFEMFNENQKAGGIEQNFGLFYPNKQPVYQISF

Figure 5. Amino acid sequence of β-1, 3-glucanase from banana

This allergen is not yet fully characterized in terms of IgE binding epitopes and it is necessary to do its detailed characterization (Heucas *et al*., 2001). Its homolog is an important latex allergen β-1, 3-glucanase (Hev b 2) from *Hevea brasilianis*, a tropical tree used as the raw material for production of latex (Grob, 2002). In addition to homology with Hev b 2, banana β-1,3-glucanase shows significant homology with the Ole e 9 allergen from olive pollen (Barre *et al*., 2009).

The aim of this thesis was to produce recombinant β-1, 3-glucanase from banana denoted as rMus a 5 for the application in component resolved diagnosis of banana allergy. For this purpose, optimization of the recombinant protein production and detailed biochemical and immunochemical characterization was performed.

2. Results

2.1. Cloning of recombinant β-1, 3-glucanase from banana

Gene of β-1, 3-glucanase designed as *Mus a 5* was isolated from total RNA of banana fruit by an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was transcribed by a RevertAidTM First Strand cDNA synthesis kit (Fermentas UAB, Vilnius, Lithuania). For amplification of the mature gene of Mus a 5, sense and antisense – specific primers with EcoR I and Xho I restriction sites 5'-*GAATTC*ATTGGTGTCTGCTACGG-3' and 5'-*CTCGAG*CTAAAAGCTTATTTGGTAGAC-3' were used, respectively. The amplified Mus a 5-encoding fragment was cloned into a pGEX-4T vector, and the construct was verified by DNA sequencing. The cloning strategy was to produce recombinant Mus a 5 (GenBank GQ268963) with glutathione-*S*-transferase as an expression tag on the N-terminal.

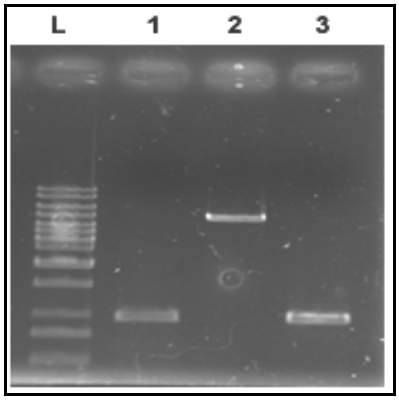

Figure 6. Agarose gel electrophoresis: L) Ladder 1 kb (Fermentas), 1) and 3) banana glucanase gene (1023 bp), 2) pGEX-4T.

The genes of banana glucanase (1023 bp) and pGEX-4T were digested with restriction enzymes XhoI and BamHI. After isolation form agarose gel the gene was ligated into the vector.

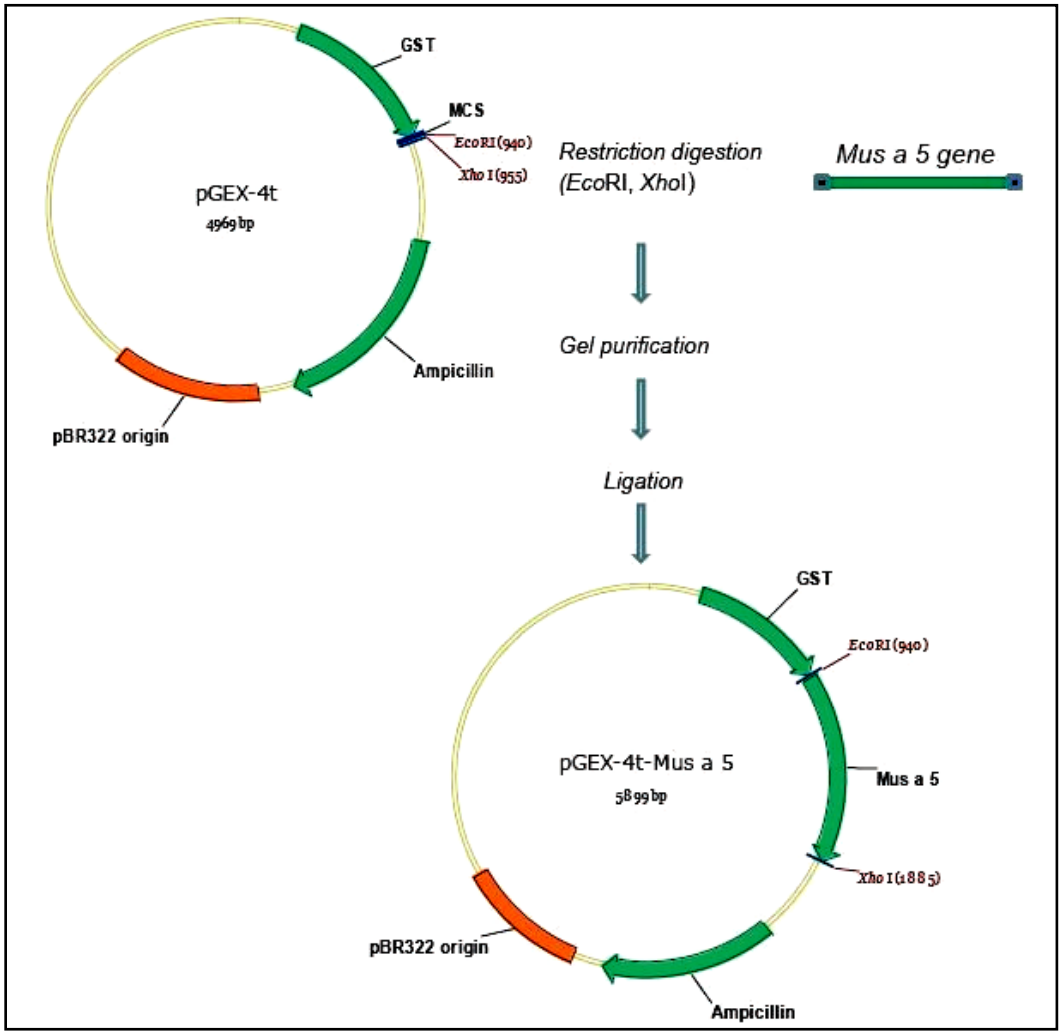

Figure 7. Cloning strategy of Mus a 5 gene into the pGEX-4T vector.

The cloning strategy introduced GST tag on the N-terminal of the GST-Mus a 5 construct (Figure 7).

2.2. Cell growth and induction of protein expression

In order to optimize protein expression of a given construct, a time course analysis of the level of protein expression is recommended. Therefore, for the optimization of rGST-Mus a 5 expression upon induction of protein synthesis, BL21 (DE3) cells were grown at different temperatures, i.e., 25, 30 and 37°C. Aliquots taken after 3, 6 and 12 h upon rGST-Mus a 5 induction were analyzed by SDS-PAG electrophoresis. Densitometric comparison of the intensity of the rGST-Mus a 5 band with all other constitutively expressed proteins per lane, revealed that the highest yield of expression was obtained after 12 h of induction of protein synthesis under the all tested temperatures (Figure 8). For the production of recombinant Mus a 5 inocula were prepared from transformed BL21 cells that were grown overnight at 37 °C in LB medium with antibiotics (100 mg L^{-1} ampicillin, 25 mg L^{-1} chloramphenicol and 25 mg L^{-1} kanamycin). The culture (0.5 mL) was introduced into 10 mL of LB medium containing the respective antibiotics. Once the absorbance (OD_{600}) reached a value of 0.6 following an initial growth phase, protein expression was induced with 1 mM IPTG (Fermentas), and the cells were grown at 25, 30 or 37 °C. Following induction of protein expression, aliquots (1 mL) were taken after 3, 6 and 12 h. The band of about 60 kDa, representing rGST-Mus a 5, is predominant in all tested samples, except in the non-induced cells with the pGEX-4T-glucanase plasmid (Figure 8, lane 1) and after 3 h of induction of protein synthesis at 25 °C (Figure 8, lane 2). Taking into consideration the presence of other proteins in the cell lysates, the optimal conditions for the protein expression and further downstream purification was protein synthesis for 12 h at 25 °C.

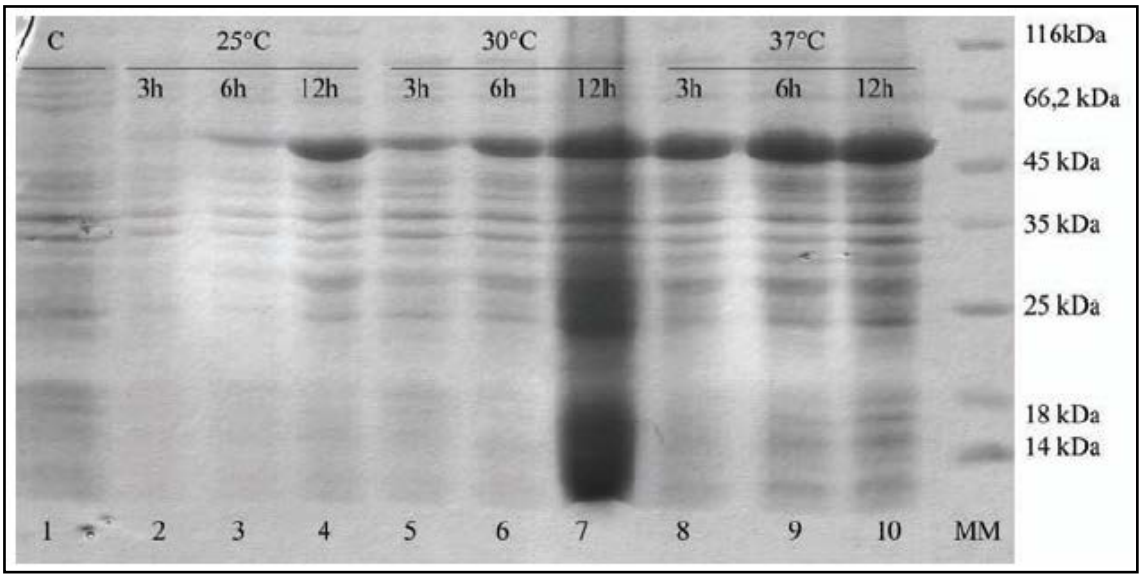

Figure 8. Time course of recombinant Mus a 5 expression at 25, 30 and 37 ℃. Expression of recombinant Mus a 5 was induced with 1 mM IPTG. Aliquots were removed at the times indicated. Proteins were visualized by Coomassie brilliant blue staining; line 1: control, MM: molecular markers.

2.3. Activity of rGST under denaturing conditions

Since a large amount of rGST-Mus a 5 was detected in inclusion bodies we tried to design an enzyme assay for monitoring expression and solubility of GST fusion protein targeted to inclusion bodies. To measure enzyme activity of the isolated rGST-Mus a 5 under denaturing conditions urea was used as a chaotropic agent. Enzyme activity was evaluated after 30 min of incubation in increasing concentrations of urea (0-8 M). It was shown that rGST retains almost 85% of activity after 30 min of incubation in buffer containing 2 M urea, while 50% of its activity was detected following incubation in 5 M urea, indicating that it could be possible to detect enzyme activity of GST-tagged proteins in denaturing conditions of 4 M urea.

In order to assess the possible application of rGST activity assay for monitoring the expression and solubility, the activity of GST expressed in bacterial cell lysate was measured. Aliquots taken during different time intervals of bacterial cell growth and IPTG-induced protein expression were centrifuged and the cell pellet was suspended either in buffer A (0 M urea, non-denaturing conditions) or buffer B (4 M urea, denaturing conditions). Following cell lysis of the samples (three freeze-thaw cycle following sonication) and centrifugation, GST activity was detected in the cell lysates under denaturing conditions. GST activity was more than two-fold higher in the samples obtained following extraction under denaturing conditions (4 M urea). The higher activity detected under denaturing conditions suggest that inclusion bodies had been formed and subsequently solubilized in 4 M urea. This enables detection of rGST both in soluble form and in the solubilized inclusion bodies (Figure 9).

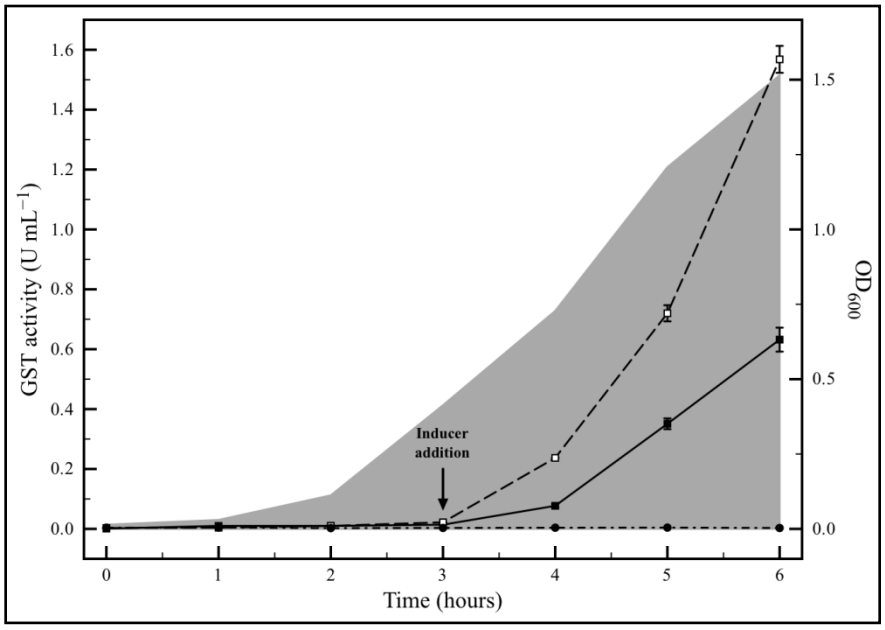

Figure 9. GST activity in bacterial cell lysate detected following extraction using buffer A with 0 M urea (—■—) and buffer B with 4M urea (- -□- -). Filled circles represent GST activity detected in control cells (activity was identical under native and denaturing conditions). Shaded area represents optical density at 600 nm during cell growth.

2.3.1. Monitoring rGST-Mus a 5 expression in inclusion bodies using GST assay

Aliquots taken during different time intervals of bacterial cell growth and IPTG-induced protein expression were centrifuged and the cell pellet was suspended either in buffer A (0M urea, non-denaturing conditions) or buffer B (4M urea, denaturing conditions). Following cell lysis of the samples (three freeze-thaw cycles following sonication) and centrifugation, GST activity was detected in the cell lysates under non-denaturing and under denaturing conditions.

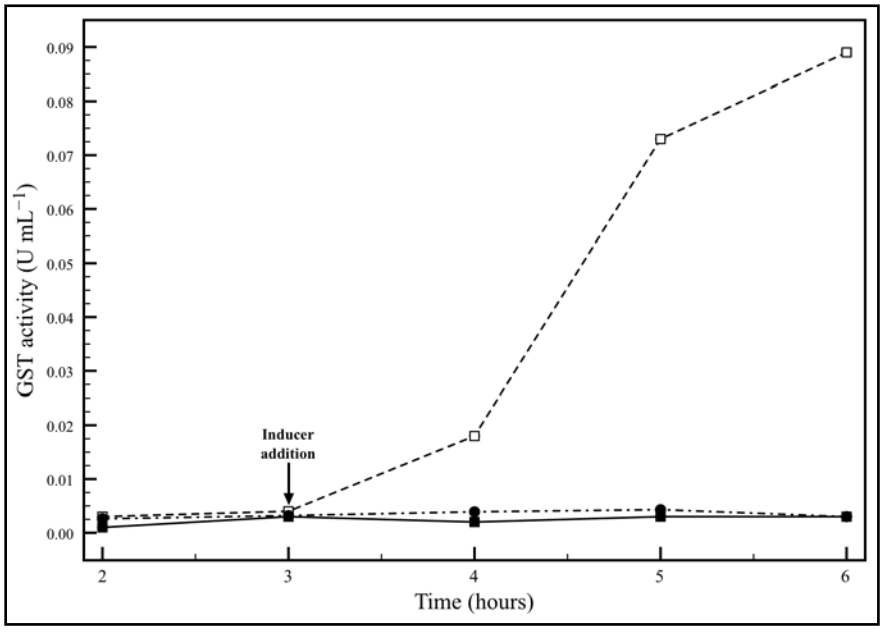

Figure 10. Detection of GST activity of GST-Mus a 5 in bacterial cell lysates prepared using buffer A with 0M urea (—■—) and buffer B with 4M urea (- -□- -). Filled circles represent GST activity detected in control cells (activities were same under native and denaturing conditions).

Under non-denaturing conditions, GST activity of the constructs was almost undetectable, implying aggregation of expressed recombinant protein in inclusion bodies under employed experimental conditions. In the following step, the pellets were solubilized in 4M urea and GST activity was detected in the GST assay with 2M urea. Protein accumulation in

inclusion bodies was confirmed by GST affinity purification protocol for rGST-Mus a 5 (Figure 10).

2.4. Isolation of *in vitro* refolded recombinant GST-Mus a 5

As the cloning strategy adds GST as a tag to the recombinant protein, the purification strategy was to employ glutathione (GSH) affinity chromatography for protein isolation. Recombinant GST-Mus a 5 was isolated from BL21 cells by glutathione affinity chromatography. Prior to affinity purification, the cells were harvested by centrifugation and after resuspension were lysed with lysozyme. Benzonase was employed to reduce the viscosity of the cell lysate caused by nucleic acids. By SDS-PAG electrophoresis and GST enzyme assay it has been revealed that the majority of produced rGST-Mus a 5 was accumulated in the insoluble fraction of the cell lysate, and its solubilization was achieved with 4 M urea. Prior to separation, denatured rGST-Mus a 5 was refolded by rapid batch dilution.

The homogeneity of the isolated rGST-Mus a 5 was assessed by SDS-PAG electrophoresis, revealing a protein band of about 60 kDa (Figure 11). The yield of the purified rGST-Mus a 5 was about 35 mg L^{-1} of LB, as calculated using the molar extinction coefficient for rGST-Mus a 5 (1.434). The extinction coefficient for the GST-Mus a 5 construct was calculated from the primary structure by ProtParam (http://expasy.org/cgi-bin/protparam).

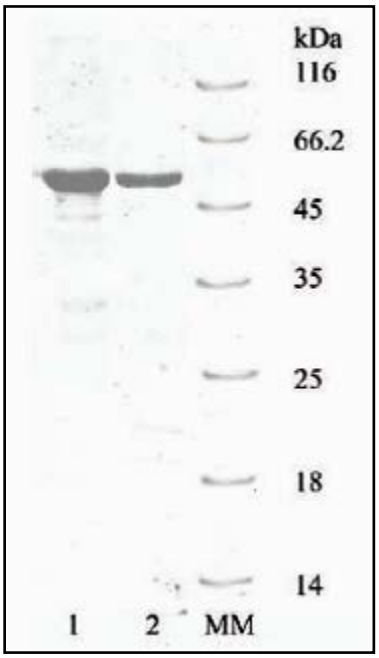

Figure 11. SDS-PAG electrophoresis of purified rGST-Mus a 5 produced in E. coli: 1) fraction of solubilized rGST-Mus a 5, 2) rGST-Mus a 5 eluted from the affinity matrix, MM) molecular markers.

2.5. Optimization of the isolation of recombinant GST-Mus a 5

Affinity chromatography is one of the most diverse and powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. It is based on highly specific biological interactions between two molecules, such as interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. These interactions, which are typically reversible, are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase (Urh, 2009). Glutathion-based affinity chromatography is very often employed method for purification recombinant GST tagged proteins. This method was employed as a first step in the purification of the recombinant GST-Mus a 5 (Figure 12).

Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides, and other charged biomolecules (Bonnerjera, 1986).

Anion exchanger	**Functional group**
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$

The reasons for the success of ion exchange are its widespread applicability, its high resolving power, its high capacity, and the simplicity and controllability of the method. Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Ion exchange separations may be carried out in a column, by a batch procedure or by expanded bed adsorption. Purification protocol for GST-Mus a 5 included separation onto an anion exchanger diethylaminoethyl (structure of DEAE functional group is given above).

Reversed Phase Chromatography (RPC) is a chromatographic method in which the adsorption of hydrophobic molecules onto a hydrophobic solid support occurs in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in desorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent is required to promote de-sorption. Reversed phase chromatography was the last step in the purification protocol of GST-Mus a 5.

After overnight protein expression at 25 °C, BL21 cells were harvested by centrifugation and were re-suspended in ice-cold distilled water (v:v, 1:4). Protein extraction was achieved by sonication, and after centrifugation supernatant was separated onto GST-Bind resin

(Novagen). Details on the experimental work are given in the Experimental part of this thesis.

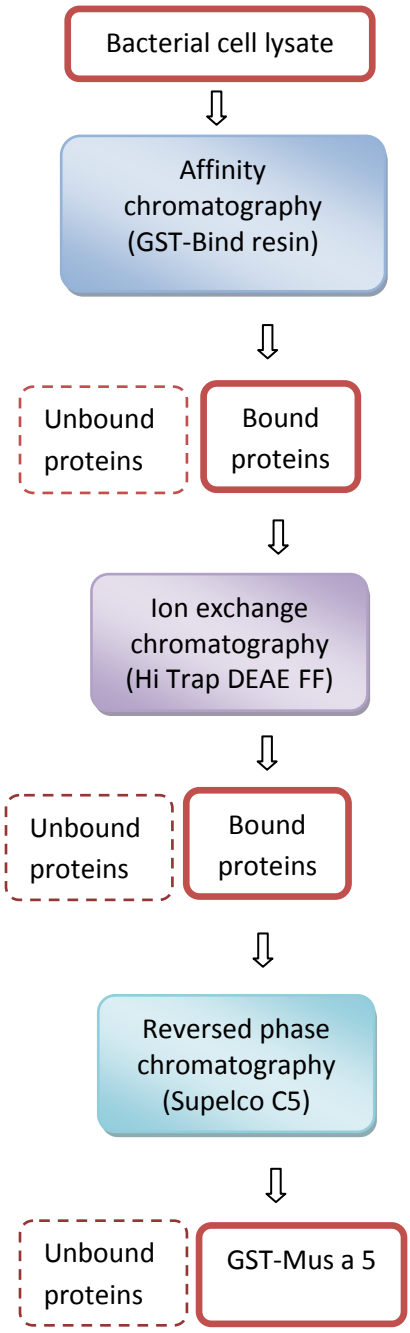

Figure 12. Scheme of the GST-Mus a 5 purification.

Elution of the bound proteins was achieved with GSH, which were afterward separated onto an ion exchange column (Hi Trap DEAE FF, Pharmacia). The final purification step was reversed phase chromatography (Supelco C5, Sigma Aldrich). Recombinant GST-Mus a 5 was eluted at 80 % of Solution B (retention time 15.05 min). All purification steps were analyzed by SDS-PAGE.

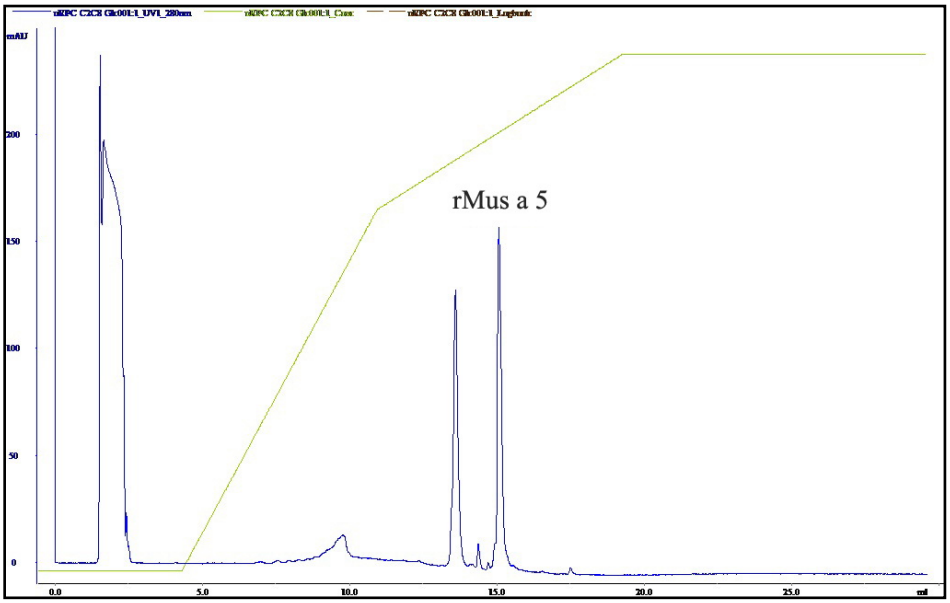

Figure 13. Chromatogram obtained after purification of GST-Mus a 5 onto reversed phase chromatography column (Supelco 5).

By optimizing the elution gradient GST-Mus a 5 was purified by reversed phase chromatography. Under the employed experimental conditions retention time for GST-Mus a 5 elution was 15 min.

By a combination of affinity, ion exchange and reversed phase chromatography GST-Mus a 5 was isolated as a single protein band in reduced SDS-PAGE of about 60 kDa (Figure 14) with a yield of 10 mg mL^{-1}.

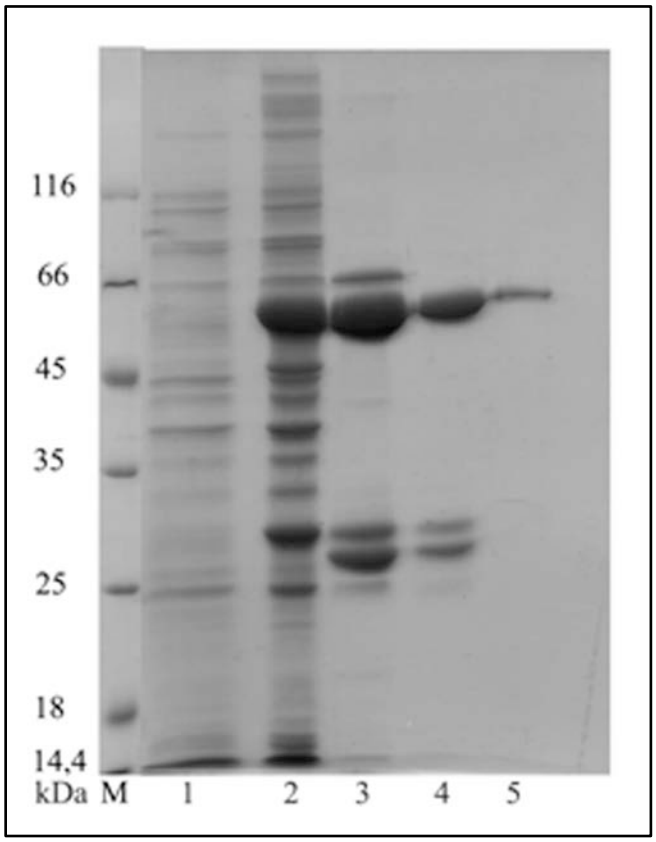

Figure 14. SDS-PAGE analysis of the purification protocol employed for GST-Mus a 5: M) molecular markers, 1) non-induced cells, 2) IPTG induced cells, 3) bound proteins eluted from the affinity column, 4) bound proteins eluted from ion-exchange column, 5) GST-Mus a 5 eluted from RP column.

2.6. Structural characterization of recombinant GST-Mus a 5

For the structural characterization of GST-Mus a 5 microsequencing was performed. The microsequencing (automated Edman degradation) is a method of sequencing amino acids in either a peptide or a protein. Phenylisothiocyanate is reacted with an uncharged N-terminal amino group, under mildly alkaline conditions, to form a cleavable derivative of N-terminal amino acid. Then, after consecutive transformations the more stable phenylthiohydantoin (PTH) amino acid derivative is formed. The PTH amino acid derivative can be identified by

using chromatography or electrophoresis. The procedure can then be repeated again to identify the next amino acid. A major drawback to this technique is that the peptides being sequenced in such manner cannot have more than 50 to 60 residues.

For Edman degradation the proteins may be provided in either solution or as a stained band of PVDF membrane derived from an electroblot. At least 10 pmoles of protein are necessary to run 10-15 cycles. For extended number of cycles it is recommend 100 pmoles of homogeneous protein. Sequencing of short peptides usually require 100-1000 pmoles. For the microsequencing GST-Mus a 5 was electrotransferred onto a PVDF membrane after 2D PAGE. The identity of the construct was confirmed as the first five amino acids determined by Edman degradation (MSPIL).

2.7. Evaluation of immunological reactivity of GST-Mus a 5

For the employment in the component-resolved allergy diagnosis a candidate allergen beside structural features should share also immunological reactivity with its natural counterpart. In this regard various immunological assays find application in the characterization of recombinant allergens. Several methods have been extensively employed for the testing of recombinant allergen immunoreactivity such as evaluation of IgG and IgE reactivity in qualitative, semiquantitative and quantitative assays.

2.7.1. IgG reactivity in immunoblot

For the evaluation of IgG reactivity of GST-Mus a 5 immunoblot and immunoblot inhibition assays were employed. IgE reactivity of GST-Mus a 5 was evaluated by ELISA. Immunoblot alternatively, Western blot, or dot-blot, is an analytical technique used to detect specific proteins in a patients' sample. The proteins to be analyzed are dispensed onto a membrane (usually nitrocellulose, nylon or PVDF), where they are probed by the antibodies of the patient. A simple secondary antibody conjugated to a reporter dye forms

the basis of the color reaction which can be read by eye (qualitative) or instrument (qualitative and quantitative). In the field of diagnostics their use is widespread as a screening tool where a patient can be profiled against multiple markers simultaneously.
Western Blotting (also called immunoblot) is a technique used for analysis of individual proteins in a protein mixture (e.g. a cell lysate). The protein mixture is applied to a gel electrophoresis (SDS-PAGE, native PAGE, isoelectric focusing, 2D gel electrophoresis, etc.) to sort the proteins by size, charge, or other differences in individual protein bands. The separated protein bands are then transferred to a membrane (e.g. nitrocellulose, nylon or PVDF), by process which is called blotting. The proteins adhere to the membrane in the same pattern as they have been separated on the gel. The proteins on this immunoblot are then accessible for antibody binding for detection.

To analyze IgG reactivity of GST-Mus a 5, rabbit polyclonal antiserum produced against natural banana glucanase was employed. GST-Mus a 5 was separated according to *pI* by isoelectric focusing (first dimension), and thereafter was separated according to the molecular mass on a SDS-PAGE (second dimension). Upon equilibration of the SDS-PA gel the protein was electrotransferred onto PVDF membrane for the detection of IgG reactivity.

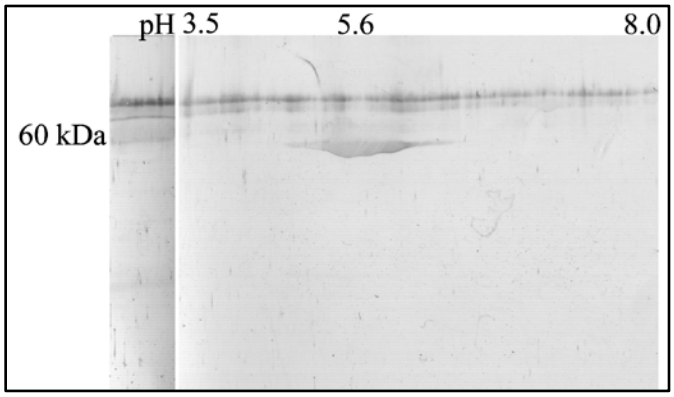

Figure 15. 2-D immunoblot analysis of GST-Mus a 5: isoelectric focusing was performed under native conditions and SDS-PAGE was performed under reduced conditions. Anti-Mus a 5 antibodies were employed for detection of GST-Mus a 5.

In 2-D immunoblot GST-Mus a 5 revealed one broad spot of about 60 kDa with p*I* about 5.7. By using anti-Mus a 5 rabbit antibodies, IgG reactivity of recombinant protein was shown in 2-D immunoblot (Figure 15) indicating presence of immunoreactive epitopes on the recombinant protein.

2.7.2. IgE reactivity of rGST-Mus a 5 in dot blot

The IgE reactivity of the rGST-Mus a 5 was examined by dot blot analysis using the sera of six banana allergic patients. To evaluate the IgE reactivity of the fusion tag, recombinant GST was also tested as a control. IgE reactivity was detected only for the rGST-Mus a 5, (Figure 16) while no IgE binding was found for GST. Although the correct protein folding should be confirmed by a thorough structural characterization, the IgE reactivity of the rGST-Mus a 5 suggests that this protein could find application as a diagnostic reagent in banana allergy.

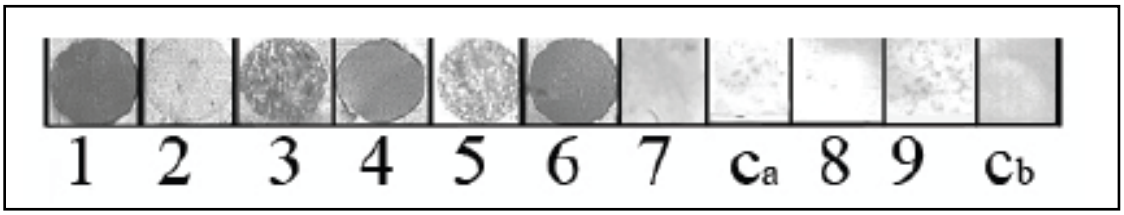

Figure 16. IgE reactivity of rGST-Mus a 5: 1-6) individual sera from banana allergic persons, 7) pool of sera from 3 healthy individuals, c_a) control of secondary antibody, IgE reactivity of rGST: 8 – pool of sera from 6 banana allergic persons, 9 – pool of sera from 3 healthy individuals and cb – control of secondary antibody.

3. Discussion

In this study the molecular and immunochemical characterization of recombinantly produced Mus a 5 allergen from banana fruit was performed. The production of recombinant proteins in a well-characterized form has become an important issue in the pharmaceutical industry (Walsh, 2010). One of the main advantages of recombinant proteins is that they can be fully characterized in terms of their physical, chemical, and immunologic properties and presented as chemically defined entities with all batches deriving from 1 master cell bank (Cromwell et al., 2011). Therefore, preparations for specific immunotherapy and diagnosis can be formulated with consistent high pharmaceutical quality to meet specifications that cannot be achieved with products based on extracts of natural source materials.

Among epitope peptides and proteins developed for recombinant protein over-production, besides His tag, GST is very often employed as a fusion partner. Recombinant fusion proteins containing the complete amino acid sequence of GST exhibit a functional GST enzymatic activity. In addition to improving solubility, GST allows detection of the expressed recombinant protein using enzyme assays and GST-specific antibodies, and also facilitates recombinant protein purification (McTigue et al., 1995). Although most GST fusion proteins are expected to be soluble, many of them, even relatively small ones (40-50 kDa), are partially or completely insoluble following lysis of prokaryotic cells (Frangioni and Neel, 1993; Hansen and Eriksen, 2007). To be able to detect accumulation of rGST-Mus a 5 in inclusion bodies a colorimetric GST activity assay under denaturating (protein solubilization in 4 M urea) conditions has been employed in this study. By monitoring GST activity, expression and accumulation of GST-Mus a 5 in inclusion bodies was detected in a rapid, sensitive, cost effective and easy to perform manner. Compared to immunoassays that are based on quantitative detection of total GST, enzyme assays measure functional

GST activity, and therefore, are more reliable for downstream protein purification. However, the limit of the employment of enzyme assay is that it only confirms correct protein folding of recombinant GST enzyme, but it does not imply correct folding and hence biological activity of the protein of interest. Urea is usually used as a weaker and less expensive denaturant in comparison to guanidin chloride. The GST activity assay performed under denaturing conditions has the potential to be employed as a rapid screening method for determining the approximate expression levels of GST fused proteins targeted to inclusion bodies which can be solubilized in lower chaotrope concentrations (up to 4M urea) and therefore facilitate the subsequent refolding steps of these proteins.

Several recombinant food allergens have been produced as GST tagged proteins, such as tropomyosins from crab (Cha f 1), lobsters (Hom a 1, Pan s 1) and shrimp (Met e 1), but also 2S albumin seed storage protein from walnut (Jug r 1) (Lorenz et al, 2001). In this study rMus a 5 was produced as a recombinant GST-tagged protein in *E. coli*. Recombinant Mus a 5 obtained from the bacterial cell lysate was purified under native conditions by affinity, ion exchange and reversed phase chromatography. Although protein expression was optimized, relatively high amount of recombinant protein was entrapped in inclusion bodies. The N-terminal sequence of the first five amino acids of purified protein was confirmed by Edman degradation. The protein revealed one spot (60 kDa, pI 5.7) which was immunoreactive with rabbit anti-Mus a 5 polyclonal antibodies in 2-D immunoblot. By using blood samples of latex allergic persons and the set of synthetic 15-mer peptides Barre et al., identified five IgE reactive epitopes along the entire amino acid sequence of Mus a 5. The first IgE reactive epitope was localized fifteen amino acids from the N terminus. As it was shown for GST-tagged tropomyosins (Lorenz, 2001) it seems that IgE reactivity of rMus a 5 was not affected by GST tag, settled on the N terminus. However, epitope mapping with a representative pool of banana allergic patient's sera should be performed to assess linear and eventual conformational IgE epitopes on Mus a 5.
Various endogenous and exogenous factors influence protein expression pattern in banana fruit (Peumans et al., 2002, Choudhury et al., 2009, Choudhury et al., 2010). Comparative analysis revealed differences in β-1,3 glucanase gene expression, glucanase activity and

fruit pulp softening rates in three naturally occurring cultivars. Also, proteins from natural biological material often exhibit microheterogeneity. Natural Mus a 5 revealed several pI isoforms in range 7.7 to 8.0 (Aleksic et al., 2012). Recombinant Mus a 5 is a homogenous protein species and its expression yield in the prokaryotic expression system was about 10 mg per liter of the cell culture.

Current *in vitro* diagnosis of food allergy is limited because of inconsistent quality of plant-food allergen extracts. Considerable knowledge obtained on food allergen characteristics can facilitate replacement of non-standardized food allergen extracts with component-resolved diagnostic approach. Although it is tested in a rather small group of banana allergic persons, recombinant Mus a 5 showed IgE reactivity comparable to nMus a 5. Therefore, further testing of clinical reactivity of rMus a 5 can approve its application in the component-resolved diagnosis of banana allergy.

4. Experimental work

4.1. Growth Media and Antibiotics

In this work growth media for growth of strains of *Escherichia coli* for multiplication of vectors and expression of target proteins were Luria-Bertani (LB), and Luria-Agar (LA).

4.1.1. Luria-Bertani (LB) medium

LB medium	Per liter
Tryptone (Torlak)	10 g
Yeast extract	5 g
NaCl	5 g
1M NaOH	1 mL
Distilled water[#]	up to 1000 mL

[#] Distilled water was added up to 900 mL and the pH was adjusted to 7.5, after which water was added up to the 1 liter.

In 0.5 liters of LM medium was prepared in a 1-liter flask and heated with stirring until chemicals were dissolved. The medium was poured into bottles with loosened caps and autoclaved 20 min at 15 lb/in^2. Before the addition of antibiotics the suspension was cooled to ~50 ºC, and the caps were tightened after the bottles cooled down to <40 ºC. The medium prepared in such a manner can be stored indefinitely at room temperature. Amplicilin was added to the final concentration of 100 μg mL^{-1} of LB.

4.1.2. Luria-Bertani (LB) agar

LB agar	Per liter
Tryptone (Torlak)	10 g
Yeast extract	5 g
NaCl	5 g
1M NaOH	1 mL
Agar (Torlak)	15 g
Distilled water	up to 1000 mL

The listed chemicals were mixed by stirring and autoclaved for 25 min. The medium was cooled down to ~50 ºC before addition of antibiotics (ampicilin, 100 μg mL^{-1}) with stirring. About 40 mL of liquid LB agar was poured per 10/cm plate (~25 to 30 plates per liter). The plates were left until agar solidifies with the lids off for about 20-30 min at room temperature. Wrapped dry plates were stored at 4 ºC.

4.2. Amplification of glucanase gene by PCR

The glucanase gene was amplified by PCR with primers carrying restriction sites for EcoRI and Xho I.

4.2.1. PCR reaction

The primers used for gene amplification were as follows:

Forward primer: 5'- GAATTCATTGGTGTCTGCTACGG -3'
Revere primer: 5'- CTCGAGCTAAAAGCTTATTTGGTAGAC -3'

The PCR reaction was set up as follows:

Buffer 10x	10 μL
10 mM dNTPs	1.0 μL
10 mM forward primer	1.0 μL
10 mM reveres primer	1.0 μL
Dream Taq Polymerase	0.5 μL
Template DNA	1.0 μL
dH_2O	85.5 μL
Total volume	100.0 μL

Water was added first, than the buffer, nucleotides (dNTP), primers, template DNA, DNA polymerase, and at the end oil to prevent evatoration.

PCR was performed under the following conditions:

Step	Temperature (ºC)	Time (sec)	No. of cycles
Initial denaturation	94	180	
Denaturation	94	45	30
Annealing	63	45	
Extension	72	45	
Final elongation	72	600	

To analyze the reaction mixture after PCR samples were analyzed by agarose gel electrophoresis.

4.2.2. Purification of PCR product

Purification of the PCR product was performed by GeneJET^{TM} PCR purification kit (Fermentas).

Reagents

Buffer PBI	450 μL
Buffer PE	750 μL
PCR reaction mixture	90 μL
dH_2O	100 μL

Procedure

1. Buffer PB1 (450 μL) was placed into QIA tube and 90 μL of sample was mixed and centrifuged at 10 000 rpm for 1 min.
2. Buffer PE (750 μL) was added to the column and the QIA tube was centrifuged at 13 000 rpm for 1 min. After centrifugation the flow through solution was discarded and the columns were centrifuged at 13 000 rpm for 1 min to dry.
3. To elute the DNA distilled water (40 μL) was added to the column and incubated for 1 min and afterwards the column was centrifuged at 13 000 rpm for 1 min.
4. Eluted samples were collected in sterilized tubes. The purified PCR products were used for further experiments.

4.3. Cloning of banana glucanase gene into pGEX-4T vector

4.3.1. Agarose gel electrophoresis

1. Materials and solutions

1. TBE buffer, 10X concentrated

Tris base [tris(hydroxymethyl)aminomethane]		108 g
Boric acid		55 g
EDTA, disodium salt		7.5 g
deionized water	up to	1000 mL

2. Agarose gel (1%)

Agarose (TopVisionTM LEGQ Agarose, Fermentas)	1g
TBE buffer	99 mL

For the preparation of agarose gel 1 g of agarose is mixed with 99 mL of TBE buffer into the glass flack. The suspension is heated until it dissolves, and boils for additional 2 min.

3. Orange loading dye (Fermentas) 10 μL loading buffer
4. DNA samples
5. DNA molecular weighs markers (ladder)
6. Ethidium bromide (10 mg mL^{-1})
7. Water bath
8. Horizontal gel electrophoresis apparatus
9. Gel casting platform
10. Gel combs (slot former)
11. DC power supply

Procedure

1. Add electrophoresis-grade agarose to 1 × TBE electrophoresis buffer. Melt in a microwave oven or autoclave, mix thoroughly, and cool to 55 °C. Pour liquid agarose solution into a sealed gel casting platform and insert comb, ensuring that there are no bubbles. Allow gel to harden.

2. Remove comb taking care not to tear the sample wells. Place the gel with the platform in tank. Add electrophoresis buffer to cover the gel ~1 mm above surface, making sure no air is trapped in the wells.

3. Add 10× loading buffer to DNA samples (final concentration 1×) and load samples with a micropipette. Also load DNA molecular weight markers (ladder) for comparison.

4. Attach leads so that DNA migrates toward the anode or positive lead. Electrophoresis was run at 1 to 10 V cm^{-1} of gel. Monitor the progress of separation process by migration of dyes in the loading buffer.

5. Turn off the power supply when bromphenolblue has migrated a distance judged sufficient for separation of the DNA fragments.

6. Stain 10 to 20 min in solution of ethidium bromide. Visualize and photograph DNA by placing gel on a UV light source.

DNA fragments of ~0.5 to 25 kb are well resolved using this method.

4.3.2. Digestion of PCR product and pGEX-4T expression vectors

For the protein expression gene of glucanase has been cloned into expression vectors pGEX-4T. PCR product and pGEX-4T were cut with EcoRI and Xho I restriction enzymes for 1.5 hours at 37 °C. The samples were centrifuged for 20 sec. at 13 000 rpm and analyzed by agarose gel electrophoresis.

Reaction mixture	pGEX-4T	PCR product
10× Tango buffer	5 μL	5 μL
Plasmid	30 μL	30 μL
EcoRI	2 μL	2 μL
Xho I	2 μL	2 μL
dH_2O	6 μL	6 μL
Incubation	1.5 h, 37 ºC	1.5 h, 37 ºC
Total volume	50.0 μL	50.0 μL

After digestion the banana glucanase gene and pGEX-4T were isolated form agarose gel. For the cloning of the glucanase into expression vector ligation mixture was prepared.

4.3.3. Ligation of banana glucanase into pGEX-4T

Ligation of the glucanase gene into pGEX-4T was performed under following conditions:

10 × Ligation buffer	1.0 μL
Insert	5.0 μL
Plasmid	2.5 μL
T4 DNA ligase (Fermentas)	0.5 μL
dH_2O	1.0 μL
Total volume	10.0 μL
Incubation	overnight, 16 ºC

4.3.4. Transformation of DH5α cells

Procedure

DH5α chemically competent cells, prepared by employing $CaCl_2$, were used for transformation with the ligation mixture of pGEX-4T with ligated PCR product.

1. The ligation mixture (10 μL) was added to the chemically competent DH5α cells (100 μL), and the mixture was incubated on ice for 10 min, than on 42 ºC for 42 sec., on ice for 5 min, and upon addition of 0.5 mL of LB into the tube was incubated for 1h at 37 ºC with agitation (200 rpm).
2. DH5α cells were transferred onto two LB agar plates containing antibiotic ampicilin (100 μg mL^{-1}) and were incubated overnight at 37 ºC.
3. Five colonies were transferred from the agar plates to LB media. All work was done in sterile conditions.

4. In five tubes with LB media (10 mL), ampicillin (10 μL, conc. 100 mg mL^{-1}) was added. Each tube was inoculated with one colony from the plate by micropitte and vortexed to mix the media and to resuspend the cells. The tubes were incubated overnight at 37 ºC with shaking.

4.3.5. Isolation of plasmid from DH5α cells

Colonies of DH5α cells transformed with pGEX-4T-glucanase were used for isolation of these plasmids. One mL of each sample was transferred to eppi tubes and centrifuged for 1 min at 13 000 rpm. Supernatants were discarded and an additional 1 mL of the samples were transferred into their respective tubes and centrifuged for 1 min at 13 000 rpm. The supernatants were discarded and the remaining pellets were used for plasmid purification.

Plasmid purification was done according to the following procedure:

1. The cell pellets were resuspended in 250 μL of the resuspention solution and vortexed.
2. Lysis solution (250 μL) was added and the tubes were inverted 4-6 times.
3. The neutralisation solution (350 μL) was added and tubes were inverted 4-6 times. The tubes were centrifuged for 5 min. at 13 000 rpm.
4. The supernatant was transferred to a blue column and centrifuged for 1 min at 13 000 rpm. The flow through was discarded.
5. Five hundred μL of wash solution was added and centrifuged for 1 min at 13 000 rpm.
6. The flow through was discarded. The washing and centrifugation was done for 1 min at 13 000 rpm.
7. The column was transferred into a new tube, and 35 μL of distilled water was added to the column. The tube was incubated for 2 min, and afterward centrifuged for 2 min.
8. Detection of plasmids was done by agarose gel electrophoresis (2.5 μL of sample with 1 μL of dye).

After UV light analysis of the agarose gel, positive clones of pGEX-4T-glucanase were identified.

4.3.6. Digestion of pGEX-glucanase vector

From positive clones the pGEX-4T-glucanase vector was isolated and digested with restriction enzymes XhoI and EcoRI. For this purpose large scale purification of the vector was performed.

Reaction mixture

10× Tango Buffer	5.0 μL
XhoI	2.0 μL
EcoRI	2.0 μL
Vector	37.0 μL
dH_2O	6.0 μL
Total volume	50.0 μL

All components were mixed at 37 ºC for two hours and afterwards the temperature was increased to 80 ºC, for 20 min to inactivate the restriction enzymes. The samples were centrifuged for 20 sec. at 10 000 rpm and thereafter were analyzed by agarose gel electrophoresis.

4.3.7. Transformation of BL21(DE3) cells with the pGEX-glucanase

BL21 (DE3) cells were transformed with the pGEX-glucanase. For transformation cells were incubated with the vector for 10 min on ice. Thereafter, 0.5 mL of LB was added into the tube, which was then incubated in a water bath at 42 ºC for 1 min, and then at 37 ºC for 60 min.

BL21 cells (0.15 mL) were spread per plate and were incubated overnight at 37 ºC. Three colonies from each plate were transferred into separate tubes containing LB media grown overnight at 37 ºC.

4.4. Induction of protein expression

Inocula were prepared from transformed BL21 cells that were grown overnight at 37 ºC in LB medium with antibiotics. The culture (0.5 mL was introduced into 10 mL of LB medium containing respective antibiotics. Once absorbance (OD_{600}) reached a value of 0.6 following an initial growth phase, protein expression was induced with 1 mM IPTG (Fermentas), and cells were grown at 25 ºC, 30 ºC, and 37 ºC, respectively. Following induction of protein expression, aliquots (1 mL) were taken after 3h, 6h, and 12 h.

4.5. Monitoring enzyme activity of GST and GST-Mus a 5 in the presence of urea

To monitor GST and GST-Mus a 5 activity in the presence of urea, BL21 cells transformed with respective plasmids (pGEX-4T and pGEX-4T-Mus a 5) were grown in LB media at 37 °C. Once optical density value of 0.4 was reached (OD_{600} 0.4), 1 mM IPTG was added in order to induce protein expression. During the course of cell growth and protein expression induction, aliquots (1 mL) were taken and cells harvested by centrifugation (5 min at 14,000 × g). The pellet was suspended either in buffer A (100 mM KH_2PO_4, pH 6.5, 100 μL) or buffer B (100 mM KH_2PO_4, pH 6.5, containing 4 M urea, 100 μL). Following three freeze-thaw cycles, suspensions were sonicated in ice water bath (20 min at 20 rms). Insoluble cell debris was removed by centrifugation (15 min at 14000 × g) and clarified supernatant was used for the enzymatic assay. For detection of GST activity in cell lysates, samples (100 μL) were mixed with 100 mM GSH (2 μL) and 2.08 mM CDNB (98 μL).

Chemically-competent *E. coli* cell lysate was used as control. Calculated activities were adjusted for values obtained from non-enzymatic controls.

4.6. Soduim dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sample Buffer (5×)

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

The protein sample was mixed with the sample buffer (4:1, v:v), and that boiled for 5 minutes.

Running Gel Solution (1×)

For different applications desired percentage of acrylamide gel should is prepared. To make up thirty mL of running gel selection of one of the following protocols (percentages) was followed, and respective ingredients were mixed. After adding TEMED and APS solution quickly polymerized into a gel.

	7%	10%	12%	15%
H_2O	15.3 mL	12.3 mL	10.2 mL	7.2 mL
1.5 M Tris-HCl, pH 8.8	7.5 mL	7.5 mL	7.5 mL	7.5 mL
20% (w/v) SDS	0.15 mL	0.15 mL	0.15 mL	0.15 mL
Acrylamide/Bis-acrylamide	6.9 mL	9.9 mL	12.0 mL	15.0 mL
(30%/0.8% w/v)				
10%(w/v) ammonium persulfate				
(APS)	0.15 mL	0.15 mL	0.15 mL	0.15 mL
TEMED	0.02 mL	0.02 mL	0.02 mL	0.02 mL

Stacking Gel Solution (4% Acrylamide)

H_2O	3.075 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL
20% (w/v) SDS	0.025 mL
Acrylamide/Bis-acrylamide(30%/0.8% w/v)	0.67 mL
10% (w/v) ammonium persulfate (APS)	0.025 mL
TEMED	0.005 mL

Electrophoresis buffer, pH 8.3

0.25 M Tris	3.0 g
0.192 M Gly	14.4 g
0.1% SDS	1.0 g
Destilled water up to	1000 mL

Staining solution for PAA gels

0.1% CBB G ili R 250	0.5 g
50 % methanol	100.0 mL
10% acetic acid	10.0 mL
Destilled wated	up to 200.0 mL

Fixing solution (50% methanol, 10% acetic acid)

methanol	500 mL
acetic acid	100 mL
Destilled wated	up to 1000 mL

Destaining solution (25 % methanol, 5 % acetic acid)

methanol	250 mL
acetic acid	50 mL
Destilled water	up to 1000 ml

Running the gel

The gel was clamped in and both buffer chambers were filled out with gel running buffer according to the instructions manual. The power leads were attached and the gel was run until the blue dye front reaches the bottom. The gel was removed from the power supply and processed further. Proteins were visualized by using Coomassie Brilliant Blue.

4.7. Optimized protocol for isolation of the recombinant GST-Mus a 5

After growing overnight cells were harvested by centrifugation (3000 × 30 min at 4 ^{o}C), re-suspended in ice-cold distilled water (1:4,v:v) and extracted by sonication (5×10 seconds at 25 W, Branson sonifier 150, Branson Ultrasonics, Danbury, Connecticut). After centrifugation (3000 × g, 30 min) PBS (1.5 M) was added to the cell supernatant (1:10, v:v), and after additional centrifugation (13000 × 5 min) protein solution was applied onto GST-Bind resin (Novagen, Darmstadt, Germany). Elution of the bound proteins was achieved with 50 mM Tris-HCl, 10 mM GSH, pH 8,0. After dialysis against 20 mM Tris pH 8,3 the recombinant protein was purified by ion exchange (Hi Trap DEAE FF, GE Healthcare, Little Chalfont, UK) chromatography on Akta Purifier (GE Healthcare, Little Chalfont, UK). The final purification step was reversed phase chromatography (Supelco C5, Sigma Aldrich, St. Louis, Missouri). The column was equilibrated with solution A (0,1% TFA), and bound proteins were eluted with a gradient of 10 CV of solution B (0,1% TFA in 90% ACN). Recombinant glucanase was eluted at 80% of solution B (retention time 15.06 mL). Purification steps were analyzed by SDS-PAGE.

4.8. N-terminal sequencing

For N-terminal sequencing recombinant glucanase was resolved on SDS-PAGE and thereafter transferred by semidry blotting onto a PVDF membrane (Serva, Heidelberg,

Germany). The membrane was subsequently washed in double-distilled water, stained (0.1 % Coomassie Blue, Serva, in 50% methanol), destained (50% methanol) and air-dried. The protein band was excised and microsequencing was kindly performed by Prof. Arnd Petersen, Borstel Research Center, Germany, by using a Procise protein sequence connected to an online PTH amino acids analyzer (PE Biosystems, Weiterstadt, Germany).

4.9. Production of polyclonal antibodies

For the production of the polyclonal antibodies to banana extract two rabbits were immunized according to the protocol described by Harlow and Lane. In brief, 0.25 mL of banana extract (0.5 mg mL^{-1}) which was prepared according to Gavrović-Jankulović et al. (2008), was mixed with 0.25 mL CFA (complete Freunds adjuvant) for the first immunization. Every 15 days, for four months, the rabbits were boosted with a mixture of 0.25 mL of the banana extract and 0.25 mL of IFA (incomplete Freunds adjuvant). Each rabbit was subcutaneously immunized with 0.5 mL of the emulsion. After four months, sera were collected and the antibodies were pooled and fractionated by ammonium sulfate (50% saturation). After dialysis against phosphate buffer saline (PBS) antibodies were aliquoted and stored in 20% glycerol at –20°C.

Antibodies against natural banana glucanase were raised in rabbits according to Harboe and Ingild, 1983, and were kindly provided by Ivana Aleksic, from the Institute for Virology, Vaccine and Sera, Torlak, Serbia.

4.10. Immunodetection

4.10.1. Immunoblot

Tris buffer saline (TBS), pH 7.6

30 mM Tris	3.63 g
150 mM NaCl	9.0 g
Destilled water	up to 1000 mL

TTBS (0.1%)

Tween 20	0.1 mL
TBS	99.9 mL

Blocking solution (5%)

skim milk	5 g
TTBS	up to 100 mL

Antibody dillution solution

0.1% solution of skim milk in TTBS

Buffer for alkaline phosphatase (AP) pH 9,6

100 mM TRIS	1.2 g
50 mM $MgCl_2$ x$5H_2O$	10.0 mg
Destilled water	up to 100.0 mL

* pH adjusted with 3M NaOH

AP substrate prepatration

Substrate 1		Substrate 2	
BCIP	50 mg	NBT	100 mg
AP buffer	20 ml	AP buffer	300 ml

Solutions of substrate 1 and substrate 2 are made before usage. One volume of substrate 1 is mixed with 9 volumes of substrate 2 and filtred.

Protocol:

• Destaining of a membrane in tTBS for10 min.

• Blocking of the membrane with 5% skim milk (in tTBS-u) 1 h at RT with shaking.

• Washing of the membrane with tTBS (3 × 10 min).

• Incubation of the membrane with the primary antibodies for 2 to 4 h at RT with shaking.

• Washing of the membrane with tTBS (3 × 10 min).

• Incubation of the membrane with the secondary antibodies for 1 h at RT with shaking.

• Washing of the membrane with tTBS (3 × 10 min).

• Incubation of the membrane with the alkaline phosphatase labeled tertiary antiboy fod 1 h at RT, with shaking.

• Washing of the membrane with tTBS (3 × 10 min).

• Washing of the membrane with TBS (1 × 10 min).

• Detection of antigen-antibody interactions with BCIP/NBT substrate for alkaline phosphatase.

4.10.2. Dot blot and 2-D immunoblot

Six sera from persons with positive clinical history to banana allergy and positive skin prick test to banana extract were used for the evaluation of IgE reactivity of rGST-Mus a 5 in dot blot. A pool of three sera from persons without banana allergy was used as a control. Purified rGST-Mus a 5 (5 μg) was applied to a nitrocellulose membrane (NC) using 96-well dot blot hybridization manifold (VWR, Vienna, Austria). To discriminate IgE reactivity of the GST tag from IgE reactivity of glucanase, rGST was applied onto the NC membrane as a control.

Membranes were blocked using 30 mM Tris buffer saline (TBS) pH 7.4, containing 5% w/v skimmed milk for 1 h at RT. Membranes were incubated with the pooled sera in 1 % skimmed milk in TBS (dilution, 1:3, v:v) overnight at RT. Goat anti-human IgE (dilution 1:10000, v:v) was used for detection of IgE binding. Alkaline phosphatase-labeled rabbit

antigoat IgE antibodies (dilution 1:30000, v:v, Sigma) were used as the tertiary antibody. IgE reactive spots were visualized with BCIP/NBT solution.

For detection of IgG reactivity of rGST-Mus a 5, rabbit antibodies against banana extract, or anti-banana glucanase rabbit antibodies were employed. After blocking, the membrane was incubated with the primary antibodies (dilution 1:5000) for 2 h at room temperature. After three washings, the membrane was incubated with alkaline phosphatase anti-rabbit antibodies (dilution 1:30000, Sigma–Aldrich, Missouri, USA).

For 2D-PAGE, recombinant glucanase (15 μg) was separated by isoelectric focusing (IEF) under native conditions, and subsequently, strip was transferred onto SDS-PA gel (4% stacking and 12% resolving gel). The pH gradient of the IEF gel was determined by reading pH of 10 mM KCl solution (1 mL) in which slices of the gel (0.5 × 1 cm) were suspended for 30 min. For immunoblot analysis, separated proteins were transferred onto a PVDF membrane (Serva, Heidelberg, Germany) under 1 mA cm^{-2} for 1 h. After blocking in 5% skim milk in TPBS (150 mM NaCl, 12 mM Na_2HPO_4, 3 mM KH_2PO_4, 0.3% BSA, 0.01% Tween 20, pH 7.4) of the membrane for two hours, recombinant glucanase was detected using anti-glucanase polyclonal rabbit antibodies (1:5000, v:v) in TPBS. Goat alkaline phosphatase-labeled anti-rabbit secondary antibodies (1:30 000, v:v, Millipore, Billerica, MA, USA) in TPBS, were used for antigen detection. Visualization of the reaction was achieved with 5-bromo-4-chloro-3-indolyl phosphate/4-nitroblue tetrazolium (BCIP/NBT, Sigma-Aldrich, St. Louis, USA).

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So far he has published three scientific papers.

Прилог 1.

Изјава о ауторству

Потписани-а _____

број уписа _____

Изјављујем

да је докторска дисертација под насловом

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_____

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, _____

_____

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора _____

Број уписа _____

Студијски програм _____

Наслов рада _____

Ментор _____

Потписани _____

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, _____

_____

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

_____

_____

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

4. Ауторство – некомерцијално – делити под истим условима

5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, _____

_____