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„Formulation development of a new hypoallergen birch pollen vaccine and its physico chemical validation by ICH conform quality control assays for a first in man clinical trial"

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1.) Abstract

Allergy is the most prevalent chronic inflammatory disease, having great socio-economic impact. Chronic lifelong dependency on symptomatic drugs is an expensive reality for the vast majority of allergy sufferers. Allergen-specific immunotherapy is the only causal and effective treatment targeting the underlying immune mechanism in a more cost-effective way. Yet, long treatment duration and risk of anaphylactic side-effects have prevented this treatment from becoming the first choice.

The EU-project BM4-SIT aims at providing an escape from its current niche position by making the treatment safer, more effective and patient friendlier, i.e. developing a therapy with negligible side-effects but improved efficacy, achieved by less injections. The concept of BM4SIT is based on two innovations: replacing current natural allergen extracts by mutated recombinant allergens having been designed to be hypo-allergenic but hyper-immunogenic, and adding an adjuvant to support rapid and effective induction of an anti-inflammatory immune response. The allergen derivative chosen to bring this concept from bench to bedside is BM41, a mutant of the culprit allergen of birch pollen allergy, Bet v 1.
The selected adjuvant is vitamin D3, which has been shown to promote active suppression of inflammation, both in vitro and in a mouse model.

One of the important goals of the EU project is to bring BM41 and vitamin D3 via the stages of GMP production, BM41 formulation development, toxicity studies, and assay development for GMP drug product production to the start of Phase II clinical trials.

Present master thesis is an essential part of that EU project, because it covered the crucial stages of BM41-drug substance assay development, BM41-drug product development (vaccine, placebo and SPT solution), BM41 potency test development and complete stability testing of interims drug product batches (vaccine, TOX study batches SPT solutions) including accelerated stability studies for the first human clinical trial.
The result was a successful complete formulation development of BM41-SPT solutions for clinical application, the development of analytical assays and corresponding characterization methods such as SDS-PAGE, Western blot, IgE binding assays, IgG response testing, BM41-specific sandwich ELISA, IgE based potency assay, RP-HPLC,

bio-burden and more for further analysis and testing of a GMP-like material at highest GMP quality standards to ensure filing of a first in man clinical study.

For stability testing of the BM41 drug substance an ICH conform stability testing protocol was established.

According to these guidelines, a long-term stability testing of the BM41 material was carried out for 15 months. The formulation and assay development led to a successful production of the BM41 drug product and ensured the timely submission of a first in man clinical study.

2.) Zusammenfassung

Eine Allergie ist die häufigste chronisch-entzündliche Erkrankung mit großen sozio-ökonomischen Auswirkungen. Lebenslange Abhängigkeit von symptomatischen Medikamenten ist teure Realität für die überwiegende Mehrheit der Allergiker. Allergen-spezifische Immuntherapie ist die einzig wirksame Behandlung, die mit dem zugrunde liegenden Mechanismus des Immunsystems interagiert. Dennoch haben lange Behandlungsdauer und das Risiko von anaphylaktischen Nebenwirkungen diese Behandlung daran gehindert, die erste Therapie-Wahl zu sein.

Das EU-Projekt BM4-SIT zielt darauf ab, aus seiner aktuellen Nischenposition zu entkommen, indem es die Behandlung sicherer, effektiver und patienten-freundlicher macht, das heißt eine Therapie mit vernachlässigbaren Nebenwirkungen, aber verbesserter Wirksamkeit, die durch weniger Injektionen erreicht wird. Das Konzept von BM4SIT basiert auf zwei Neuerungen: das Ersetzen aktueller, natürlicher Allergen-Extrakte durch mutierte, rekombinante Allergene, die als hypoallergen, aber hyper-immunogen bezeichnet werden. Durch die Zugabe eines Adjuvans wird eine schnelle und wirksame Immunantwort induziert. Das in dieser Diplomarbeit untersuchte und verwendete Protein ist BM41, eine Mutante des Hauptallergens der Birke, Bet v 1, welches das klinisch relevante Hauptallergen für das Auftreten der Birkenpollenallergie ist.

Als immunstimmulierendes Adjuvans wurde in diesem Projekt Vitamin D3 ausgewählt. In mehreren Studien konnte *in vitro* als auch im Mausmodell gezeigt werden, dass Vitamin D3 aktiv die Suppression von Entzündungen begünstigen kann.

Eines der wichtigen Ziele des EU-BM4-SIT Projekts ist es, BM41 und Vitamin D3 mittels GMP-Wirkstoffproduktion, die BM41-Formulierungsentwicklung, Toxizitätsstudien und Assay-Entwicklungen für die GMP-Impfstoffproduktion bis zur Einreichung der ersten Phase der klinischen Studie zu bringen.

Die vorliegende Masterarbeit ist ein wesentlicher Bestandteil dieses EU-Projektes, da sie alle kritischen Phasen der Entwicklung der BM41 Wirksubstanz, bis zum finalen

Arzneimittelproduktes (Impfstoff, Placebo und SPT-Lösungen), sowie der Stabilitätsstudien und Toxizitätsstudien für die Freigabe für die erste klinische Studie behandelt und beschrieben hat.

Das Ergebnis war die erfolgreiche Entwicklung von analytischen Assays und Charakterisierungsmethoden, wie SDS-PAGE, Western Blot, IgE-Bindungstests, BM41-spezifische Sandwich-ELISA, RP-HPLC, Bio-Burden für die spätere Analyse und Prüfung eines GMP-ähnlichen Materials nach höchsten GMP Qualitätsstandards.

Für die Stabilitätstests der BM41-Arzneimittelsubstanz wurde ein ICH-konformes Stabilitätsprüfprotokoll etabliert. Nach diesen Richtlinien wurde eine Langzeitstabilität des BM41-Materials für 15 Monate durchgeführt. Die BM41 Wirkstoff Formulierung und Assay-Entwicklung führten zu einer erfolgreichen Produktion des BM41-Impfstoffes und Hauttestlösungen und garantieren somit eine rechtzeitige Einreichung einer ersten klinischen Studie.

3.) Acknowledgements

This thesis becomes a reality with the kind support and help of many individuals, I would like to extend my sincere thanks to all of them.

I would like to express my special gratitude and thanks to Dr. Frank Stolz and Dr. Angela Neubauer for their guidance and constant supervision, as well as for imparting their knowledge and expertise.

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„Das erste, das der Mensch im Leben vorfindet, das letzte, wonach er die Hand ausstreckt, das kostbarste, was er im Leben besitzt, ist die Familie.“

Adolph Kolping (1813 – 1865), German theologian

And thank you mom and dad for your unconditional love and support. Your smile gives me the strength I need to realize my dreams.

Christine, Günther, Joachim and Cora - I love you, you are teaching me for life.

4.) Table of Contents

5.) Abbreviations

(D)PBS	(Dulbecco's) Phosphate buffered saline
µm	Mikro meter
AB	AntiBody
AP	Alkaline Phosphate
APCs	Antigen presenting cells
API	Active Pharmaceutical Ingredient
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CAP	Cellulose acetate membrane precipitin
CASO-Agar	Trypticase soy agar
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
CFA	Complete Freunds Adjuvant
Cfu	Colony Forming Unit
DoA	Degree of Adsorption
DP	Drug Product
DS	Drug Substance
E. coli	Escherichia Coli
ELISA	Enzyme Linked Immuno Sorbent Assay
F_{ab}	Fragment antigen binding
HCDC	High Cell Density Culture
HCl	Hydrogenchloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IC50	Half maximal Inhibitory Concentration
ICH	International Council for Harmonisation of Technical Requirements
for	Pharmaceuticals for Human Use
IFA	Incomplete Freunds Adjuvant
IgA	Immunglobulin A
IgD	Immunglobulin D
IgE	Immunglobulin E

IgG	Immunglobulin G
IgM	Immunglobulin M
IL	Interleukine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
L	Liter
MHC	Major histocompatibility complex
mm	Milli meter
MW	Molecular weight
NaCl	Natrium chloride
NHS	Normal Human Serum
nm	Nano meter
OD	Optical Density
PAF	Platelet-activating factor
PAGE	Polyacrylamide-gel-electrophoresis
PBS	Phosphate Buffered Saline
PGD_2	Prostaglandin D_2
pH	Potentia hydrogenii
RD	Relative density
Rf	Relative migration distance
RNA	Ribonucleic acid
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscope
SI	T-cell stimulation index
SIT	Specific immunotherapy
SPT	Skin Prick Test
TGF beta	Transforming Growth Factor beta
TGS	Tris-Glycine-SDS
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrose factor
WFI	Water for Injection

6.) Introduction

6.1 Allergy

Allergies (Hypersensitivities) are among the most common chronic conditions worldwide. Allergic diseases affecting the upper and lower respiratory tract, such as allergic rhinitis and rhino conjunctivitis and allergic asthma, which are the most prevalent chronic inflammatory diseases[1]. Across the world, their prevalence has increased significantly over the past decades as has been established in the International Studies of Asthma and Allergies in Childhood (ISAAC) and the European Respiratory Health Surveys (ECRHS). Based on these studies and confirmed by the World Health Organization (WHO), the current estimates are that up to 30 % of the population suffer from allergic rhino-conjunctivitis and 20 % from asthma, thereby clearly being the most common chronic inflammatory disease affecting the health of the population of Europe. Allergic respiratory diseases have a very significant impact on the quality of life of patients. Not surprisingly, allergies are therefore also an immense financial burden to society, both direct (diagnosis and treatment costs) and indirect (loss of school and work days).[2]

Allergies are characterized by an overreaction of the immune system to proteins, called allergens (these allergens are for healthy people harmless substances) and a simultaneous production of Immunoglobulin E (IgE) antibodies.

6.2 Antibodies (Immunoglobulin, Ig)

In general antibodies are described as large molecules (figure 1) that are recruited by the immune system to identify and neutralize foreign objects like bacteria and viruses. They are glycoproteins, produced by B-cells (plasma cells), which react with antigens and therefore they are one of the most important parts of the immune system.[3]

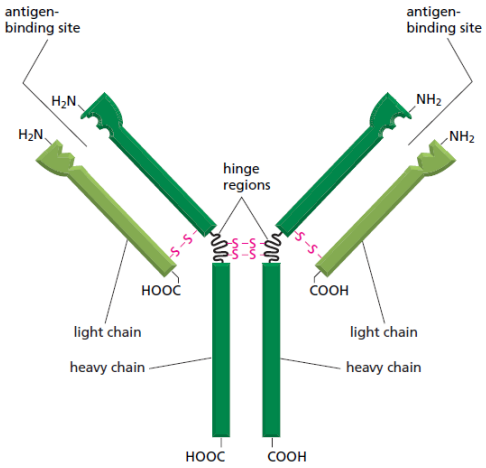


Figure 1. Bivalent antibody molecule taken from[3].

a) Structure

Every human antibody consists of 4 polypeptides: two heavy chains and two light chains which are connected via non-covalent bindings to each other (e.g. disulphide bridges). As a result this chains form a Y-shaped molecule. The tip of the Y consists of a variable and constant region: the variable region interacts with antigens and enables a specific adaption to a certain antigen (approximately 10^7-10^8 different variations).[4]

Treating the antigen with papain (protease) results in cleavage of the Y shape into 3 fragments: 2 F_{ab} Fragments ("ab" for antigen binding, the tip of the Y structure) and the F_c Fragment ("fragment crystallisable region", the tail of the Y structure) as demonstrated in figure 2. The F_{ab} Fragment contains the variable section to bind a specific target (antigen).

The Fc Fragment reacts with the so called Fc Receptor on the surface of cells of the immune system and can also bind some proteins of the complement system.[5]

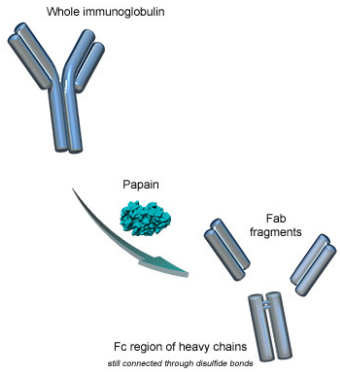


Figure 2: Papain cleavage of antibody into F_{ab} and Fc fragments.[6]

b) Isotypes

In the human serum 5 different isotypes of immunoglobulins are known. This division is based on a variation of the antibodies heavy chain (table 1):

	IgG	IgA	IgM	IgD	IgE
Mass	150 kDa	160 kDa	900 kDa	184 kDa	190 kDa
Heavy chain	γ	α	μ	δ	ε
Light Chain	κ/λ	κ/λ	κ/λ	κ/λ	κ/λ
Content in human plasma [g/l]	8-18	0.9-4.5	0.6-2.8	0.003-0.4	$1\text{-}14\text{x}10^{-4}$

Table 1. Five different isotypes of human immunoglobulin modified according to [4]

IgG

This group of antibodies represent the major part of immunoglobulins in the human serum. IgG antibodies are the only immunoglobulins which facilitate the passage through the human placenta and therefore can provide a foetus with immunity. IgG binds to mycotoxins and microorganisms. This formed immune complex can further be phagocytised by macrophages. IgG's can also activate the cleavage of the complement system to eliminate invaders. [7]

IgA

This kind of antibody protects the human mucosa (respiratory, urogenital tract) from bacterial infections.[7]

IgM

Are pentameric antibody structures secreted from B-cells in an early stage of infection to activate the complement system. IgM's can also take a membrane-bound shape and act like a B-cell-receptor.[7]

IgD

IgD is co-expressed with IgM and a surface protein of the majority of mature B-cells prior to antigenic stimulation and functions as a transmembrane receptor. The biological function of IgD is yet not clearly understood.[8]

IgE

Plays a major role in allergic reactions. IgE bind to allergens and trigger histamine and cytokine release from mast cells (IgE binds to the F_c receptor of mast cells).

The great variability of the antibodies is triggered by genetic recombination. Different gene segments (V, J, D) encode for the variable region of each immunoglobulin. The segments get randomly selected and combined.[7]

Allergy includes several hypersensitivity reactions with different immunological reactions which Coombs and Gell classified into four types. In general the immunologic reaction can be divided into antibody mediated which applies for type I-III allergy, and cell mediated which applies for type IV allergy.[9]

7.) Hypersensitivity

In 1902 Charles Richet und Paul Portier tried to immunize dogs with small doses of a protein of a jellyfish. After 26 days and the third injection, the dogs died unexpectedly within 25 minutes. Richet and Portier recognized that not the small doses of proteins killed the dogs, instead, the proteins were only the trigger of an explosive reaction of the dogs immune system.[10] This was one of the first demonstration of a reaction called "hypersensitivity".

Allergens are also responsible for the activation of IgE-binding mast cells in the exposed tissue. This action can lead to different kind of responses that are known as hypersensitivities and are characteristics of an allergy. Gell and Coombs classified the hypersensitivities in 4 different types underlying their different immune reactions:

7.1 Different types of hypersensitivities

There are 4 different types of hypersensitivity to distinguish (figure 3):

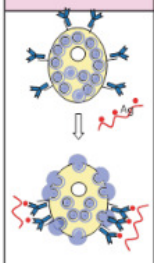
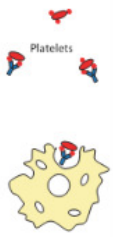
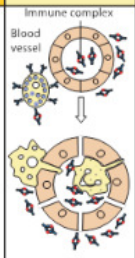
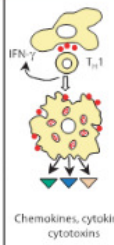
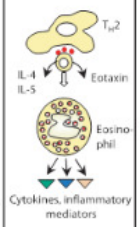
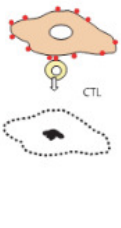
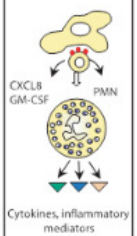
	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG	IgG	IIFN-γ, TNF-α (T_H1 cells)	IL-5, IL-4/IL-13 (T_H2 cells)	Perforin/ granzyme B (CTL)	CXCL8, GM-CSF (T cells)
Antigen	Soluble antigen	Cell- or matrix-associated antigen	Soluble antigen	Antigen presented by cells or direct T-cell stimulation	Antigen presented by cells or direct T-cell stimulation	Cell-associated antigen or direct T-cell stimulation	Soluble antigen presented by cells or direct T-cell stimulation
Effector	Mast cell activation	FcR+ cells (phagocytes, NK cells)	FcR+ cells Complement	Macrophage activation	Eosinophils	T cells	Neutrophils
							
Example of hypersensitivity reaction	Allergic rhinitis, asthma, systemic anaphylaxis	Hemolytic anemia, thrombocytopenia (e.g., penicillin)	Serum sickness, Arthus reaction	Tuberculin reaction, contact dermatitis (with IVc)	Chronic asthma, chronic allergic rhinitis Maculopapular exanthema with eosinophilia	Contact dermatitis Maculopapular and bullous exanthema Hepatitis	AGEP Behçet's disease

Figure 3. Classification of hypersensitivity reactions

Cellular and soluble immune components implicated in hypersensitivity responses.[11]

Type I, II and III are antibody mediated, type IV is a cell mediated process.

Type I:

It's commonly called "allergy". The type I hypersensitivity is mediated by IgE antibodies produced by plasma cells (B-cells) in response to stimulation of Th2 cells (type 2 T helper cells) by antigens and subsequently called allergens like house dust, pollens, or insects. This form of allergy can be systemic (systemic anaphylaxis) or localized to a specific target, tissue or organ (allergic rhinitis, asthma).[12 13]

Type II:

Is the "cytotoxic" kind of hypersensitivity and involves IgG or IgM antibody-mediated reactions (antibody dependent cytotoxicity). These reactions affect a variety of organs and tissues. The IgG or IgM antibodies react with cell surface antigens in order to activate the complement system.[12 13]

Type III:

Is known as the immune complex hypersensitivity. Immune complexes are aggregations of soluble antigen and IgG antibodies. These Immune complexes induce tissue damage due to inflammatory response and activation of the complement system.[12 13]

Type IV:

Is a non-antibody induced T-cell mediated late reaction to antigens (chemicals, metals, haptens). The type IV hypersensitivity is the only type that does not involve antibodies, it is rather a type of cell-mediated-response to an antigen: The CD4+ helper T-cells get the target antigen in a complex with MHC II on the surface of antigen-presenting cells. A typical type IV reaction is the allergic contact dermatitis which results in epidermal necrosis and inflammation.[12 13 5]

7.2 Close-up: Type I Hypersensitivity

As described above, the type I hypersensitivity is an immediate antibody mediated reaction of the immune system to antigens. This type of hypersensitivity is the most prevalent of hypersensitivity diseases and is divided in to the following 3 phases (figure 4):

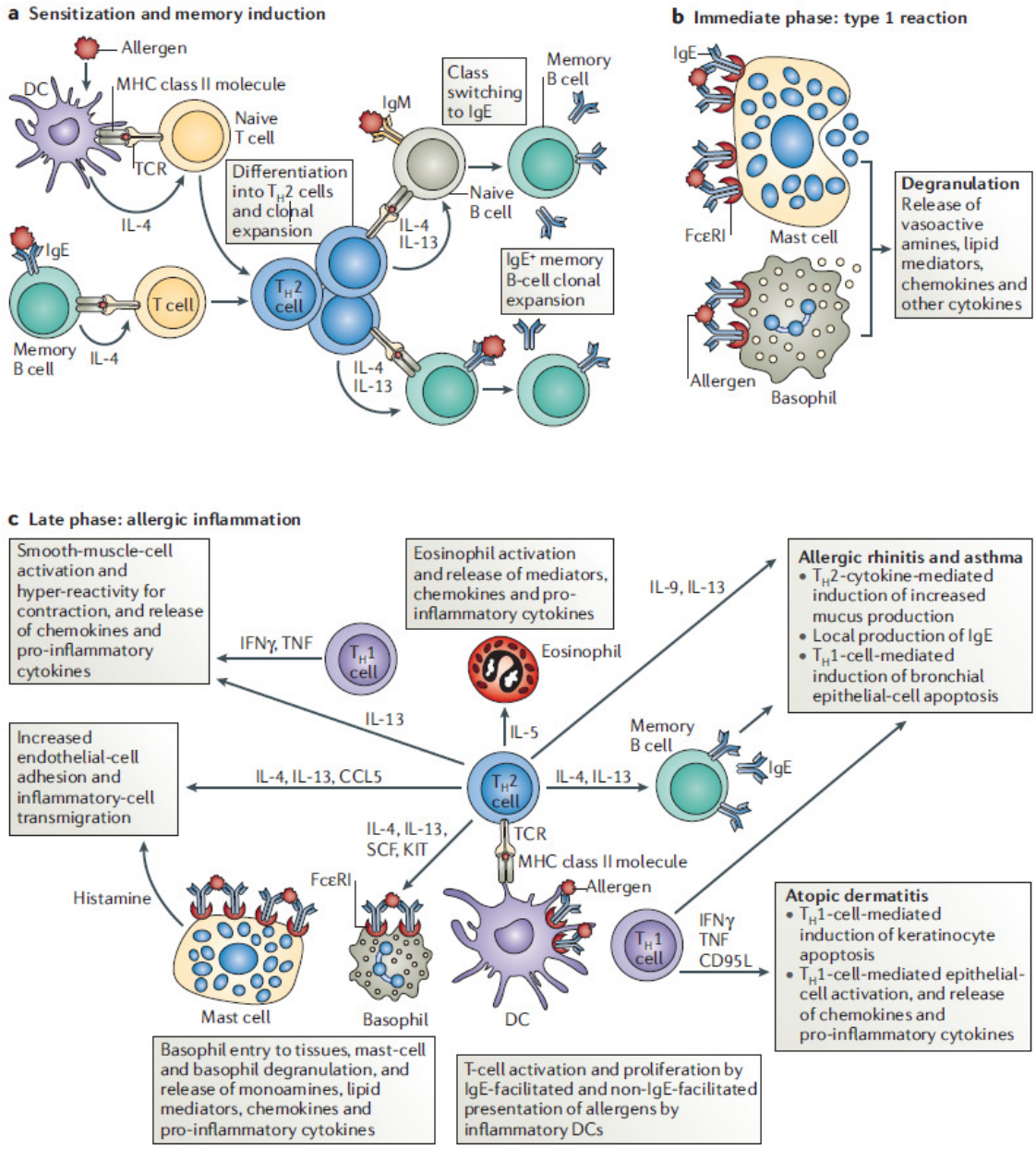


Figure 4. Immunological mechanisms of type I Hypersensitivity[13]

A) Sensitization and memory induction

Dendritic cells and macrophages are so called antigen presenting cells (APCs). APCs pick up the allergen and immigrate into lymph nodes. In the lymph nodes the APC present the antigen peptide, which is bound to class II MHC molecules, of $CD4^+$ naïve

T-Helper-cells. These T_H cells express specific T-cell receptors against the bound peptide.

Naive T-Helper cells proliferate into T_H2 Cells Interleukins like IL-4, IL-5, IL-13 support this immunological switch. Especially cytokine IL-4 stimulates T_H2 cell development by activating transcriptions factors like STAT6 or GATA-3, which play a central role in exerting IL4 mediated biological responses or in T-cell development and endothelial cell biology. [14] [13]A typical B-cell class switch is driven by an activated CD4+ T_H2 cell interaction with B-lymphocytes and leads to an IL-4 release which activates B-cell clonal expansion. As a consequence activated B-Cells differentiate into plasma cells and start to produce IgE instead of IgM that are specific for the antigens which invade the body.[4] [5]

B) The immediate phase of an allergic reaction type 1

The immediate phase of an allergic reaction type 1 starts just some minutes after the repeated exposure to an allergen. Mast-cells get activated by binding IgE to a receptor called FcεRI-receptor (figure 5). The FcεRI –receptor is characterized by a high affinity for IgE and has been detected on eosinophils, mast cells and basophils, as well as dermal macrophages and activated monocytes. In contrast to the FcεRI -receptor, many immune cells express the FcεRII-receptor (CD23), which has been shown to have a low affinity for IgE binding. Both receptor facilitate antigen uptake by APC's and furthermore augment immune responds.[15]

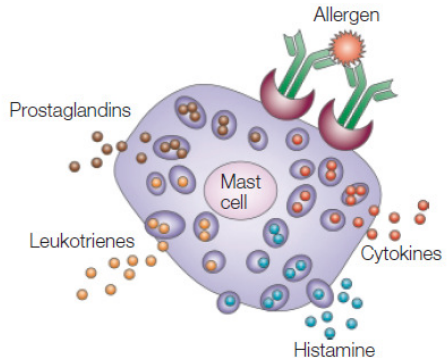


Figure 5. Degranulation of a mast-cell after crosslinking of the antigen[16]

Crosslinking of the antigen which is bound by the FcεRI-Receptor on the surface of mast-cells leads to degranulation and secretion of the preformed granule contents, so called proinflammatory mediators like Prostaglandins, Leukotriens, Cytokines and Histamines. [13] [16] [17]

An important role also play Histamines. They are vasoactive amines which bind to different cell types that express distinct classes of histamine receptors (H_1, H_2, H_3). Binding of histamine to endothelium cells causes contraction which leads to increased vascular permeability. Histamine causes vasodilation and vascular leak. These effects cause wheal and flare as a response of an immediate hypersensitivity. [18]

Many different cytokines are produced by mast cells and basophils. Well known are Tumor Necrose Factor (TNF), IL-1, IL-3, IL-4, IL-5, IL-6, IL-13, CCL3 (chemokine C-C motif ligand 3), CCL4 (chemokine C-C motif ligand 4) and granulocyte-monocyte colony-stimulating factor (GM-CSF). They are responsible for differentiation of the specific T-Helper cells. Differentiated T_H2 – cells, mast cells and basophils produce cytokines which are highly proinflammatory and recruit other cells to respond to infections. [18] [19]

Prostaglandins, leukotriens and platelet-activating factor (PAF) are lipid mediators like prostaglandin D_2 (PGD_2), leukotriens or PAF. They get *de novo* synthesized by mast cells after their activation. Lipid mediators cause bronchoconstriction and an increase of permeability. PAF also works as a platelet aggregator and activates leucocytes. High proinflammatory leukotriens and PAF play a crucial role in development of asthma.[19]

C) Late phase reaction

This phase is characterized by accumulation of inflammatory cells like leukocytes, neutrophils, eosinophils, basophils and TH2 cells. Also interleukins, cytokines or leukotriens play a crucial role.

This accumulation of different immune reactive cells provides several inflammatory effects. [13]

8.) Pollenallergy

Pollen are one of the most common causes of allergies in Europe and in the United States. Plants produce microscopic pollen for reproduction. These pollen get distributed through the air (or insects) to ensure fertilization of other plants of the same species. If these tiny particles get released from trees, weeds and grasses they can infiltrate human's mucous membranes and cause allergic symptoms like allergic rhinitis (hay fever) or in worst case asthma or a systemic anaphylaxis.[20]

8.1 Birch (*Betula, spp.*)

Birch are wind-pollinated early flowering plants (April to May) and in boreal regions a major initiator of pollinosis. The Common Silver Birch (*B. verrucosa*) is the prevalent European species, but absent in southernmost parts of Europe. Because of cultural factors like importing Birch for urban parklands it gradually spreads into the Mediterranean regions (e.g. Northern Italy). The wind-pollinated Birch (figure 6, 7 and 8) shed enormous quantities of pollen: a single catkin can produce 6 million grains. These highly allergenic pollen impact human health by causing seasonal hay fever, pollen-related asthma and other allergic diseases (such as cross-reactive food allergies).[21] Pollen levels are determined by the weather (high and low years for the abundance of pollen and subsequent seed). The most common form of seasonal respiratory diseases in Europe is caused by pollen allergies.[22]

Figure 6. Betula populifolia: leaves and catkin[23]

Figure 7. Typical white tree bark of Betula pendula[24]

Figure 8. Forest with Betula sp[25]

8.2 The Birch Pollen and its allergens

The pollen grains of Betula are triangular and have a well ringed and shouldered pore (figure 9). Their size ranges from 15 to 30 µm.[20] [26]

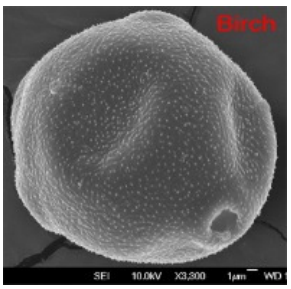


Figure 9. SEM photo of a betula pendula grain[27]

Birch pollen contain various antigens, the major birch pollen allergen is called Bet v 1, a 17-kDa protein which belongs to the class of intracellular pathogenesis-related proteins on the basis of sequence similarity.[28] Bet v 1-homologous proteins were identified in a broad range of plant derived food, such as apple, providing molecular evidence for cross-reactivity of tree pollen allergy and allergy to food plant origin.[29] Besides Bet v 1 there are other proteins known like the cytoskeleton-associated profilins [Bet v 2] which are also potential candidates for cross-reactivity.[29] [30]

The following allergens have been characterised:

- Bet v 1, a 17 kDa protein, a ribonuclease and a PR-10 protein
- Bet v 2, a 15 kDa, profilin
- Bet v 3, a 24 kDa calcium-binding protein
- Bet v 4, a 9 kDa calcium-binding protein
- Bet v 5, a 35 kDa isoflavone reductase-related protein
- Bet v 6, a 30-35 kDa protein, PCBER (Phenylcoumaran benzylic ether reductase)
- Bet v 7, a 18 kDa protein, cyclophilin
- Bet v 11 [31] [20]

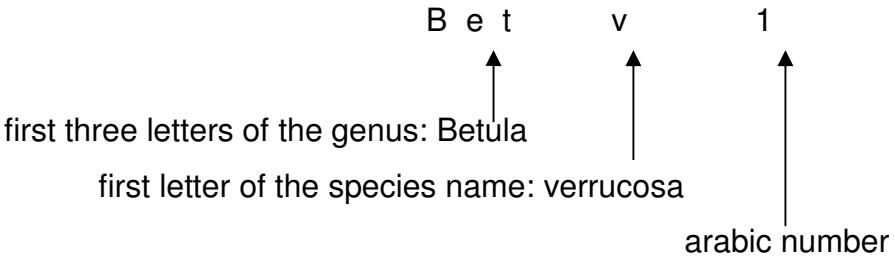
8.3 Bet v 1 protein

Bet v 1 causes type I allergies especially in early spring. Type I (or IgE-mediated) allergies affect up to five people in Europe and USA. Typical symptoms are asthma, hay fever, dermatitis and, sometimes anaphylactic shock. Contact with this allergens results in sensitization; often contact leads to a cross-linking reaction of IgE on mast cells and a subsequently release of histamine and therefore the typical symptoms of an allergic reaction.[32]

8.4 A Nomenclature system for allergens

A nomenclature system has been invented for allergens that are responsible for IgE-mediated atopic allergies in humans.[33]

Designation:



Tuft and Blumenstein reported in 1942 for the first time a cross reactivity of patients, suffering from pollinosis, to plant-derived food allergy.[34] This cross reactivity is nowadays well-known and topic of several studies. Patients suffering from birch pollen allergy often derive an oral allergy syndrome (OAS): the consumption of raw fruits or vegetables lead to an IgE-mediated food allergy. The underlying trigger of these oral allergy syndromes is that some allergens (e.g. Bet v 1 and Mal d 1, the major allergen of apple) have 55.6 % identity at the nucleic acid level which leads to a IgE cross-reactivity of Bet v 1 and Mal d 1.[35] Bet v 1 and Mal d 1 belong to a class of proteins called PR proteins (pathogenesis-related proteins).[36] Other proteins belonging to this family include the major pollen allergens:

- Api G I (*Apium graveolens*)
- Cor a I (*Corylus avellana*)
- Mal d I (*Malus domestica*)
- Ara h 8 (*Arachis hypogaea*)[37]

8.5 Structure and biological function of Bet v 1

The protein structure and site of the major T-cell epitope have been discovered by NMR technology.[38] [39]

The Bet v 1 protein (figure 10) consists of:

- 7 anti-parallel beta-strands
- 3 alpha-helices[40]

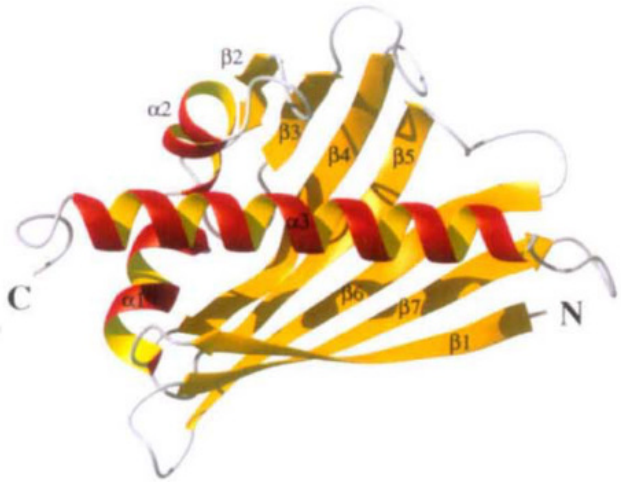


Figure 10: X-ray structure of Bet v 1[40]

Four strands represent the global fold, 2 strands of the helices form a C-terminal amphipathic helical motif (T-cell epitope). The Bet v 1 protein has also a large hydrophobic cavity, which may represent a ligand binding site. [41]

Biological function of Bet v 1

The biological function of Bet v 1 is not fully explained yet. It has been shown, that Bet v 1 has a hydrophobic pocket which can bind many ligands (siderophores, flavenols). Bet v 1 belongs to the class of pathogenesis-related (PR) proteins, which are expressed under presence of infections and stressful conditions.[36] In addition Bet v 1 has been shown to mount a Th2-response which is crucial in allergy development.[42]

9.) BM41- the hypoallergen protein

Our main objective of the EU-BM4-SIT project was to create an innovative causal therapy for the birch pollen allergy; a safe and rapid induction of an anti-inflammatory immune response using a mutant hypoallergen and an appropriate adjuvant. The allergen derivative chosen to bring this concept from bench to bedside is BM41, a mutant of the culprit allergen of birch pollen allergy (Bet v 1).[43]

By using a recombinant technology, an even more extreme hypoallergenic but hyper immunogenic profile was achieved in the designed mutant BM41, the protein, which is used for SIT in the BM4SIT project. An exchange of just five amino acids of Bet v 1.0101 was shown to result in a stably unfolded but monomeric soluble protein that is hardly recognized by human IgE.[43] [44] The properties of BM41 are:

- BM41 induces high levels of IgG (blocking antibodies) that cross-react with the corresponding wild-type allergen Bet v 1a
- BM41 showed strong immunogenicity by a high T-cell stimulation index (SI)
- Low binding of human IgE in serological assays
- Reduced ability to activate basophil cells and induced mediator release
- Low capacity to induce skin reactions in birch pollen allergic individuals

BM41 was produced recombinant in *E. coli* BL21(DE3) and purified according to established protocols.

The starting basis of this project part was a gene and the corresponding amino acid sequence of BM41.

ATGGGTGTTTTCAATTACGAAACTGAGACCACCTCTGTTATCCCACAGC
TCGACTGTTCAAGGCCTTTATCCTTGATGGCGTAATCTCTTTCCAAAGGT
GCACCCCAAGCCATTAGCAGTGTTGAAAACATTGAAGGAAATGGAGGG
CCTGGAACCATTAAGAAGATCAGCTTTCCCGAAGGCTTCCCTTTCAAGT
ACGTGAAGGACAGAGTTGATGAGGTGGACCACACAAACTTCAAATACA
ATTACAGCGTGATCGAGGGCGGTCCCATAGGCGACACATTGGAGAGA
TCTCCAACGAGATAAAGATAGTGGCAACCCCTAGTGGAAGCACCATCA
AGAGTATCAGCAACAAGTACCACACCAAAGGTGACCATGAGGTGAAGG
CAGAGCAGGTTAAGGCAAGTAAAGAAATGGGCGAGACACTTTTGAGGG
CCGTTGAGAGCTACCTCTTGGCACACTCCGATGCCTACAACTAA

BM41 amino acid sequence (without start-methionine):
G V F N Y E T E T T S V I P A A R L F K A F I L D G D N L F P K V A P Q A I S S V
E N I E G N G G P G T I K K I S F P E G F P F K Y V K D R V D E V D H T N F K Y N
Y S V I E G G P I G D T L E K I S N E I K I V A T P S G S T I K S I S N K Y H T K G D
H E V K A E Q V K A S K E M G E T L L R A V E S Y L L A H S D A Y N
The region of the exchange sequence of BM41 (figure 11) is highlighted by a gray box (compared to Bet v 1 (figure 12), isoform 0101, exchanged amino acids – from epitope sequence of Mal d 1, isoform 0108 is highlighted by blue letters).

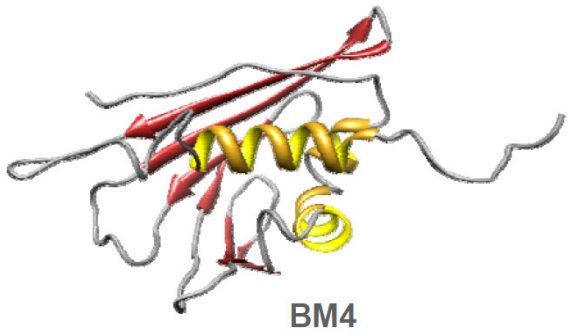


Figure 11. Three dimensional structure of BM41[43]

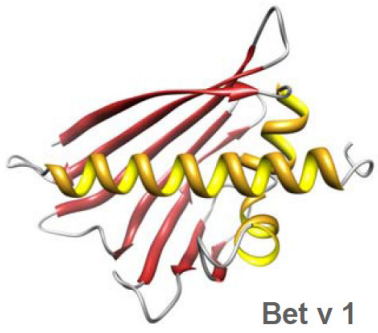


Figure 12. Three dimensional structure of Bet v 1[43]

The sequences (original nucleotide sequence and target protein sequence) were provided from the University of Salzburg (Dr. Michael Wallner, Department of Molecular

Biology). Construction of the plasmid (E. coli codon optimization of BM41 sequence, gene-synthesis of codon optimized BM41 and subcloning into expression vector pET-28b(+) (figure 13) was outsourced to Thermo Fisher (Geneart). The produced Master Cell Bank (MCB) has been designated with an internal batch number. The cell bank was produced and characterized according to GMP regulatory guidelines.

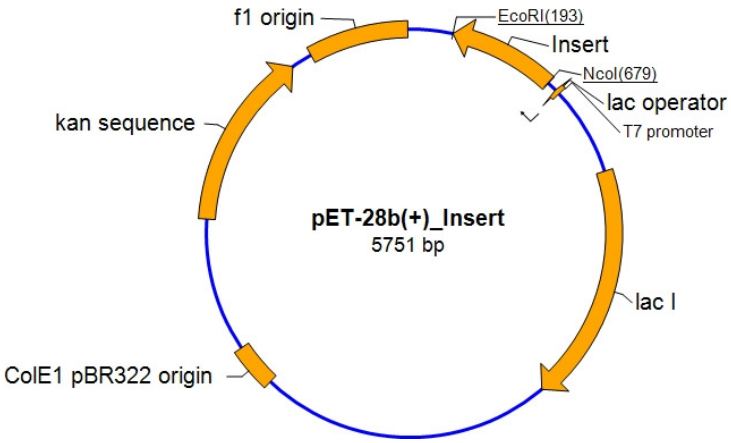


Figure 13. Plasmid map of pBM41 (BM41 in expression vector pET-28b(+)).

Function of Expression System

The pET-28b(+) vector (Merck Milipore Cat# 69865-3) contains several important elements:

- lacI gene which codes for the lac repressor protein
- T7 promoter which is specific to only T7 RNA polymerase
- lac operator which can block transcription
- polylinker
- Kanamycin resistance gene
- f1 and ColE1 origin of replication

In the pET expression system, the transcription of the recombinant gene is under the control of T7 promotor. The host cell for this expression system is a bacterium, which has been genetically engineered to incorporate the gene for T7 RNA polymerase, the lac promoter and the lac operator in its genome (lysogenic DE3 lambda prophage). When lactose or the analogue IPTG is present inside the cell, transcription of the T7 RNA polymerase is activated and the recombinant gene under the control of T7 promotor will be transcribed and further translated to protein.

The new recombinant molecule showed reduced allergenicity if compared to the corresponding wild-type allergen (table 2).

Safety	Allergenic activity / Hypoallergenicity	➔ low/no binding of human IgE ➔ reduced ability to activate basophil cells and induce mediator release in biological assays ➔ low capacity to induce skin reactions
Efficacy	Immunogenicity	High levels of IgG
	T-cell activity (human T-cell recognition)	High T-cell stimulation index (SI) in proliferation assay using T-cells (PBMC) from birch pollen allergic patents

Table 2. Characteristics of the hypoallergen mutant variant BM41

It has been shown in different clinical trials (by Biomay's licenses) that a vaccine comprising Bet v 1 or a Bet v 1 derivative as the single active component can effectively replace birch pollen extract in the therapy of birch pollen allergy. [44]

At the moment the only available treatment that targets the mode of action of allergy is SIT.[45]

9.1 Allergen Specific Immuno Therapy (SIT)

Pollen is one the most common causes of allergic respiratory diseases worldwide. For the treatment of allergic rhinitis and asthma symptomatic drugs are used very often. Immunotherapy for treatment of allergies was first developed by Noon and Freeman in St. Mary's Hospital London in the early 20th century. The term immunotherapy stands for the treatment of an allergic disease by suppressing the immune respond. [46] [47]

This treatment is administered mainly to patients with allergic rhinitis or rhino conjunctivitis without or with mild asthma. Interestingly, it has been demonstrated that SIT can prevent the frequent development of allergic rhinitis into allergic asthma.[48] The treatment is supressing the allergen-specific inflammatory immune responses (IgE, Th2, and Th17) by induction of protective allergen-specific non-/anti-inflammatory responses (IgG_4, IgA and Tregs). [49]

In SIT identified patient-specific allergenic extracts are given to the patient to achieve clinical tolerance and to reduce further allergic symptoms. SIT can be administered by injections or – the more prevalence art of performing SIT – by sublingual route. Typically patients receive injections starting at a very low dose of allergen, increasing this dose gradually (updosing) until maintenance dose is achieved. This final-dose injections need to be repeated 4-6 weeks for three to five years. SIT induces changes in T-cell and antibody respond to the allergenic predisposition as follows:[50] [51]

- Reduction of IgE and induction of IgG antibodies
- Reduced recruitment of effector cells
- Altered T-Helper cell shift (TH2 to TH1)
- Induction of T-reg cells (which damp the respond to allergen exposure) [52]

But SIT only works in selected patients (fatal adverse effects occurred in patients with asthma) and the vaccines which are used are still unstandardized extracts of allergens. One big disadvantage of SIT is that it can still provoke a systemic allergic reaction. [53] An approach to improve this side-effects is the use of recombinant allergen derivatives (instead of unstandardized extracts) which are designed to have a reduced IgE reactivity and a retained T-cell reactivity.[54] IgE-binding to specific IgE-epitopes on allergens is only possible, if the protein is correctly folded in its tertiary structure. These IgE

epitopes are the underlying target by producing a recombinant allergen via introducing mutations in this specific protein sequence. Alternatively, conformational IgE epitopes may be destroyed by fragmentations or oligomerization of the allergen.[55]

SIT blocking antibodies

One purpose of SIT is to modulate the immune response against allergens by inducing immunological changes during the course of SIT.[56]

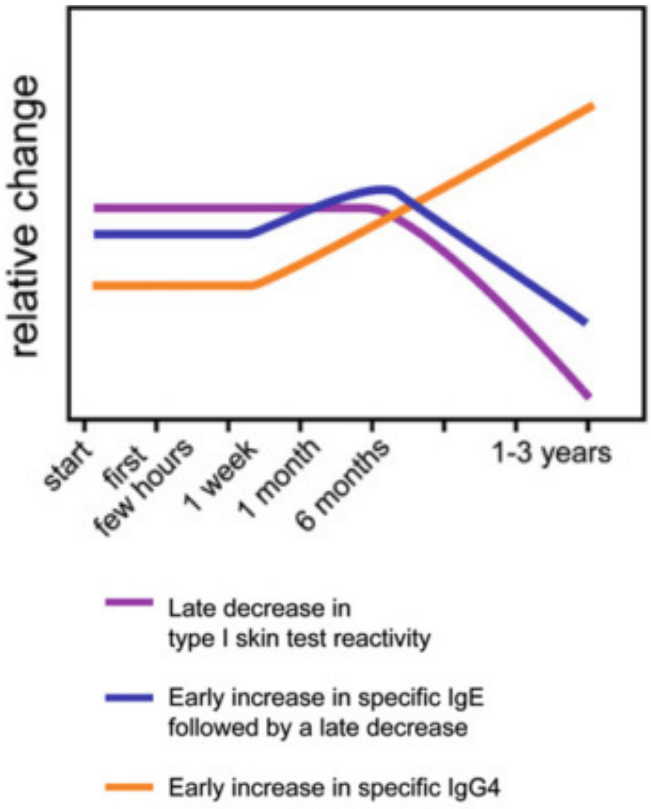


Figure 14: Modulation of immune response during the course of SIT [52]

The main changes SIT induces takes place in allergen-specific IgE and IgG subclasses (figure 14). SIT increases in an early stage the IgG (IgG_4) levels. IgG binds to the same epitopes as IgE and captures the antigen before the IgE. This results in a so called "blocking effect": The effector cell-bound and trigger of an allergic reaction immunoglobulin E cannot reach the allergen and therefore no activation of mast cells and basophils takes place.[52]

After first administration of SIT an increase of specific IgE levels followed by a late decrease and vice versa an early increase in specific IgG could be observed. SIT also leads to a late decrease in type I skin test reactivity as well as a decrease of tissue mast cells and eosinophils.[52] The two hallmarks of effective SIT are the induction of

allergen-specific, non-inflammatory IgG_4 and IgA antibodies and of anti-inflammatory regulatory T-cells, cytokines IL-10 and TGF beta.[57]

9.2 BM4SIT EU Project

BM4SIT stands for "**B**et v 1 **M**utant for [**4**] **S**pecific **I**mmuno **T**herapy". The concept of BM4SIT is to administer a mutant of Bet v 1, BM41, designed to reduce allergenic activity but at the same time enhance immunogenic properties, i.e. SIT with a molecule with a negligible risk of allergic side-effects that more effectively induces a protective allergen specific IgG_4 and IgA antibody response.

The overall objectives of the BM4SIT project is to provide a significantly safer treatment of birch pollen allergy and to achieve a higher efficacy more rapidly using following major building stones:

- improved safety by using the derivative of Bet v 1, BM41, with hypoallergenic properties
- enhanced efficacy by using the derivative of Bet v 1, BM41, with enhanced immunogenicity
- more rapid efficacy by using vitamin D_3 as adjuvant promoting anti-inflammatory responses

The target of BM4SIT is to bring two innovations from pre-clinical proof-of-concept to clinical proof-of-concept. The BM4SIT project is built up along two independent innovation tracks that come together in a joint first-in-man clinical trial (figure 15).

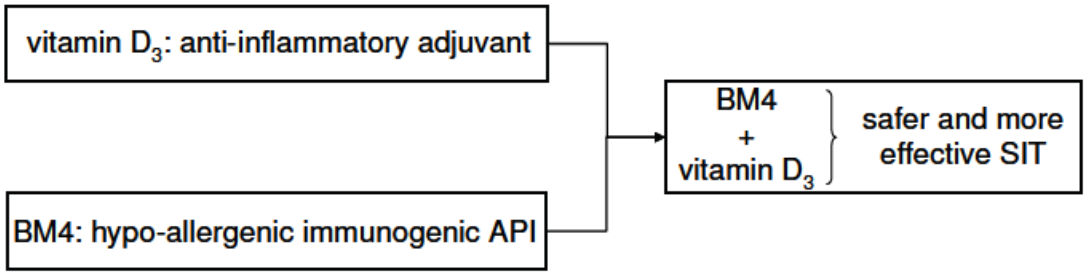


Figure 15: Tracks and Targets of BM4SIT project

The major milestones of the BM4SIT project are

- Production of a GMP-like batch of the drug substance (BM41) and formulated drug products for performance of preclinical toxicity and stability studies
- Production of a GMP batch of the drug substance (BM41) and of the formulated drug products for first-in-man evaluation of their performance in immunotherapy and skin testing
- Achieving a successful outcome of the pre-clinical toxicity (acute and repeated) and stability studies for the drug products
- Identification of an effective dose of BM41 and of vitamin D3
- Proven safety of the BM41 drug products.
- Clinical evaluation (safety and efficacy) of a combination of BM41 and vitamin D3

9.3 Specific Objectives of my Master Thesis

The objectives of present master thesis were to pave the way for successful producing the proposed BM41 GMP drug substances and BM41 GMP drug products (vaccine and placebo) as well as skin prick solutions (SPT solution). These GMP produced products are complying with GMP regulations and GCP requirements needed for human clinical trials. Therefore the aim of my thesis was to achieve the following measurable deliverables:

- full established QC assays and corresponding protocols for BM41 drug products including a new potency assay, compliant with GLP requirements, the
- development, formulation and testing of SPT solutions and
- to evaluate and successfully prove the stability of drug products (vaccine and SPT solution) using interims real-time stability studies

Many different assays had to be reviewed or established for stability testing of drug products to provide evidence on how the quality of the new hypoallergenic drug product varies with time under the influence of temperature for safety and efficacy.

For later analysis and testing of the GMP-material analytical characterization methods like SDS-Page, Western blot, IgE-binding potency test, BM41-specific sandwich ELISA, RP-HPLC, bio burden, UV/concentration and even more assays were used in order to perform an ICH conform stability study for future registration of the new product in the European Union, Japan and the United States.

10.) Material and Methods

10.1 Laboratory equipment

Equipment	Manufacturer	Serial Nr:	Lot/Cat/Int.Nr.
Pipette (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl)	Corning	058261199 158250094 158240037 158230344 158220125	LPP055 LPP053 LPP049 LPP051 LPP048
Multipette plus	Eppendorf	K24786C	LPP079
Pipetboy	Integra		
Eppendorf tubes (1.5 mL, 2 mL)	Greiner bio-one	616201 623201	E10041AX E10040U
Tips (10 µl, 200 µl, 1000 µl)	Corning	800.4092.1110	4115
Pipette (5 mL, 10 mL, 25 mL, 50 mL)	Greiner bio-one	606180 607180 760180 768180	14040871 14062051 14041241 14062431
Centrifuge	Eppendorf 5424 VWR Mini Star	5424DI557607	LTZ008
SDS chamber	Bio Rad	135BR0028268	LPS026
SDS power supply	Bio Rad	041BR87525	LPS021
UV-Imager	Bio Rad	735BR03974	LDE002
RO water supply	AB Vienna Labpart-ner		PRW001
Magnetic stirrer	Heidolph MR3000	090615712	LMR018
pH-meter	VWR	13020795	LPH005
Autoclave	Fedegari	4027E	PAF001
Refrigerator	Carel Master Cella		PKR001
Freezer	Siemens	GS28S01	PTK008
Deep freezer	New Brunswick	007-001-001	UTK008

Laminar hood	Ehret	Biosafe 7-130-2	FGM007

Equipment	Manufacturer	Serial Nr:	Lot/Cat/Int.Nr.
Vortexer	VWR	Labdancer S40	PVG008
Heater	Eppendorf	535540423	LTM001
Ice Machine	Scottsman		
Erelnmayer flasks	VWR	I214/II34	
Parafilm	Parafilm	PM/996	
Photometer	Eppendorf	613125371	LPM007
Criterion Tris-HCl Protein Gel	BioRad	345/0020	
Precision Plus Protein Standards	BioRad	1610374	
Falcon (50 mL, 15 mL)	Greiner	188271	
iMark Microplate Absorbance Reader	Bio Rad	16242	
Incubator 37 °C	Binder		LPS007
Incubator 59 °C	Binder		LPS005
96 wells solid plate NUNC MaxiSorp plate	Thermo Fisher scientific	442404	
96 well solid plate for BCA assay	Thermo Fisher scientific	15045	
Plate shaker	Heidolph	543/42205/00/3	PVG 004
50 mL reservoirs	Roth	E830.1	
Transblot Turbo	Bio Rad	16242	
Blotting Membrane	Bio Rad		LPS001
Water-bath	GFL		LWS004
Micro-cuvette	Eppendorf	952010069	C152656N
HPLC	Agilent 1260		LPS028
Zorbax 300 SB-C3 Narrow Bore	Agilent	AG883750-909	

Equipment	Manufacturer	Serial Nr:	Lot/Cat/Int.Nr.
Zorbax 300 SB-C3 Narrow Bore Guard Column	Agilent	AG821125-924	
Rapid flow Filter Unit 50mm dia. 0,2 µm	Nalgene	565-0020	
CASO-Agar and Sabouraud-Glucose-Agar	Biomerieux	1003994330 1004822950	

10.2 Chemicals

Chemical	Manufacturer	Lot/Cat./Int. Nr.	Molecular weight (gram/mol) or [%]
TGS	Bio Rad	161/0772	10x
TBE	Bio Rad	161/0770	10x
WFI	Fresenius	19HC30GA	n.a.
NaCl	Merck	K45678224424	58.44
HCl	VWR	14F190006	37%
Isopropanol	BDH Prolabo	08I290027	60.1
Brilliant Blue R 250	Roth	256242048	854.03
NaOH	Merck	321/44	40.0
Glycerol	Merck	Z255191201	100%
HEPES	Sigma Aldrich	H3375-500G	99.5%
TrisHCl	Serva	08100	157.6
TrisBase	VWR	392	121.14
Magnesiumchlo-ride-Hexahydrate [$Cl_2Mg\ 6H_2O$]	Sigma Aldrich	BCBJ1724V	203.30
Acetic acid	Roth	384218607	96%
Beta-mercaptoeth-anol	Fluka Chemika	239-06	78.13
BSA	Roth	272187596	98%
PBS	Alfa AESAR	J61917	10x
Tween 20	Sigma Aldrich	9005-64-5	20%
TMB Substrate	Sigma Aldrich	061M5358V	
Ethanol	Roth	344218091	96%
SDS	Fluka Chemika	372998/1 30798	97%
Skim Milk Powder	Sigma Aldrich	SZBF3420V	
NBT/BCIP	Roche	13913700	20 tablets
Alum	Brenntag	4624	
BM41	In house at Biomay		620µg/mL
Bradford Reagent	Sigma Life Science	SLBP3810V	

Chemical	Manufacturer	Lot/Cat./Int. Nr.	Molecular weight (gram/mol) or [%]
QuantiPro BCA Assay Kit	Sigma Aldrich	QPBCA	
Phenol Test	Spectroquant, Merck	HC574971	
Phenol crystalized	Prolabo	20596.297	
H_2SO_4	VWR	7664-93-9	96 %
Na_2CO_3	VWR Prolabo	28248.367	40
$NaHCO_3$	Spectroquant, Merck	K46165723503	84.01
Glycerol	VWR Prolabo	14E050028	92,09
Bet v 1 wt protein	In house at Bi-omay	22	
Acetonitril Li-Chrosolv	Spectroquant, Merck	I758430	
Trifluoroacetic acid HPLC	Fisher Thermo Scientific	1157803	

10.3 Antibodies

Antibody	Source	Lot/Cat./Int. Nr.	Concentration
Goat anti-human IgE HRP	KPL	074-1004	1 mg x mL^{-1}
Bet v 1 IgG mouse F1 antibody	Medical University of Vienna		1.08 mg x mL^{-1}
Mouse anti-BM4 F8	InVivo Bio Tech	AK2775/01	1 mg x mL^{-1}
Donkey anti-rabbit IgG HRP	GE Healthcare	9551642	
Goat anti-mouse IgG HRP	Jackson ImmunoResearch	127442	0.8 mg x mL^{-1}
Goat anti-mouse IgG antibody AP	Sigma-Aldrich	SLBB9232	1.3 mg x mL^{-1}

11.) Methods

11.1 Assessment of the Visual Appearance and Colour

The visual nature of the DP is assessed by visual inspection after swaying of the product vial. 3 vials are inspected at each time point. Observations regarding appearance, color, and aggregates are documented.

11.2 Measurement of pH

The pH measurement is performed at RT (25 °C) by using a micro electrode SympHony pH-Meter, which is always calibrated on the day before sample measurements. The pH of the measurement of all solutions must be within the range 6.0 and 8.0. Measurement of pH using a microelectrode (SympHony pH-Meter, LPH-005) and a 3-point calibration of the electrode on the day of the measurement.

11.3 Protein Concentration

a) BCA protein assay QuantiPro BCA Assay Kit

This kit is used to measure (at A562 nm) total protein concentration compared to a protein standard. Quantitation is based on 2 steps:

The Biuret reaction: Reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (figure 16), with the highly sensitive and selective colourimetric detection of the cuprous cation (Cu^{+}). Two molecules of bicinoninic acid chelate with each of the reduced cuprous cation and form a purple-colored complex which absorbs at 562 nm.

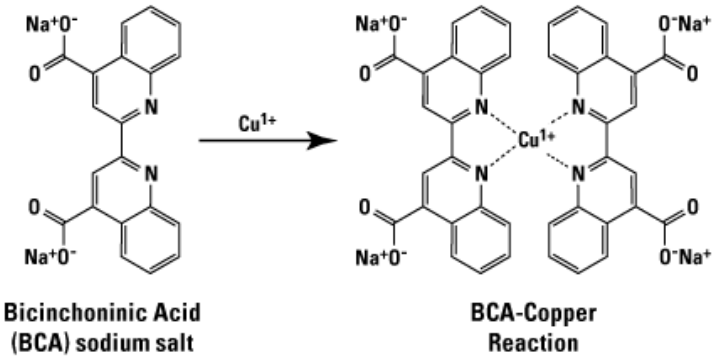


Figure 16. The reaction of BCA with cuprous ion.

Two molecules of BCA bind to each molecule of copper that had been reduced by a peptide-mediated biuret reaction [58]

Procedure: A series of dilutions of standard protein (= unformulated BM41 stock solution; c = 620 µg x mL^{-1}) was prepared using the same buffer in which the sample is in (final concentration of Glycerol and HEPES in sample and standard protein 12.5 % Glycerol and 2.5 mM HEPES). SPT samples are diluted 1:4 in WFI. The unformulated BM41 Standard gets diluted in Glycerol/HEPES. As a control we used albumin [c=2mg/mL], diluted 1:100 with Glycerol/HEPES and as Blank we used the Glycerol/HEPES Buffer. The BCA protein assay was carried out in 96 well solid plates for BCA assay (Thermo Fisher Scientific). Standards, sample, blank and the control (albumin) were pipetted (400 µL) into a 1.5 mL Eppendorf tube and 400 µL of working reagent was added (QA : QuantiPro BCA QB : Copper sulphate = 25 : 25 : 1) and manually mixed. 300 µL of each solution were pipetted in duplicates. The plate was incubated for 25 minutes at 57.5°C (+/- 2.5°C) before measuring the absorbance at 562 nm on a plate reader (BioRad).

The standard curve and sample concentration calculations were evaluated with Excel 2013.

b) Bradford

The Bradford Reagent (Sigma Life Science) is used to measure (at A595 nm) total protein concentration compared to a protein standard. This reagent is a colorimetric protein assay, based on the color shift of the dye Coomassie Brilliant Blue under acid conditions.

Procedure: A series of dilutions of standard protein (= unformulated BM41 stock solution; c = 620 µg x mL^{-1}) was prepared using WFI. Samples were diluted 1:10 in WFI. Standards, sample and blank (WFI) were pipetted (400 µL) into a cuvette (Eppendorf) and 400 µL of working reagent was added and manually mixed. The cuvettes were incubated for 5, 15, 30 minutes at room-temperature before measuring the absorbance at 595 nm at a photometer (Eppendorf).
The standard curve and sample concentration calculations were evaluated with Excel 2013.

c) Degree of Adsorption (BCA)

A BCA-Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Protein absorbance is measured at 562 nm that is nearly linear with increasing protein concentrations over a broad working range. The degree of adsorption of a BM41 formulation was calculated by assessing the amount of protein in the supernatant of the vaccine after centrifugation using a BM41 standard curve.

Procedure: A series of dilutions of standard protein (= unformulated BM41 stock solution; c = 620 µg x mL^{-1}) was prepared using WFI. Samples were centrifuged (Eppendorf 5424) at 14.500 rpm for 10 minutes. The BCA protein assay was carried out in 96 well solid plates for BCA assay (Thermo Fisher Scientific). Standards, sample and blank were pipetted (100 µL) in duplicates and 100 µL of working reagent was added (QA: QuantiPro BCA QB : Copper sulphate = 25 : 25 : 1) into predefined wells of the 96 well plate. The plate was incubated for 30 minutes at 57.5°C (+/- 2.5°C) before measuring the absorbance at 562 nm on a plate reader (BioRad). The standard curve and sample concentration calculations were evaluated with Excel 2013.

11.4 Bioburden

For the determination of colony count of aerobic microorganisms and fungis in test samples CASO-Agar and Sabouraud-Glucose-Agar from Biomerieux are used. The assay involves pipetting and striking of 2 aliquots from each test sample on CASO-Agar plates and Sabouraud-Glucose-Agar plates from Biomerieux and subsequent incubation at 32,5°C (aerobic microorganisms) or 22,5°C (fungis) for 5 days. After incubation agar plates are checked for possible growth of bacteria and fungies. Possible colonies are counted and the mean of colony number is calculated and documented.

11.5 SDS Polyacrylamide-gel-electrophoresis (SDS-PAGE)

For separation and identification of proteins according to their molecular weight a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been performed. SDS (=Sodium Dodecyl Sulfate) is an anionic detergent which can be bound be the denaturated polypeptides. SDS can be bound by the polypeptides and masks the initial charge of the protein. Because of this complex the protein becomes negatively charged in proportion to their molecular weight. The proteins can now be separated by size due to the fact

that they move at different speeds through the gel. A molecular marker (BioRad Precision Plus Protein Standards) of known molecular weight has been used as a reference and for identification of the protein.[59]

For reducing conditions SDS in combination with β-mercaptoethanol, a reducing agent, helps to denature quaternary, tertiary and secondary structures of proteins.

Procedure: Analytical SDS-PAGE was performed using Criterion Tris-HCl Protein Gel (BioRad, 18 well) under reducing (sample buffer with beta-mercaptoethanol) and/or non-reducing conditions. Samples were diluted (0.5 µg) in sample buffer/WFI, heated for denaturation (5 minutes at 96 °C) and spin down. The electrophoretic apparatus (BioRad) is filled with 1 x TGS (Stock 10 x TGS BioRad) and the SDS-Page is carried out at 200 Volt for 55 minutes.

Sample Buffer
8.75 % SDS
0.22M Tris-Base
3.5 % beta-mercapto-ethanol
25 % Glycerin
0.05 % bromphenol-blue
pH 6.8 adjust with 25 % HCl

11.6 Coomassie Blue staining

Finally the proteins were visualized by Coomassie Blue staining. The detection depends on a non-specific binding of the dye to aromatic amino acids of the protein under acidic conditions. The staining process is depending on temperature. During destaining the not-bond dye gets removed so that only visible protein bands appear on a clear background.

Procedure: The gel got stained for 1 hour at 60°C with coomassie blue staining-solution. For destaining we added 3 times a destaining solution every 20 minutes at 60 °C and wash the gel when clear bands are visible and the background appears clear.

Coomassie blue staining solution:	Destaining solution:
500 mL Isopropanol	200 mL acetic acid 96 %
2 g Brilliant Blue R 250	1800 mL WFI
200 mL acetic acid 100%	
1300 mL WFI	

11.7 Western Blotting

Western Blotting was used to detect the BM41 protein by using specific antibodies after separation of the protein via SDS-Page. For transferring the BM41 protein on to blotting membrane (BioRad) electro blotting was performed (TurboBlot BioRad). For detection of the BM41 protein a monoclonal mouse antiBM4 antibody (InVivo BioTech) was applied as primary antibody. Detection was done by alkaline phosphatase (AP) conjugated goat anti-mouse IgG antibody (diluted 1 : 10.000 in blocking buffer, Sigma-Aldrich).

Procedure:

SDS Page was performed according to established protocol to separate the proteins. Electro Blotting was performed according to manufactures protocol (TurboBlot Bio-Rad). The membrane got blocked in blocking solution for 1 hour. The membrane was incubated with the primary antibody mouse antiBM4 antibody (diluted 1:30.000 in blocking solution) provided by InVivo BioTech over night at 4°C. On the next day the membrane got washed 3x with TBS-T for 10 minutes. The blot was incubated with a secondary antibody anti mouse IgG AP conjugated (Sigma Aldrich, diluted 1:10.000 in blocking buffer) for 2 hours at room-temperature followed by a 3 times washing step with TBS-T for 10 minutes. After incubation with the appropriate secondary antibody, bound antibodies were first incubated for 10 minutes with AP-Buffer and then visualized with staining solution. The reaction was stopped by washing with WFI.

10x TBS:
61 g TrisBase
88 g NaCl
adjust with 25 % HCl to pH 7.4 +/-0.1
ad 1 L with WFI

Staining Solution:
1 x NBT/BCIP tablet
10 mL WFI

AP Buffer:
12.11 g TrisBase
5.84 g NaCl
1.02 g Magnesiumchloride-Hexahydrate
adjust with 25 % HCl to pH 9.5 +/-0.1
ad 1 L with WFI

Blocking Solution:
9 g Non fat dry milk
300 mL TBS-Tween

1x TBS-Tween:
100 mL TBS
900 mL WFI
5 g Tween-20

- Mouse antiBM4 IgG antibody 1 : 30.000 (InVivo BioTech)
- AP conjugated goat anti-mouse IgG antibody 1 : 10.000 (Sigma-Aldrich)

11.8 Phenol

For the measurement of phenol content the Assay Kit for Phenol testing of Spectroquant, Merck was used. It measures phenol and its ortho- and meta substituted compounds in a phenol containing sample solution. The results are based on a standard curve. Assay was performed by using a modified manufacturer's protocol and further improved in house at Biomay by establishing a Phenol based standard curve.

Procedure:

A series of dilutions of standard phenol solution (5 % Phenol solution) was prepared using WFI. Samples were centrifuged (Eppendorf 5424) at 14.500 rpm for 10 minutes. The phenol assay was carried out in 96 well solid plates (Thermo Fisher Scientific). Samples and the standard were diluted 1:1000. To each dilution the working reagent was added according to manufacturer's protocol and incubated for 10 minutes. WFI was used as a blank. Sample, blank and different diluted standards were pipetted (100 µL) in duplicates into predefined wells of the 96 well plate. The absorbance was read

at 490 nm on a plate reader (BioRad). The standard curve and sample concentration calculations were evaluated with Excel 2013.

Phenol Solution:
5 g Phenol (Prolabo, crystallized)
100 mL WFI

11.9 ELISA

Enzyme-linked immunosorbent assay (ELISA) is an immunologic method which originally uses enzyme-linked immunoglobulins to detect proteins which are immobilized on an inert surface (96 well ELISA plate). The linked enzyme (secondary antibody) usually catalyzes a colorimetric reaction by which the antibody-antigen interaction can be visualized by adding the enzyme specific substrate (secondary antibodies are usually labeled with alkaline phosphate ore horseradish peroxidase). The amount of the bound antibody is proportional to the amount of the present antigen on the plate.

a) IgG

This method was chosen to evaluate the amount of allergen specific antibodies produced by rabbits in response to immunization with the BM41 protein.

Procedure:

ELISA plates (96 wells solid plate NUNC MaxiSorp) were coated with 2 µg of the unformulated BM41 (c = 620 µg x mL^{-1}, diluted in coating buffer) over night at 4°C. On the next day, coating buffer had to be discarded and the plate was washed 3 times with 100 µL PBST. The plate was blocked with 100 µL blocking buffer for 2 hours at room temperature. A series of dilutions of the serum of the immunized rabbit (= primary antibody) was prepared. The plate was incubated with the different dilutions for 2 hours at room-temperature. After 2 hours the serum had to be discarded and the plate was washed 3 times with 100 µL PBST again. For detection the plate was incubated with a secondary HRP labelled antibody (donkey anti-rabbit IgG antibody, diluted 1:10.000 in dilution buffer) for 2 hours and visualized by using TMB substrate. The reaction was stopped using 2N H_2SO_4 and measurement was taken at 450 nm by iMark Microplate Absorbance Reader. The standard curve and calculations were evaluated with Excel 2013.

Coating Buffer (1 x DPBS):	1x PBS-Tween (PBST):
100 mL 10 x DPBS	100 mL PBS
900 mL WFI	900 mL WFI
	5 g Tween-20 (0.05 % Tween)
Blocking Buffer:	Dilution Buffer:
1 g BSA	50 mL PBST
100 mL PBST	50 mL Blocking Buffer

- Charles River Rabbit anti BM41 Sera d=70 final bleeding different dilutions
- Donkey anti-rabbit IgG antibody 1 : 10.000 (GE Healthcare)

b) Hypoallergenicity

The aim of this test was to analyse the reduced IgE binding capacity of a hypoallergen mutant vaccine (BM41) in contrast to Bet v 1, the wildtype allergen.

Procedure:

ELISA plates (96 wells solid plate NUNC MaxiSorp) were coated with 2 µg of the un-formulated BM41 (c = 620 µg x mL^{-1}, diluted in coating buffer) and the wildtype allergen Bet v 1 (c = 1080 µg x mL^{-1}, diluted in coating buffer) over night at 4 °C. Predefined wells were only coated with coating buffer but without protein (negative control). On the next day, coating buffer had to be discarded and the plate was washed 3 times with 100 µL PBST. The plate was blocked with 250 µL blocking buffer for 2 hours at room temperature. 10 different sera of patients, suffering from a birch pollen allergy were pipetted in the predefined wells as well as 2 additive negative controls:

- Normal Human Serum (NHS)
  A healthy patient who does not suffer from any allergy. Bet v 1 and/or BM41 protein should not be able to bind any IgE from the human patients' serum, therefore no signal should be detected.
- Coating Buffer
  In several defined wells we pipetted coating buffer instead of human sera from birch allergic patients. No binding of IgE to coating buffer should occur in these wells.

For positive control 2 wells which were coated with Bet v 1 protein were incubated with the specific anti Bet v 1 IgG mouse F1 antibody (diluted 1 : 1000 in dilution buffer) and 2 wells which were coated with BM41 protein were incubated with a specific mouse anti BM41 IgG antibody (diluted 1 : 35.000 in dilution buffer) (figure 17).

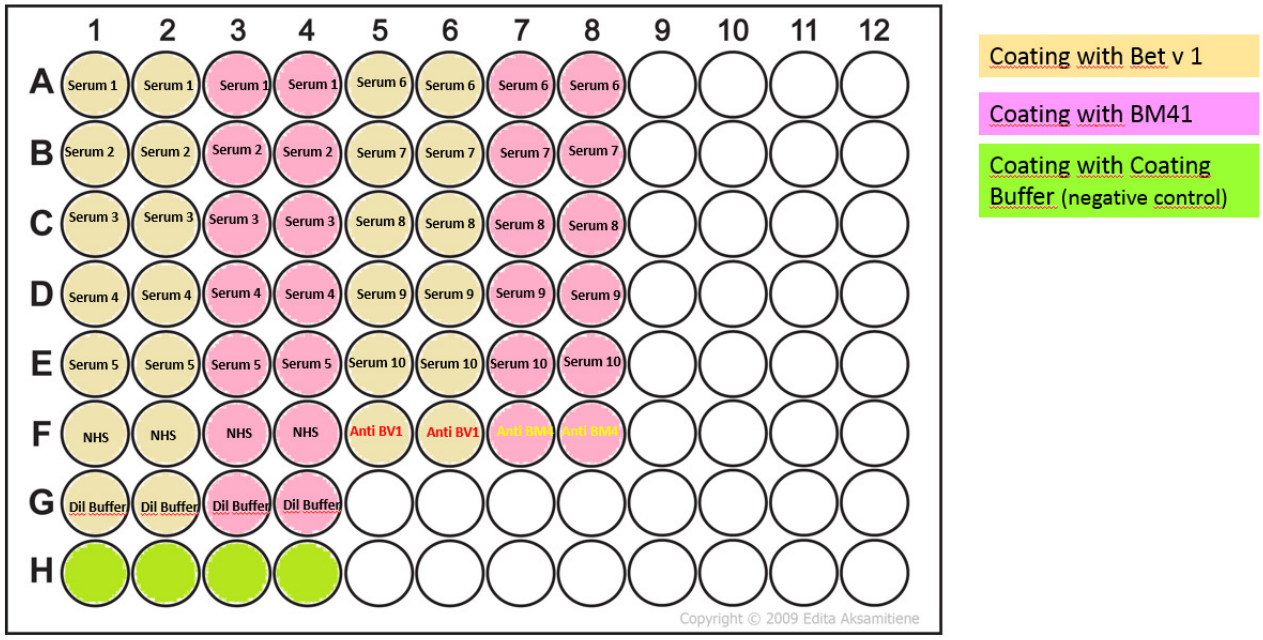


Figure 17. Plate Design for Hypoallergenicity ELISA

The plate was coated with wildtype Bet v 1, BM41, coating buffer and incubated with different patients sera, dilution buffer and the specific anti Bet v 1 and anti BM41 antibodies.

The maxisorb ELISA plate was incubated with human sera from allergic and nonallergic patients at room-temperature for 2 hours. For positive control anti-Bet v1 and anti-BM41 antibodies were used. After incubation sera were discarded and the plate was washed 3 times with 100 µL PBST again. For detection of primary antibodies secondary HRP labelled antibodies (anti-human IgE diluted 1 : 10.000 in dilution buffer or anti-mouse IgG diluted 1 : 50.000 in dilution buffer) were used. After 2 hours detection signals were visualized by using TMB substrate. The reaction was stopped using 2N H_2SO_4 and measurement was taken at 450 nm by iMark Microplate Absorbance Reader. The standard curve and calculations were evaluated with Excel 2013.

Coating Buffer:	1x PBS-Tween (PBST):
300 mL Na_2CO_3 [0.25 M]	100 mL PBS
700 mL $NaHCO_3$ [0.25 M]	900 mL WFI
adjust with 25 % HCl to pH 9.6 +/-0.1	5 g Tween-20 (0.05 % Tween)
Blocking Buffer:	Dilution Buffer:
1 g BSA	200 mL PBST
100 mL PBST	1 g BSA

- Sera from highly birch pollen allergic patients
- Bet v 1 IgG mouse F1 1 : 1000 (in house)
- Mouse antiBM4 IgG antibody 1 : 35.000 (InVivo BioTech)
- Goat anti-mouse IgG HRP (Jackson ImmunoResearch) 1 : 50.000
- Goat anti-human IgE HRP 1 : 10.000 (KPL)

c) Potency Assay

For the BM4-SIT project it was crucial to evaluate the potency of non GMP produced drug products BM41-STA-20150819 (BM41) during interims stability testing at storage temperature of 2℃-8℃ for several month. As a consequence the purpose of this BM41-potency assay was to provide detailed information about the impact of temperature (2℃ - 8℃) over a period of time on the potency by determination of EC_{50} values of BM41-product samples (BM41-vaccine) in order evaluate its suitability for a planned clinical study.

The BM4SIT potency assay works like an inhibition ELISA (enzyme linked immunosorbent assay). This method is based on the assumption that incubation of a given quantity of the reference positive serum with the sample containing antiBM41 antigen will allow the formation of antibody-antigen complexes in a directly proportional manner. If this antibody-sample mixture (= inhibition mix) is then incubated in an ELISA plate coated with BM41, only free antibodies will be able to react with the immobilized antigen on the plate. The antibodies against BM41 in the reference serum has performed a serum-antigen complex and cannot bind the immobilized antigen and are subsequently washed away. The antibodies left to react with the antigen can then be determined by incubation with TMB Substrate. The degree of residual reactivity in the

binding of the reference sera is reciprocal to the concentration of antigen in the sample. Our goal was to perform a reproducible potency assay to obtain a sigmoidal dose-response curve for determination of IC50 with GraphPad Prism to compare “Gold-standard” (unformulated BM41) and formulated BM41 drug product (DP).

Procedure:

ELISA plates (96 wells solid plate NUNC MaxiSorp) were coated with 2 µg of the unformulated BM41 (c = 620 µg x mL^{-1}, diluted in coating buffer) over night at 4 °C. Inhibition mixes were prepared as followed and incubated over night at 4 °C (table 3 and table 4)).

Inhibitionmixes:

Preparation of Sera Dilutions:

Charles River Rabbit anti BM41 Serum d=70 final bleeding 1:35.000

V1: 1:10.000 → 1µl + 10 mL Dilution Buffer

V2: 1:35.000 → 1µl + 10 mL Dilution Buffer

V3: 1:116 → 1µl + 115 µl Dilution Buffer

The anti BM41 serum has to be diluted 1 : 35.000 in every inhibition mix.

Inhibitionmix BM41 Sample:

	Concentration [µg/mL]	Volume [µl]	Pipetting
1	160	300	300µL BM41 + 1µl V3
2	80	300	300µl BM41 + 171µl V1 + 129µl DB
3	40	300	300µl (2) + 300µl V2
4	20	300	300µl (3) + 300µl V2
5	10	300	300µl (4) + 300µl V2
6	5	300	300µl (5) + 300µl V2
7	2.5	300	300µl (6) + 300µl V2
8	1.25	600	300µl (7) + 300µl V2
9	0.625	300	300µl (8) + 300µl V2 Discard 300µl (optional)

Table 3. Pipetting Protocol for inhibition mix BM41 sample.

Inhibitionmix BM41 (Goldstandard):

	Concentration [µg/mL]	Volume [µl]	Pipetting
1	160	300	154.84 Gold + 171µl V1 + 274.16 DB
2	80	300	300µl (1) + 300µl V2
3	40	300	300µl (2) + 300µl V2
4	20	300	300µl (3) + 300µl V2
5	10	300	300µl (4) + 300µl V2
6	5	300	300µl (5) + 300µl V2
7	2.5	300	300µl (6) + 300µl V2
8	1.25	300	300µl (7) + 300µl V2
9	0.625	300	300µl (8) + 300µl V2 Discard 300µl (optional)

Table 4. Pipetting Protocol for inhibition mix unformulated BM41 (goldstandard)

On the next day, coating buffer was discarded and the plate was washed 3 times with 100 µL PBST. The plate was blocked with 100 µL blocking buffer for 2 hours at room temperature. After removing the blocking solution, a 100 µL positive control of serum-dilution V2 (high signal, 0 % inhibition) was pipetted into predefined wells in triplicates and for determination of background 100 µL of blocking solution was pipetted intriplicates. The plate was incubated with the different inhibition mixes for 1 hour at 37 °C (sealed plate with parafilm).

After 1 hour the inhibition mixes was discarded and the plate was washed 3 times with 200 µL PBST again. For detection the plate was incubated with a secondary HRP labelled antibody (donkey anti-rabbit IgG antibody, diluted 1:10.000 in dilution buffer) for 1 hour at 37 °C and visualized by using TMB substrate. The reaction was stopped using 2N H_2SO_4 and measurement was taken at 450 nm by iMark Microplate Absorbance Reader. The standard curve and calculations were evaluated with Excel 2013 and GraphPad Prism 6.

Coating Buffer (1 x DPBS):

100 mL 10 x DPBS

900 mL WFI

1x PBS-Tween (PBST):

100 mL PBS

900 mL WFI

5 g Tween-20 (0.05 % Tween)

Blocking Buffer:

1 g BSA

100 mL PBST

Antibodies:

- Charles River Rabbit anti BM41 Sera d=70 final bleeding 1:35.000
- Secondary AB: donkey anti-rabbit IgG HRP Antibody 1:10.000 (GE Healthcare)

11.10 RP-HPLC

The Reversed Phase HPLC was used for analyzing recombinant proteins and for quality control. In theory the opposite of normal phase, or Reversed Phase Chromatography, results from the adsorption of hydrophobic molecules onto a hydrophobic solid support 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 that is required to promote desorption.

Procedure:

Technical Specifications:

- RP-HPLC was performed on Agilent 1260 Infinity LC
- Separating Column: Agilent Zobrax 300SB-C3 (silica microspheres) narrow bore (2.1 * 150 mm, 5Micron)
- Guard Column: Agilent Zorbax 300SB-C3 narrow bore guard column (2.1*12.5 mm 5 Micron)

- Eluent B: HPLC Gradient Grade Acetonitrile + 0.1% Trifluoroacetic acid (0.2 mL/minute)
- Eluent C: LiChrosolv HPLC Water + 0.1% Trifluoroacetic acid (0.2 ml/minute)
- Sample Injection Volume: 10µl = 6.2µg protein
- Maximum runtime of analysis = 55 minutes
- Temperature of the column oven = 25 ℃
- Temperature of autosampler = 4 ℃ (samples are stored at 4 ℃)
- Detection at 280 nm

Injection schedule of eluting gradients are shown in table 5:

Time (minutes)	% eluent B	% eluent C
0	5	95
50	50	50

Table 5. HPLC injection schedule of eluting gradients

Injection schedule of washing steps are shown in table 6:

Time (minutes)	% eluent B	% eluent C
0	5	95
1	100	0
10	100	0
15	5	95

Table 6. HPLC injection schedule of washing steps

The UV_{280nm} chromatogram were evaluated according to predefined parameter (slope sensitivity, peak width, area reject, height reject).

The identity of the sample was checked by comparing the retention times (rt) of the reference substance and the sample (acceptance criteria: maximum aberration 2 %).

12.) Results

12.1 Formulation of BM41 vaccine and placebo

The aim of BM41 drug substance formulation development was to prepare a final BM41 stability batch, and to place it on stability at 5±3℃ (4 ℃) in order to evaluate the suitability of BM41 drug product for interims stability studies and subsequent human clinical application.

An optimal BM41 formulation was developed by adsorbing the BM41 to aluminium hydroxide (Alhydrogel, Brenntag 2 %) in 10mM HEPES buffer at pH 7.4. For better tolerance the formulation mixes were adjusted to a 0.9% NaCl concentration. Aluminium-containing adjuvants showed better allergenic properties than standard vaccines.[60] There exist different theories of mode of action, why aluminium hydroxide triggers the protective immunity. But none of these theories fully explain how the adjuvant promotes protective antibody responses.[61]

The formulated BM41 was termed BM41-STA-20150819 and prepared using following composition as shown in table 7:

Ingredients	Target quantity	Function
BM41 protein	160µg	Active substance
NaCl	9 mg	Isotonic aid
Aluminium hydroxide	2 mg	Adsorbant
HEPES	10mM, pH= 7.4	Buffer

Table 7. Composition of formulation of BM41 drug product for stability testing

Components were mixed and incubated at room temperature for 60 minutes to achieve a high adsorption to aluminium hydroxide.

Human clinical formulation should ideally contain 80 µg BM41 protein and a minimum of 1.25 mg aluminium hydroxide in dose of 500 µl.

Same procedure was used to formulate placebo samples for stability testing but without API (BM41) as shown in table 8.

Ingredients	Target quantity	Function
NaCl	9 mg	Isotonic aid
Aluminium hydroxide	2 mg	Adsorbant
HEPES	10 mM, pH= 7.4	Buffer

Table 8. Composition of formulation of Placebo samples for stability testing

The degree of adsorption was evaluated by Quanti Pro BCA protein assay (Sigma) according to established protocols.

12.2 Stability studies of BM 41 drug product and placebo

3 mL Aliquots of the BM41-STA-20150819 were placed on stability at 2-8 ℃ and on room temperature with continuous monitoring of the temperature. The batches were tested as follows (table 9):

Analytical Test		Time points [month]							
	t=0	t=1	t=3	t=6	t=9	t=12	t=15	t=18	t=20
Visual Appearance	X	X	X	X	X	X	X	X	X
pH measurement	X	X	X	X	X	X	X	X	X
Protein concentration in supernatant (stability of adsorption to alum)	X	X	X	X	X	X	X	X	X
Inhibition ELISA (Potency Test)	X	X	X	X	X	X	X	X	X
Bioburden	X								X
Hypoallergenicity								X	

Table 9. Time schedule for stability testing of BM41-STA-20150819

12.3 Specifications

The results of the initial characterization of the DP according to test parameters and acceptance criteria shown in table 10 were as follows:

Parameter	**Acceptance Criteria**
Characteristics	
Appearance	Suspension
Colour	White
pH	6.0 – 8.0
Bioburden	< 1 cfu/mL.
Protein in supernatant (BCA)	≤ 16 µg/ml
Potency (Inhibition ELISA)	For information only

Table 10. Specifications for stability testing of BM41-STA-20150819

12.4 Results of Stability Testing of BM41 vaccine and placebo

a) Visual Appearance

The visual nature of the DP is assessed by visual inspection after swaying of the product vial. 3 vials were inspected at each time point. Observations regarding appearance, colour, and aggregates were documented (table 11).

Parameter	Acceptance Parameter	Compliance
Clouding	very clouding	Yes
Colour	White	Yes
Particle in Solution	No	Yes

Table 11. Results of visual appearance of stability testing

b) pH measurement

The pH measurement of BM41-STA-20150819 was performed at RT (25℃) by using a micro electrode SympHony pH-Meter, which was always calibrated on the day before sample measurements. Results in figure 17 and table 12 demonstrated clearly that pH values of BM41-STA-20150819 were always constant over months, indicating high stability and therefore in line with the specifications (pH= 6.0 - 8.0) shown in table 10.

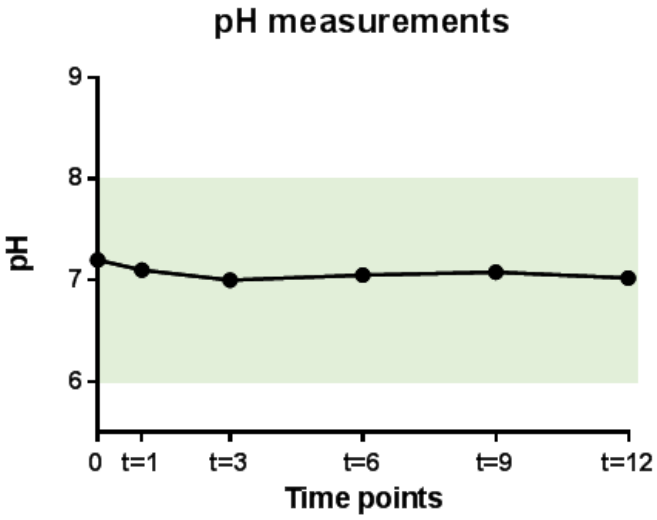


Figure 18. Results of pH measurements of BM41 drug product.

pH measurements at time point t=0 until t=12 months of stability testing of BM41-STA-20150819 are shown.

Time point	pH
t=0	7.20
t=1	7.10
=3	7.00
=6	7.05
t=9	7.08
t=12	7.02

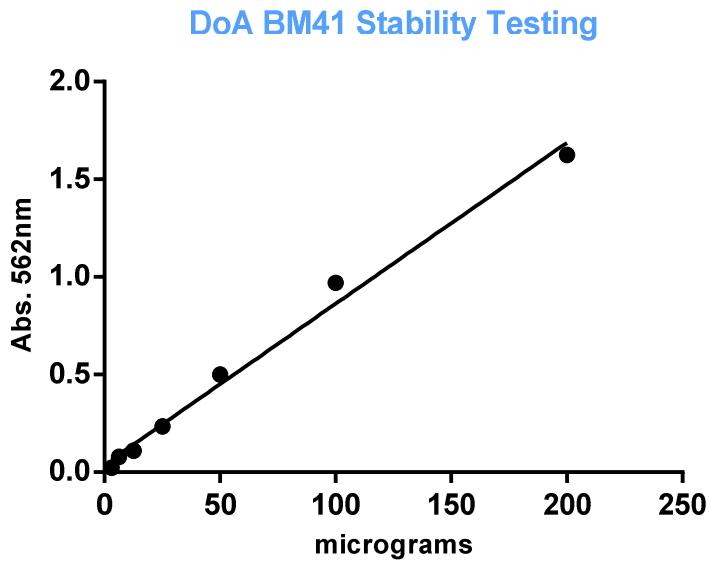
Table 12. Results of pH measurements

of time point t=0 to time point t=12 months of stability testing of BM41-STA-20150819.

c) Protein concentration in supernatant

DoA stands for the degree of adsorption and was an index of the amount of the BM41 protein [µg/µl] was bound to the aluminium hydroxide. The samples were centrifuged for 10 minutes at 14500 rpm to ensure spinning the aluminiumhydroxid-protein-complex down. As a consequence the DoA in % was used to describe the ratio between adsorbed and non-adsorbed BM41 protein in the supernatant.

For stability testing at different time points the degree of adsorption of BM4-STA-20150819 was measured in 3 different vials (V1, V2, V2) and a sample which was stored at room temperature. The DoA was determined by BCA Protein Assay. Exemplarily for all measurements a corresponding standard curve of the BCA assay and the results of the stability testing of BM41-STA-20150819 are shown in figure 19.



	micrograms	Abs. 562nm
BM4-STA-20150819 c=160µg/mL V1	-0.566	0.034
BM4-STA-20150819 c=160µg/mL V2	2.711	0.060
BM4-STA-20150819 c=160µg/mL V3	0.163	0.040
BM4-STA-20150819 c=160µg/mL RT	6.109	0.089

Figure 19. Result of Degree of Adsorption Analysis of BM41-STA-20150819

For stability testing of BM41-STA-20150819 3 vials stored at 4 °C and one vial stored at room-temperature were tested. This figure shows the standard curve of one DoA stability testing and the measured absorbance at 562 nm as well as the corresponding calculated micrograms of protein in the supernatant.

Calculation of DoA:

DoA = 100 – (protein in supernatant [µg/mL] x 100 / protein quantity of the sample [µg/mL], table 13)

Sample	DoA	Protein in supernatant	Specification
BM4-STA-20150819 V1	100 %	0 µg/mL	≤ 16 µg/ml
BM4-STA-20150819 V2	98.31 %	2.71 µg/mL	≤ 16 µg/ml
BM4-STA-20150819 V3	99.90 %	0.163 µg/mL	≤ 16 µg/ml
BM4-STA-20150819 RT	96.18 %	6.109 µg/mL	≤ 16 µg/ml

Table 13. Calculation Results of DoA of BM41-STA-20150819 stability testing

For stability testing of BM41-STA-20150819 3 vials stored at 4 °C and one vial stored at room-temperature were tested. The table represents the calculated degree of adsorptions calculated in Excel as explained above.

The summarized observed data of all DoA measurements is indicated below in figure 20.

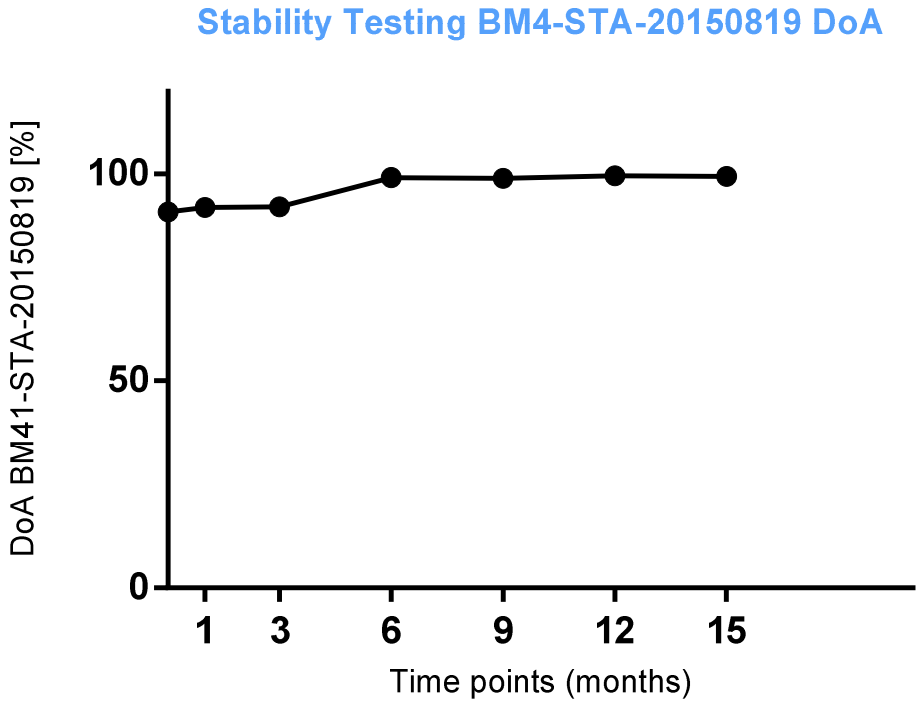


Figure 20. Results of all DoA measurements of BM41-STA-20150819

For stability testing of BM41-STA-20150819 three vials stored at 4 °C and one vial stored at room-temperature were tested. This figure shows the degree of adsorption in percent of BM41-STA-20150819 stability testing.

All measurements at the different time points were within the predefined specification ≤16 µg/ml. The protein BM41 built a stable complex with the alum compound.
In addition the BCA results in figure 20 and table 13 showed precisely that almost no de-adsorption of BM41 protein from aluminium hydroxide was detectable over the time period of 15 months resulting in DoAs from 90 % - 100 %. The calculated DoA values corresponded to a protein concentration of only 0-14 µg/µl BM41 and were therefore in line with the specifications (≤ 16 µg/ml) shown in table 10.

d) Potency Assay (Inhibition ELISA)

New regulatory demands require the use of a potency assay for alum adsorbed final products. For clinical trials and for future registration of the product it is essential to develop an Inhibition ELISA to measure the potency of (different batches of) the final product. Using rabbit anti-sera raised against the drug product or drug substance, an Inhibition ELISA had been set up that can measure potency.

In the established potency assay a BM41 “gold standard” was coated on a 96 well plate and afterwards incubated with an αBM4rabbit sera mix. The sera were pre-incubated with different BM41 drug product samples, which need to be tested, and a “gold standard” of BM41 reference protein. Intact BM41 samples should give a high signal of inhibition whereas degraded samples were expected to give a low signal of inhibition.

For developing the Potency Assay at Biomay we instructed Charles River Laboratories to immunize a rabbit (female New Zealand) with the protein BM41 to obtain a fresh αBM4rabbit serum:
A BM41 solution (2.5 mL) with a concentration of 0.2 mg/mL BM41 protein in 10mM HEPES Buffer pH 7.4 was sent to Charles River Laboratories for formulation with CFA and IFA (500µL protein + 500µL adjuvant CFA/IFA) to immunize one New Zealand rabbit as shown in table 14.

BM41-Dose per injection: 0.1 mg

Vaccination schedule for immunization

Day 0	**Pre-Bleeding + 1. Injection**
Day 28	Injection
Day 38	Test Bleeding
Day 42	Injection
Day 52	Test Bleeding
Day 56	Injection
Day 70	Final Bleeding

Table 14. Vaccination schedule for rabbit immunization by Charles River Laboratories

Determination of IgG response of BM41 immunized New Zealand rabbits

For determination of the IgG response of BM41 immunized rabbits we diluted the BM41 sera from day 0, 38, 52 and 70 from 1 : 10 to 1 : 781250 and analysed them via IgG Elisa according to Biomay's protocol.

A 96 ELISA well plate was coated with 2 µg of the BM41 protein and incubated with different dilutions of the antiBM4 rabbit sera. Detection was performed by using a donkey anti-rabbit IgG HRP labelled secondary antibody and the TMB Solution (Sigma Aldrich). IgG levels of rabbit antiBM4 sera are shown in figure 21:

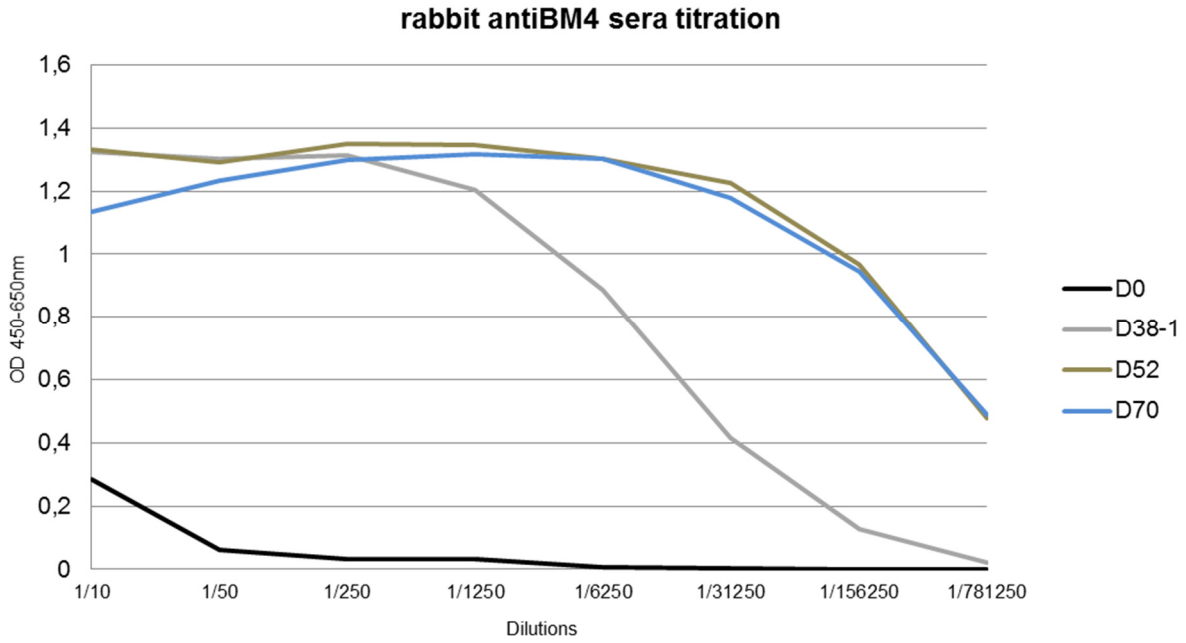


Figure 21. Rabbit antiBM4 sera titration using different dilutions

This figure shows the IgG antibody levels after immunization with the BM41 antigen in increasing dilutions of the rabbit serum. Line blue and green which represent sera from day 52 and day 72 showed high OD values. As expected at day 0 (first injection, indicated in black) was at the baseline (no antibodies were present at this time).

The ELISA results showed that BM41 rabbit sera samples from day 52 and day 70, which were diluted from 1:35.000 to 1:100.000, were suitable for the potency assay development because they demonstrated the highest OD values. In contrast sera sample from day 38 showed a poorly IgG response, resulting in a low OD of 0.22. As expected our negative control from day 0 (pre-immune serum) resulted in a flat line.

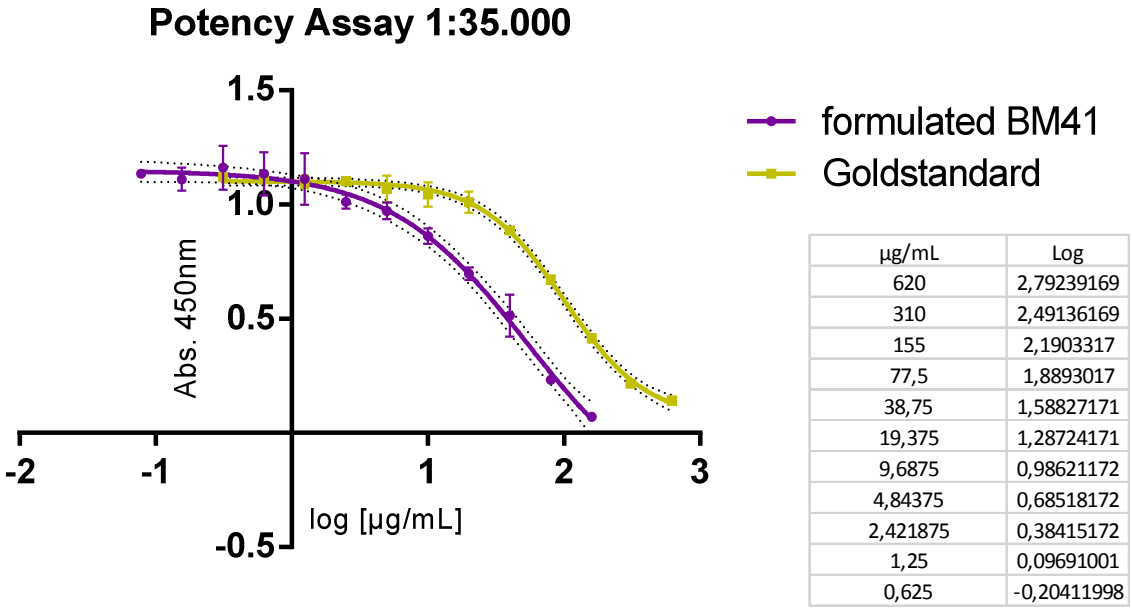
Referring to a potency assay protocol provided by University of Salzburg (Dr. Michael Wallner, Department of Molecular Biology) we tried to obtain a sigmoidal curve by analysing our data with GraphPad. The sigmoidal curve is necessary for the IC50 analysis of the drug product. Our aim was to reach the following expectations:

- Reaching a sigmoidal curve for the unformulated BM41 (“goldstandard”) and the formulated BM41
- Calculating an IC50 with GraphPad Prism Version 6
- Providing detailed information about the impact of temperature and time on the potency
- Evaluating the suitability for a clinical study
- Confirming the measurements by getting reproductive results (the potency assay will be performed on 3 consecutive days under the same conditions)

For the initial experiments 12 dilutions (table 15) of inhibition antigen have been selected in order to obtain a sigmoidal curve, which is necessary for the IC50 analysis. The potency of the formulated BM41 was compared with the physico-chemically characterized unformulated BM41 gold standard stored at -20 °C in house at Biomay. The IC50 was determined by using GraphPad Prism 6 software. Measurements were performed in duplicates.

Dilution	Sample: formulated BM41 [c=160µg/mL]	Goldstandard: unformulated BM41 [c=620µg/mL]
1:2	160 µg/mL	620 µg/mL
1:2	80 µg/mL	310 µg/mL
1:2	40 µg/mL	155 µg/mL
1:2	20 µg/mL	77.5 µg/mL
1:2	10 µg/mL	38.75 µg/mL
1:2	5 µg/mL	19.375 µg/mL
1:2	2.5 µg/mL	9.6 µg/mL
1:2	1.25 µg/mL	4.2 µg/mL
1:2	0.625 µg/mL	2.42 µg/mL
1:2	0.3125 µg/mL	1.2 µg/mL
1:2	0.156 µg/mL	0.6 µg/mL
1:2	0.078 µg/mL	0.3 µg/mL

Table 15. Dilution and pipetting protocol for the initial potency assay experiment



µg/mL	Log
620	2,79239169
310	2,49136169
155	2,1903317
77,5	1,8893017
38,75	1,58827171
19,375	1,28724171
9,6875	0,98621172
4,84375	0,68518172
2,421875	0,38415172
1,25	0,09691001
0,625	-0,20411998

Figure 22. Result of initial potency assay including curve fit.

This figure shows the initial potency assay which was performed according to table 13 with using the αBM4 rabbit sera mix in a dilution of 1:35.000. 12 values were used to obtain a sigmoidal curve for the IC50 data analysis. The line in gold represents the goldstandard BM41 protein, the formulated BM41 drug product is indicated in violet.

Both curves showed a sigmoidal characteristic course but in the range of higher diluted values (very low concentration of formulated and unformulated sample) a linear behaviour can be seen (figure 22). Due to this effect we decided to exclude some of the highest dilution steps (table 16) and repeated the assay using the same conditions again.

Dilution	Sample: formulated BM41 [c=160µg/mL]	Goldstandard: unformulated BM41 [c=620µg/mL]
1:2		620 µg/mL
1:2		310 µg/mL
1:2	160 µg/mL	155 µg/mL
1:2	80 µg/mL	77.5 µg/mL
1:2	40 µg/mL	38.75 µg/mL
1:2	20 µg/mL	19.375 µg/mL
1:2	10 µg/mL	9.6 µg/mL
1:2	5 µg/mL	4.2 µg/mL
1:2	2.5 µg/mL	2.42 µg/mL
1:2	1.25 µg/mL	1.2 µg/mL

Table 16. Dilution and pipetting protocol for the second potency assay experiment

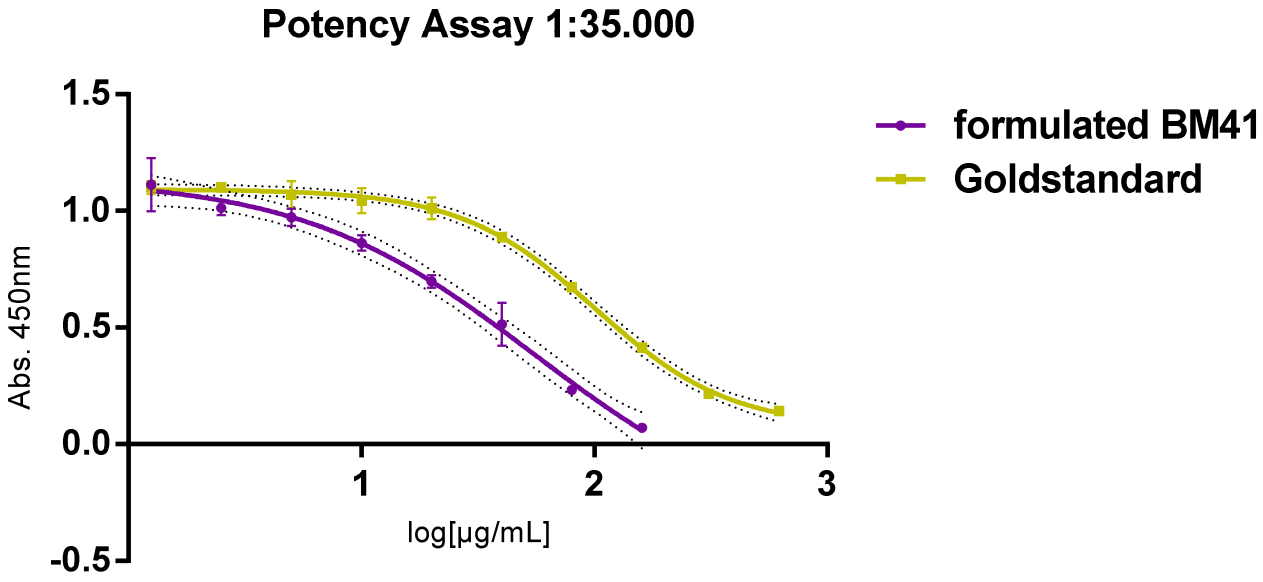


Figure 23. Result of 2nd potency assay including curve fit.

This figure shows a potency assay which was performed according to table 16 with using the αBM4 rabbit sera mix in a dilution of 1:35.000. For the sample we used 8 different dilutions (indicated in violet) and 10 dilutions were selected for the BM41 gold standard (indicated in gold). The values were used to obtain a sigmoidal curve for the IC50 data analysis. The line in gold represents the goldstandard BM41 protein, the formulated BM41 drug product is indicated in violet.

The inhibition results of the goldstandard showed again a sigmoidal behaviour but the curve of the formulated BM41 did not reach the inhibition plateau at the highest concentration (figure 23). The highest concentration of c=160 µg/mL of the formulated BM41 was too low so that the antibodies of the antiBM4 sera were still in excess and the antibody-antigen-reaction could not reach the plateau of the reaction. So we tried to dilute the antiBM4 sera 1:100.000 using the same conditions but changed again the dilution steps:

Dilution	Sample: formulated BM41 [c=160µg/mL]	Goldstandard: unformulated BM41 [c=620µg/mL]
1:2		620 µg/mL
1:2		310 µg/mL
1:2	160 µg/mL	155 µg/mL
1:2	80 µg/mL	77.5 µg/mL
1:2	40 µg/mL	38.75 µg/mL
1:2	20 µg/mL	19.375 µg/mL
1:2	10 µg/mL	9.6 µg/mL
1:2	5 µg/mL	4.2 µg/mL
1:2	2.5 µg/mL	2.42 µg/mL
1:2	1.25 µg/mL	1.2 µg/mL
1:2	0.6250 µg/mL	0.6 µg/mL

Table 17. Dilution and pipetting protocol for potency assay using antiBM4 sera 1:100.000

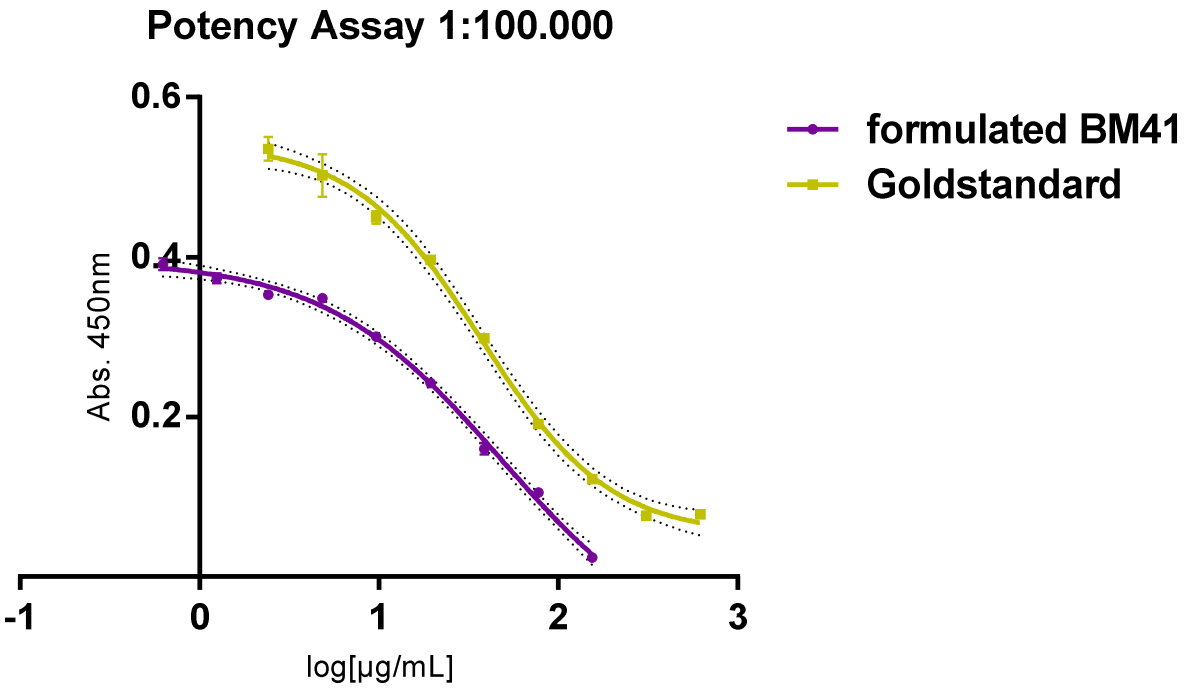


Figure 24. Result potency assay including curve fit.

This figure shows the potency assay which was performed according to table 17 using the αBM4 rabbit sera mix in a dilution of 1:100.000. Different values were used to obtain a sigmoidal curve for the IC50 data analysis. The line in gold represents the goldstandard BM41 protein, the formulated BM41 drug product is indicated in violet.

Using a higher diluted antiBM4 sera (1:100.000) unfortunately resulted in a very low absorption indicating that the limit of detection was reached. Also the behaviour of the formulated BM41 drug product sample could not be improved: no plateau could be reached at the highest concentration. But the IC50 calculation is also possible without reaching the plateau by using Graphpad Prism curve fit.

For further potency assays we decided to use 9 dilution steps. We started at 160 µg/mL and diluted down to 0.625 µg/mL for the formulated BM41 drug substance, as well as for the BM41 protein alone (goldstandard). In addition we prepared a 1:35.000 dilution of the Charles River rabbit anti BM41 sera at day 70 (final bleeding) (table 17).

To exclude that the Charles River rabbit anti BM41 sera reacted with any other substrate we performed an inhibition assay, using, instead of the formulated BM41 sample, a freshly formulated BSA sample as a negative control (same concentration of Alum and BSA protein as the formulated BM41 samples). The results are shown in figure 25.

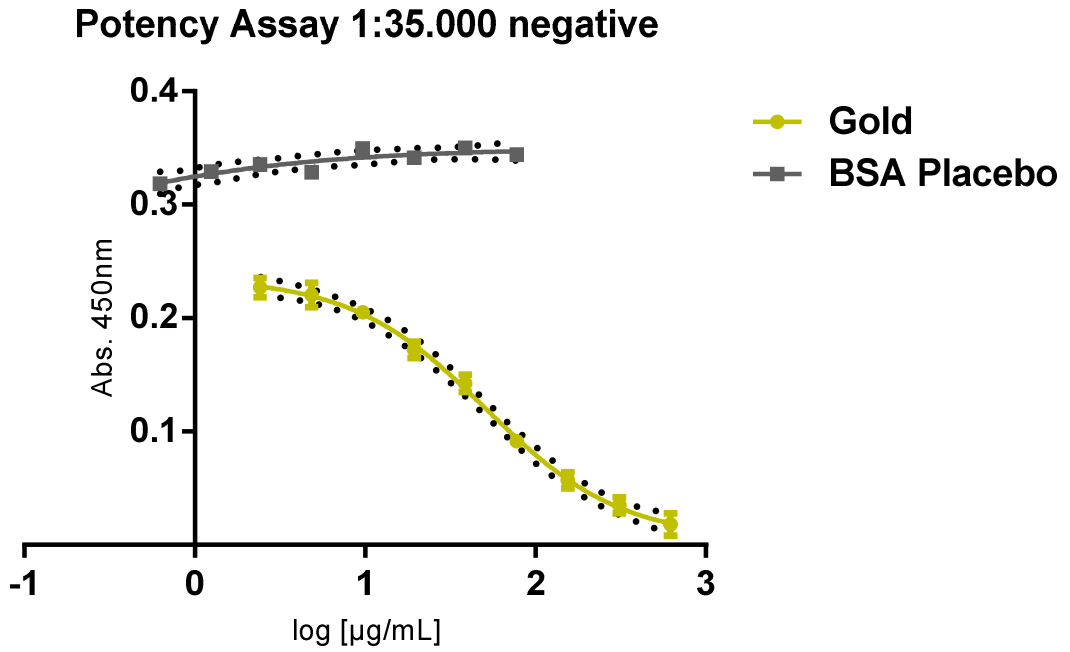


Figure 25. Result of potency assay including curve fit negative control (placebo)

This figure shows the potency assay which was performed using the αBM4 rabbit sera mix in a dilution of 1:35.000. Instead of a sample with an active pharmaceutical ingredient we measured a placebo sample as a negative control indicated in grey. The line in gold represents the goldstandard BM41 protein.

The result of this assay nicely showed that the rabbit anti BM41 sera did not bind to the BSA sample and therefore no inhibition signal is detectable.
As a proof of concept we repeated the assay on 3 consecutive days, using the same conditions (figure 26, 27, 28).

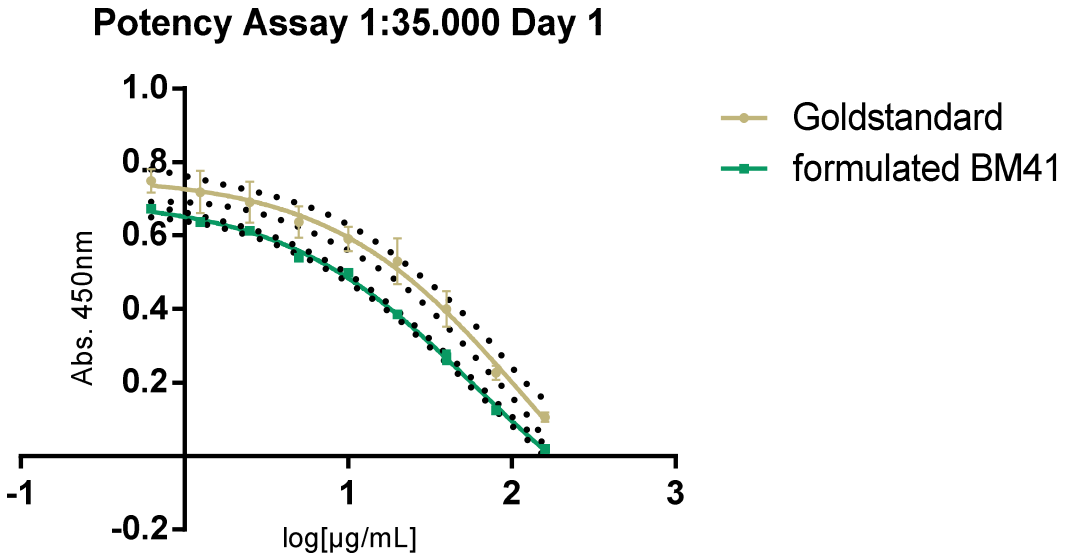


Figure 26. Proof of concept, result of potency assay of Day 1

The gold standard (unformulated BM41) is indicated in gold and the formulated BM41 sample in a green line.

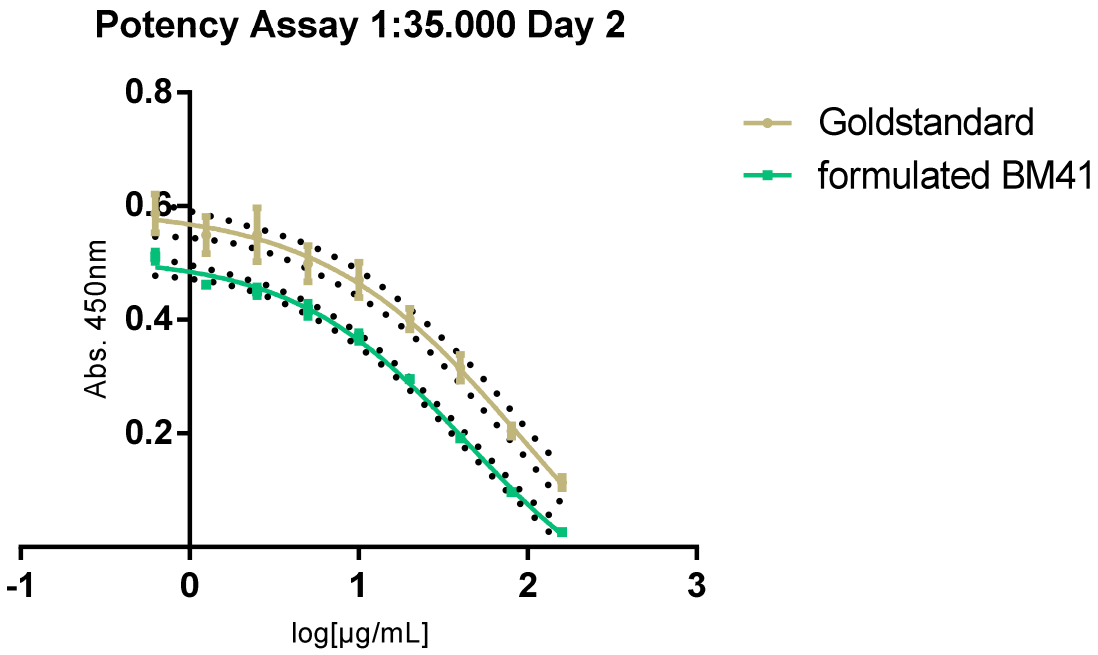


Figure 27. Proof of concept, result of potency assay of Day 2

The gold standard (unformulated BM41) is indicated in gold and the formulated BM41 sample in a green line.

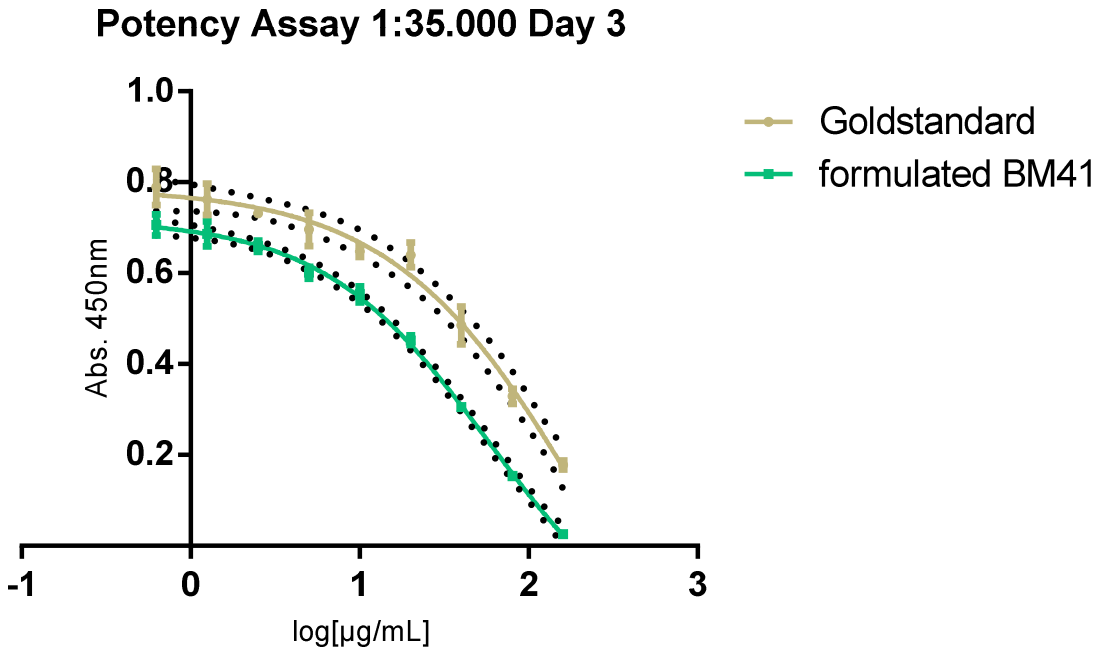


Figure 28. Proof of concept, result of potency assay of Day 3

Figures 27, 28 and 29 show the BM41 potency assay which was performed according to the established protocol on 3 different days using the αBM4 rabbit sera mix in a dilution 1:35.000. 9 values were used to obtain a sigmoidal curve for the IC50 data analysis. The gold standard is indicated in gold line and the formulated BM41 sample in green.

Subsequently it was possible to establish a new protocol for the IC50 determination of the BM41 drug product.

The results of 3 independently performed assays from day 1, 2 and 3, which were calculated by using the logarithm to the base10, showed that the inhibition signals of the formulated BM41 samples were consistent and reproducible. Calculation of IC50 values demonstrated clearly the robustness of the assay (table 18)

Assay	log IC50 value
Day 1	1.764
Day 2	1.638
Day 3	1.766

Table 18. Calculated IC50 Results of potency assay

Table 18 shows the calculated logIC50 inhibition values for day 1, 2 and 3 (GraphPad Prism) of tested BM41drug products.

e) Hypoallergenicity (IgE ELISA)

The aim of this hypoallergenicity test was to analyse the reduced IgE binding capacity of a hypoallergen mutant vaccine (BM41) in contrast to the wildtype allergen (Bet v 1). As a consequence the BM41 protein should have bound significantly less IgE antibodies than the wildtype allergen.

The ELISA has been performed according to Biomay's standard operating procedure (SOP). Therefore ELISA plates were coated with 2 µg of the hypoallergen mutant protein (BM41) and wildtype protein (Bet v 1). For analysing the binding capacity we tested 10 different sera of patients suffering from a birch allergy (the sera were preselected using CAP test). In addition we used NHS and coating buffer as negative controls.

As a positive control we detected the wild-type allergen and the hypoallergen with the specific antibodies (anti-BM41 and anti-Bet v 1 antibodies).

For a positive result in a hypoallergenicity test the following specifications are used as a reference (table 19):

Parameter	Acceptance Parameter
OD_{405} Sera with high CAP	>1.0
OD_{405} NHS	<0.2
OD_{405} negative control	<0.2
OD_{405} Bet v1	>1
OD_{405} BM41	>1

Table 19. Reference values for hypoallergenicity ELISA for Stability Testing of BM41 are shown.

The results of the hypoallergenicity sensitive IgE ELISA demonstrated clearly that the hypoallergen variant BM41 strongly reduced IgE binding (75-97%) to birch allergic patients in comparison to wildtype allergen Bet v 1. All results are shown in table 20, 21 and in figure 29.

		OD_{405nm}			
		Bet v 1	**BM41**	**%OD_{405nm} BM41**	**% Reduction of IgE binding capacity**
Serum 1	V2 102 0011_I	1,489	0,0385	**2,59**	**97,41**
Serum 2	V2 103 0007_I	1,292	0,043	**3,33**	**96,67**
Serum 3	V2 104 0004_I	1,7615	0,269	**15,27**	**84,73**
Serum 4	V3 105 006_I	1,7685	0,1205	**6,81**	**93,19**
Serum 5	V2 201 0005_I	2,195	0,531	**24,19**	**75,81**
Serum 6	V2 201-0031	1,639	0,053	**3,23**	**96,77**
Serum 7	V2 201-0044	1,8095	0,3055	**16,88**	**83,12**
Serum 8	V3 202 0020_I	1,746	0,1165	**6,67**	**93,33**
Serum 9	V2 401 0020_I	1,351	0,0335	**2,48**	**97,52**
Serum 10	V2 601 0027_I	1,0935	0,081	**7,41**	**92,59**
NHS	V2 601 0001_I				91,11

Table 20. Results of OD405nm measurements of hypoallergenicity ELISA of Stability Testing of BM41 drug product and calculated reduction of IgE binding capacity of BM41 protein to different human birch pollen allergic sera.

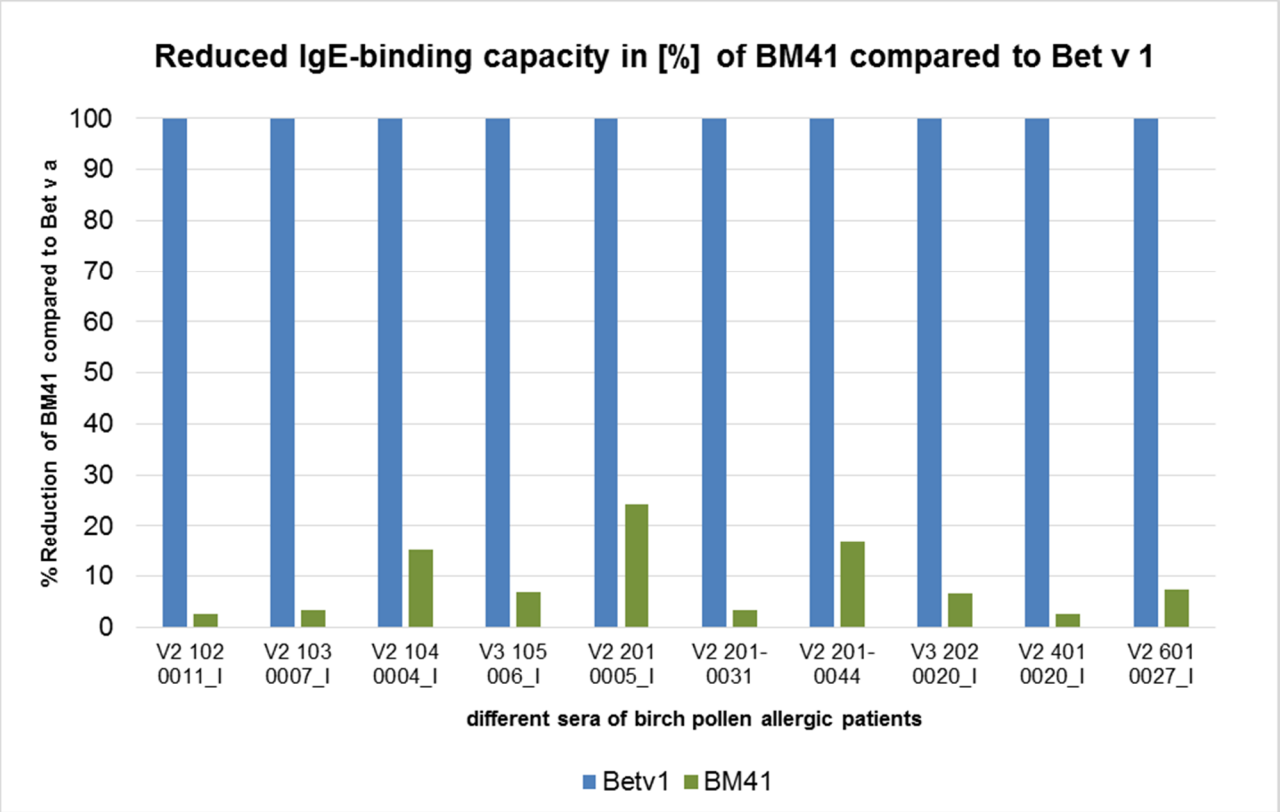


Figure 29 shows the reduced IgE binding capacity of a hypoallergen mutant vaccine (BM41) in contrast to the wildtype allergen (Bet v 1).

Green bars indicate a dramatically IgE reduction in % of BM41 compared to reference WT protein Bet v 1, which is indicated by blue bars. Values have been normalized resulting in a IgE binding capacity of Betv 1 of 100%. As a consequence the IgE binding capacity of BM41 protein was reduced to at least 76%.

Parameter	Acceptance Parameter	Result
OD_{405} Sera with high CAP	>1.0	1.61
OD_{405} NHS	<0.2	0.04
OD_{405} negative control	<0.2	0.02
OD_{405} Bet v1	>1	1.44
OD_{405} BM41	>1	1.24

Table 21. Results and Acceptance Parameter of Hypoallergenicity ELISA of BM41 Batch 20150819

f) Bioburden

For the determination of colony count of aerobic microorganisms and fungis in BM4-STA-20150819 samples CASO-Agar and Sabouraud-Glucose-Agar from Biomerieux were used. This assay was carried out according to established protocol at time point t=0. No bacterial growth or contamination with fungies could be detected.

The next observation will be at time point t=20 months.

12.5 Formulation of a Skin Prick Test Solution and stability testing

The procedure of skin prick testing is an essential method to diagnose IgE-mediated allergic reactions in patients with rhinoconjunctivitis, asthma, urticaria, anaphylaxis, atopic eczema and suspected food or drug allergy. Skin Prick Testing helps to confirm the diagnosis of a suspected type I allergy.[62] [63] [64]

4 different skin testing solutions (A, B, C, D) were prepared for the stability testing of the active pharmaceutical ingredient (table 22, 23, 24 and 25):

Solution A		
Ingredient	*Function*	*Concentration*
BM41 [620µg/mL]	Active substance	100µg/mL
Glycerol 99%	Stabilization	50 %
Albumin	Protein Stabilizer	0.12mg/mL
Phenol 5 %	Preservative	0.5%
HEPES [45mM]	Buffer	10mM, pH 7.4

Table 22. Chemical composition of SPT Solution A

Solution A contained one critical ingredient, phenol which optimally should be avoided in human applications. Phenol is a common agent to reduce bacterial growth.[65]
The second, but not critical additive was Albumin. Albumin [0.12mg/mL] is used for stabilizing the active substance and to reduce nonspecific binding of the allergen protein to the inner glass wall of vials. This is particularly important for diluted vaccines.

Furthermore additional solutions (B, C, D) were formulated; the critical substances like phenol and/or albumin were no components of that solutions. Monitoring of the 4 solutions was performed by testing changes of pH, stability and measuring the concentration of allergen protein at different time points (table 29).

Solution B		
Ingredient	*Function*	*Concentration*
BM41 [620µg/mL]	Active substance	100µg/mL
Glycerol 99%	Stabilization	50 %
Albumin	Protein Stabilizer	0.12mg/mL
WFI		
HEPES [45mM]	Buffer	10mM, pH 7.4

Table 23. Chemical composition of SPT Solution B

Solution C		
Ingredient	*Function*	*Concentration*
BM41 [620µg/mL]	Active substance	100µg/mL
Glycerol 99%	Stabilization	50 %
WFI		
Phenol 5 %	Preservative	0.5%
HEPES [45mM]	Buffer	10mM, pH 7.4

Table 24. Chemical composition of SPT Solution C

Solution D		
Ingredient	*Function*	*Concentration*
BM41 [620µg/mL]	Active substance	100µg/mL
Glycerol 99%	Stabilization	50 %
WFI		
HEPES [45mM]	Buffer	10mM, pH 7.4

Table 25. Chemical composition of SPT Solution D

Finally solution D was the suitable solution for future human applications. Because of safety reasons we decided to exclude phenol in our formulations.

The solutions were sterile filtrated using the Nalgene Rapid flow Filter Unit 50mm dia, 0.2 µm. Previous measurements of the BM41 unformulated standard (c=620 µg/mL) with Eppendorf BioPhotometer demonstrated clearly that the 2µm Nalgene filter was the perfect choice for sterile filtration of BM41 protein. Almost no loss of active substance was determined (table 26, 27 and 28).

BM41 before filtration:

E280 measurement 1	0.349
E280 measurement 2	0.364
E280 measurement 3	0.361
Mean	0.358

Table 26. E280 measurements of BM41 before filtration

For calculating the concentration, we had to observe the extinction coefficient. Using ExPASy ProtParam[66] we observed an extinction coefficient about 0.598. For further photometric measurements this coefficient was used for calculation of the protein concentration.

BM41 after filtration with Nalgene 0.2µm:

E280 measurement 1	0.349
E280 measurement 2	0.358
E280 measurement 3	0.355
Mean	0.354

Table 27. E280 measurements of BM41 after filtration

Calculations of concentration using following quotation: $c_{[mg/mL]} = A / \varepsilon \times L$ *(L=1 cm)*[67]

Before filtration:	$c_{[mg/mL]}$ = 0.358 / 0.598 = 0.599 mg/mL
After filtration:	$c_{[mg/mL]}$ = 0.354 / 0.598 = 0.592 mg/mL
Difference [%]	1.17 %

Table 28. Calculation of concentration of BM41 after and before filtration

Sterile filtration with a 0.2 µm filter of the BM41 protein resulted in only 1.17% (mean of 3 measurements) loss of BM41 protein after filtration.

12.6 Stability Testing of Skin Prick Test Solutions A, B, C, D

Table 29 shows the time schedule of stability testing of Skin Prick Test Solutions:

		Methods						
Time	Sample	pH	SDS Page Reduced/non reduced	Western Blot	Proteinquantifi-cation (Bradford)	HPLC	phenol content	Biobur-den
t=0	A, B, C, D	X	X	X	X	X	X	X
t=2 week	A, B, C, D	X	X		X		X	
t=4 weeks	A, B, C, D	X	X		X		X	
t=6 weeks	A, B, C, D	X	X		X		X	
t=8 weeks	A, B, C, D	X	X	X	X	X	X	
t=12 weeks	D	X	X		X		X	
t=16 weeks	D	X	X		X		X	
t=20 weeks	D	X	X		X		X	
t=24 weeks	D	X	X	X	X	X	X	X
t=0/8/24weeks -20 °C	D		X		X			

Table 29. Time Schedule of Stability Testing of Skin Prick Test Solutions A, B, C, D

A sample stored at -20°C would have been additionally tested in case a 4°C or 25°C (RT) sample has showed considerable change (compared to t=0) in stability. At time point t=12 weeks only solution D has been tested because this solution should further be used for human applications (for reason of safety).

12.7 Specifications of stability testing of SPT solutions

The stability testing was performed according to the following specifications:

Analytical Test	**Specifications**
pH	6.0 – 8.0
Protein concentration	≤ 100 µg/mL
SDS Page reduced/non reduced	Protein identification, purity
Bioburden	< 1 cfu / 1mL suspension
Western Blot	identification
HPLC	Protein identification, purity
Phenol content	4-5mg/mL

Table 30. Specifications for Stability Testing of Skin Prick Test Solutions A, B, C, D

12.8 Results of Skin Prick Test Solution stability testing

a) pH measurement

The pH measurement was performed at RT (25℃) by using a micro electrode Sym-pHony pH-Meter, which was always calibrated on the day before sample measurements. The pH of the measurement solution must be within the range 6.0 and 8.0.

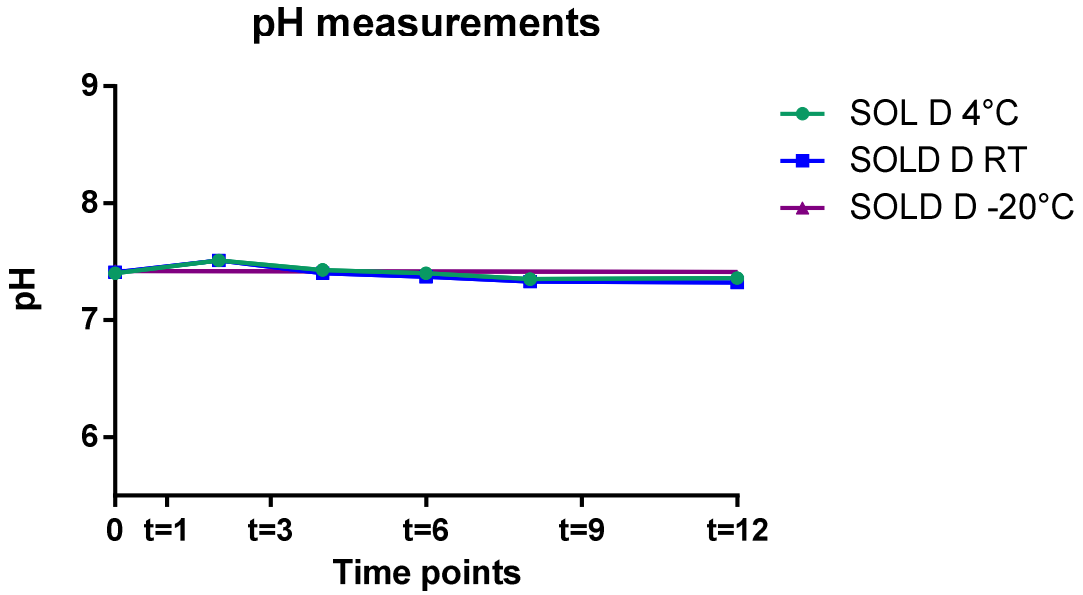


Figure 30. Results of pH measurements of SPT Stability Testing

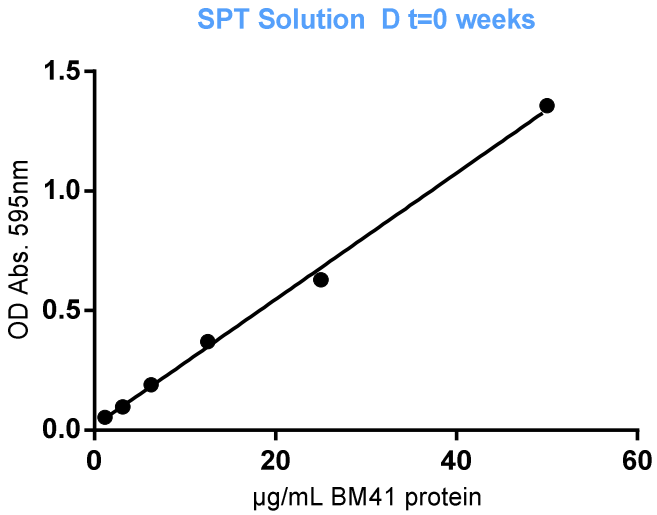
This figure shows the results of the pH measurements of Solution D (4℃ green, room temperature blue and -20 ℃ violet) of time points t=0 to t=12 weeks. Data were obtained by using GraphPad Prism software 6.0.

The results of solution D showed a constant pH value after a time period of 12 weeks for all temperature conditions (figure 30).

b) Protein concentration

To determine if the active ingredient (BM41 protein) non-specifically binds to the glass vials we tried to determine the protein concentration of different samples with BCA - and Bradford Assay. At first we measured the BM41 protein content with the BCA Quanti Pro Assay Kit (Sigma-Aldrich) but the glycerol compounds of the solutions interfered strongly with the assay.[68] Because of the interfering phenol and glycerol compounds we decided to measure the protein concentration with Bradford Reagent (figure 31).

After 8 weeks of measurement of solutions A-D we decided to go for solution D, which did not contain albumin and phenol. As a standard we used the unformulated BM41 (c = 620 µg/mL) and prepared a standard curve by using 5 different dilutions. To minimize the interfering background caused by glycerol we diluted the samples 1:10.



	µg/mL BM41 protein	Optical Density
Sol D RT t=0 weeks	11.598	0.325
Sol D 4°C t=0 weeks	9.969	0.282
Sol D -20°C t=0 weeks	11.484	0.322

Figure 31. Results of Bradford Assay of Stability Testing

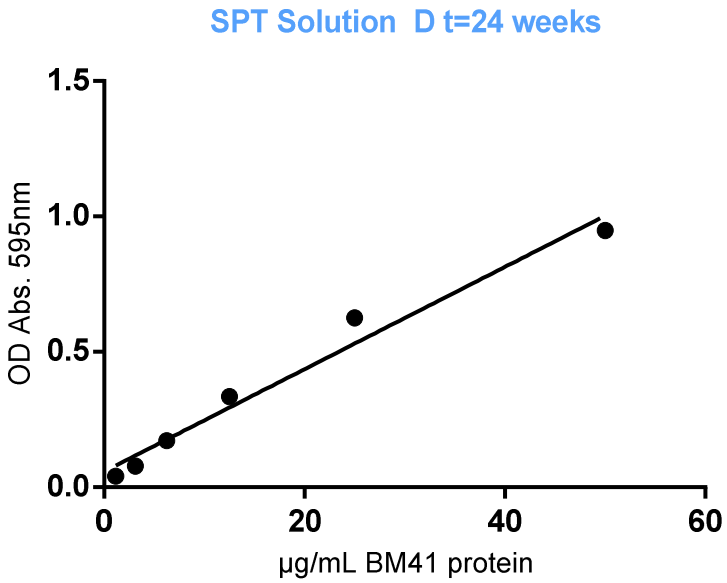
For stability testing Skin Prick Test Solution D (4°C, room temperature and -20 °C) were tested. This figure shows the standard curve of the Bradford Assay and the measured absorbance at 562 nm as well as the corresponding calculated micrograms of protein in the sample at time point t=0 weeks. Data were obtained by using GraphPad Prism software 6.0.

In total calculated BM41 concentrations:

Solution D RT: 11.598 x 10 = 115.98 µg

Solution D 4°C: 9.969 x 10 = 99.69 µg

Solution D -20°C: 11.484 x 10 = 114.84 µg



	µg/mL BM41 protein	Optical Density
Sol D RT t=24 weeks	13.346	0.310
Sol D 4℃ t=24 weeks	12.340	0.291
Sol D -20℃ t=24 weeks	12.128	0.287

Figure 32. Results of Bradford Assay of SPT stability testing

For stability testing Skin Prick Test Solution D (4℃, room temperature and -20 ℃) were tested. This figure shows the standard curve of the Bradford Assay and the measured absorbance at 562 nm as well as the corresponding calculated micrograms of protein in the sample at time point t=24 weeks. Data were obtained by using GraphPad Prism software 6.0.

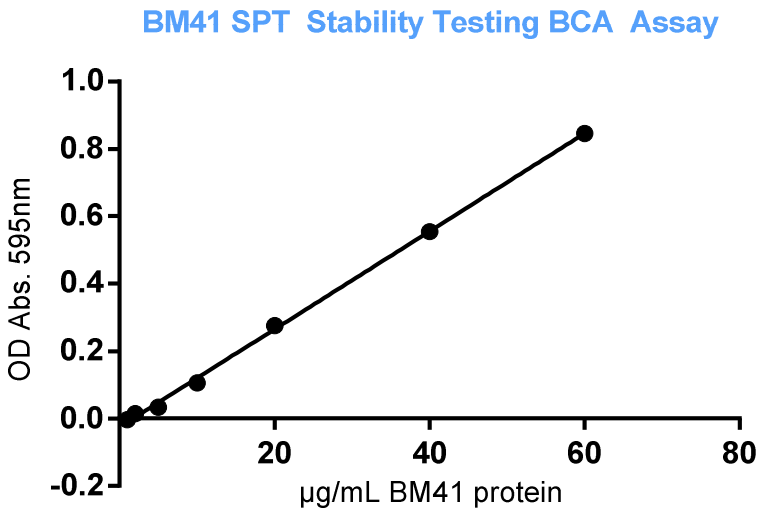
The final results of Bradford measurements at t=24 weeks at different temperatures are shown below:

Solution D RT: 13.346 x 10 = 133.46 µg
Solution D 4℃: 12.340 x 10 = 123.40 µg
Solution D -20℃: 12.128 x 10 = 121.28 µg

Referring to the results of Bradford measurements at t=24 weeks (figure 32) at different temperatures we realized that protein concentration values were not in accordance with the BM41 protein amount of 100µg/mL used for preparation of solution D. As a consequence we planned to establish a new and more precise way to measure the protein concentration.

The most import result of the Bradford measurement was that no real reduction of BM41 protein in solution D was measurable over the period of 24 weeks. In addition we could proof that unspecific bindings of the BM41 protein to the inner side of the glass vials could be excluded.

For our improvement of the BM41 protein measurements the BCA assay was the method of choice (figure 33):



	µg/mL BM41 protein	Optical Density
BM41 SPT	23.764	0.319
BSA control	19.803	0.262

Figure 33. BCA Assay of BM41 Skin Prick Test Solution

This figure shows the standard curve of the BCA Assay of SPT Stability Testing (BM41 protein) and the measured absorbance at 565 nm as well as the corresponding calculated micrograms of BM41 protein in the sample. Data were obtained by using GraphPad Prism software 6.0.

The BM41-SPT samples and the unformulated BM41 standard was diluted in a way that the final concentration of Glycerol and HEPES was equal (12.5 % Glycerol and 2.5 mM HEPES).

SPT samples were diluted 1:4 in WFI. The unformulated BM41 protein, which was used as a standard, was diluted in a Glycerol/HEPES solution. As a control we used albumin [c=2mg/mL], diluted 1:100 with Glycerol/HEPES as well.

In total calculated BM41 concentrations:
BM41 Solution: 23.764 µg x 4 = 95.05 µg
Albumin Control: 19.803 µg x 100 = 1.98 mg

For a positive result in a BCA SPT stability protein measurement the following specifications were used as a reference:

- Retrieval rate of Albumin Control: 85 – 115 %
- Relative Standard Deviation of Sample: < 20 %
- Protein concentration [mg/mL]: 0.07 – 0.15

The study demonstrated that all measurements, obtained by using the new established Bradford SPT stability protein assay, were in range and valid.

c) SDS Page reduced/nonreduced

SDS Page was performed by using 10-20 % Tris-HCL (BioRad) and stopped after 55 minutes at 200 Volt. SPT samples (BM41) were diluted (0.5 µg) in sample buffer/WFI (bromphenol-blue and for the reducing conditions beta-mercapto-ethanol) and heated for denaturation (5 minutes at 96 °C).

t=0 weeks – reduced SDS Page

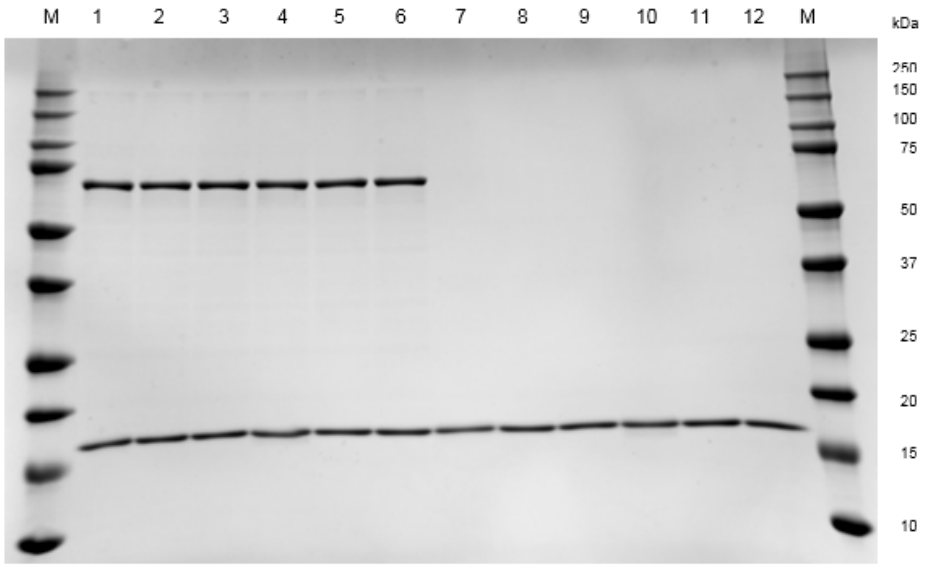


Figure 34. Reduced SDS Page of SPT Stability Testing at time point t=0 weeks

Containing solution A RT (lane 1), solution A 4 ℃ (lane 2), solution A -20 ℃ (lane 3), solution B RT (lane 4), solution B 4 ℃ (lane 5), solution B -20 ℃ (lane 6), solution C RT (lane 7), solution C 4 ℃ (lane 8), solution C -20 ℃ (lane 9), solution D RT (lane 10), solution D 4 ℃ (lane 11), solution D -20 ℃ (lane 12).

t= 0 weeks – nonreduced SDS Page

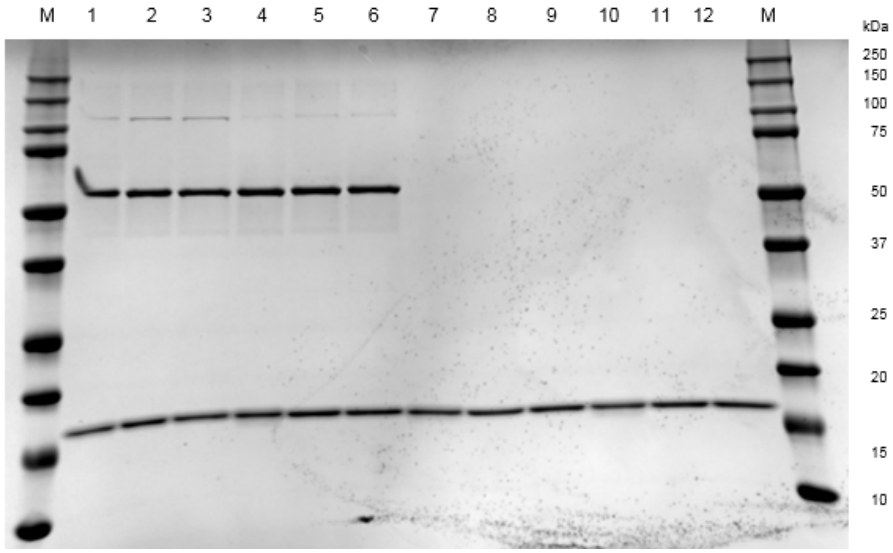


Figure 35. Non-reduced SDS Page of SPT Stability Testing at time point t=0 weeks

Containing solution A RT (lane 1), solution A 4 ℃ (lane 2), solution A -20 ℃ (lane 3), solution B RT (lane 4), solution B 4 ℃ (lane 5), solution B -20 ℃ (lane 6), solution C RT (lane 7), solution C 4 ℃ (lane 8), solution C -20 ℃ (lane 9), solution D RT (lane 10), solution D 4 ℃ (lane 11), solution D -20 ℃ (lane 12).

All detected bands confirmed the expected BM41 protein at 17.4 kDa (figure 34 and figure 35). The additional band detected on lane 1, 2, 3, 4, 5, 6 (under reduced and non-reduced conditions) showed the additive Albumin (at 66 kDa).[69]

t= 24 weeks – reduced and nonreduced SDS Page

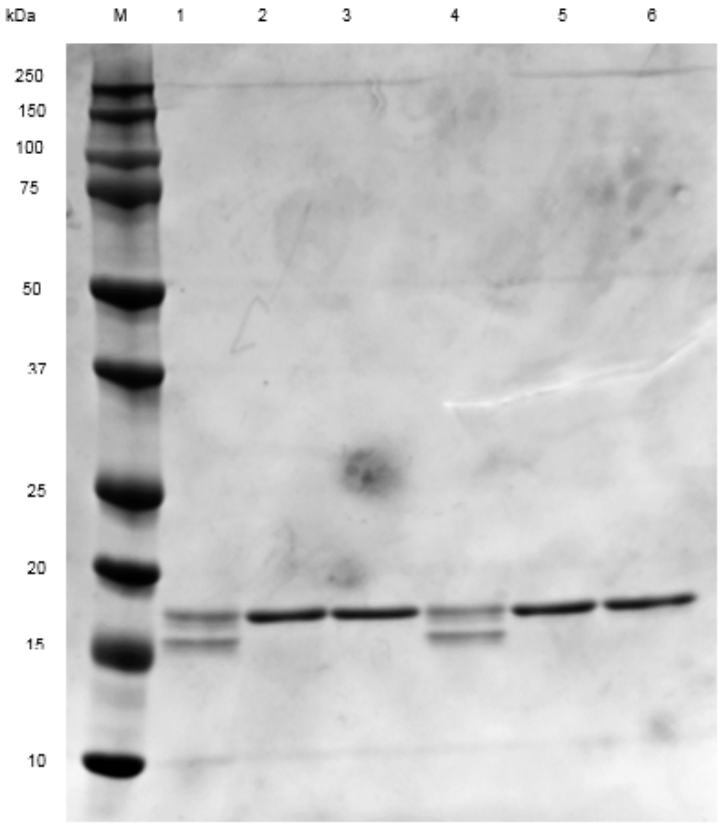
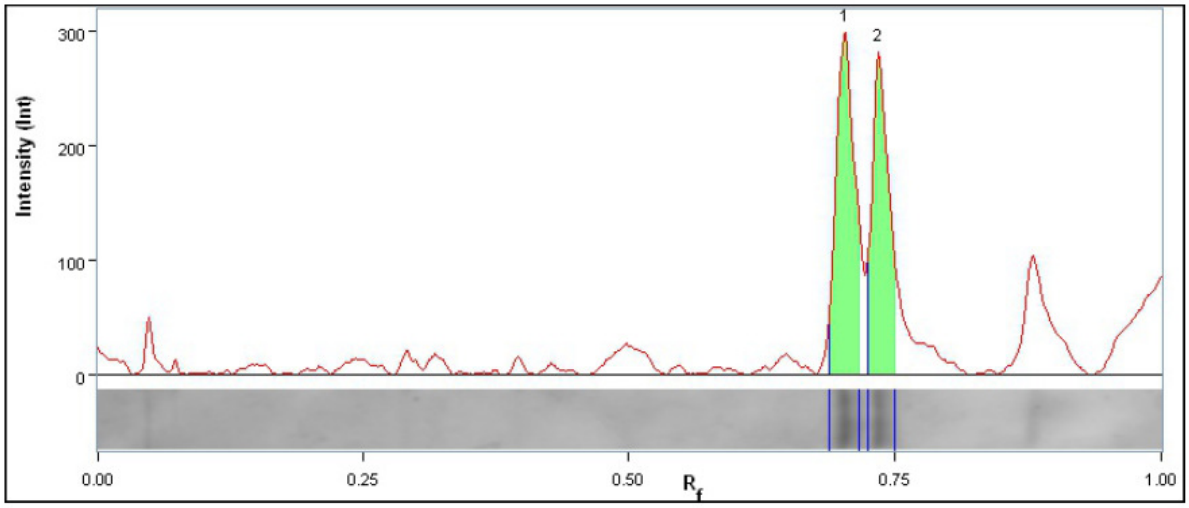


Figure 36. Reduced and non-reduced SDS Page of SPT Sol D t=24 weeks

containing solution D RT reduced (lane 1), solution D 4 °C reduced (lane 2), solution D -20°C reduced (lane 3), solution D RT non-reduced (lane 4), solution D 4 °C non-reduced (lane 5), solution D -20°C reduced (lane 6),

After 24 weeks, the BM41 protein on 4 °C and -20 °C was still stable but interestingly at room temperature a protein degradation was well visible (figure 36). Quantitation of the degraded protein after electrophoresis was made by producing a linear relationship between log MW and Rf.

Rf (relative migration distance) = distance to band / distance to dye front:



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		N/A	0,703	245.898	N/A	N/A	53,0	24,2
2		N/A	0,734	218.310	N/A	N/A	47,0	21,5

Figure 37. Quantitation and analysis of the degraded BM41 protein in SPT sample D (RT)

This figure shows the analysis of the degraded protein BM41 after electrophoresis on Lane 1 SPT Solution D stored at room-temperature under reduced conditions.

Two bands were detected: 53 % and 47 %. This clearly demonstrated that the BM41 protein degraded into 2 fragments of similar size (figure 37).

d) Bioburden

For the determination of the colony count of aerobic microorganisms and fungis in SPT samples CASO-Agar and Sabouraud-Glucose-Agar from Biomerieux were used. The assay was carried out according to established protocol. After incubation for 5 days the plates were checked, counted for possible growth of bacteria and fungies and of each of the agar plates a photograph for documentary was taken.

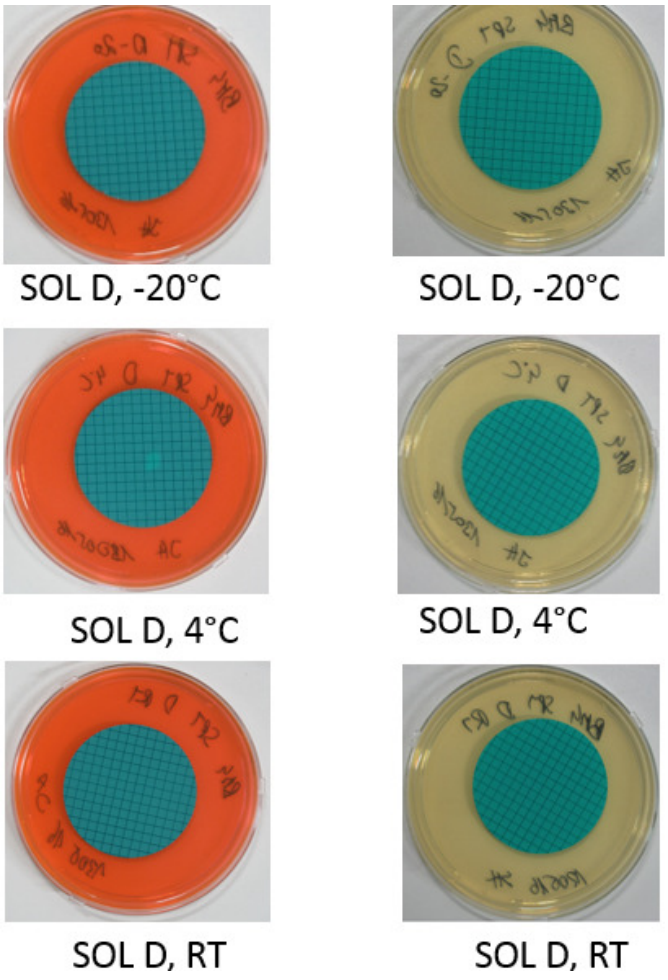


Figure 38. Photograph documentary of Bioburden Testing

Figure 38 shows the negative CASO-Agar and Sabouraud-Glucose-Agar Plates for detection of microbiological contamination of Solution D (-20 ℃, 4 ℃ and room-temperature).

None of the solutions showed bacterial or fungi growth, demonstrating that the solutions were sterile (figure 38).

e) Westernblot

Western blotting was used to detect the BM41 protein by using specific antibodies (anti BM41 antibody) after separation of the protein via SDS-Page. To obtain a monoclonal antibody for BM41 we instructed the company InVivo BioTech Services GmbH to produce a monoclonal mouse anti BM41 antibody. The antibody was purified by InVivo BioTech with protein G and delivered in 10 x 1mg/mL aliquots.

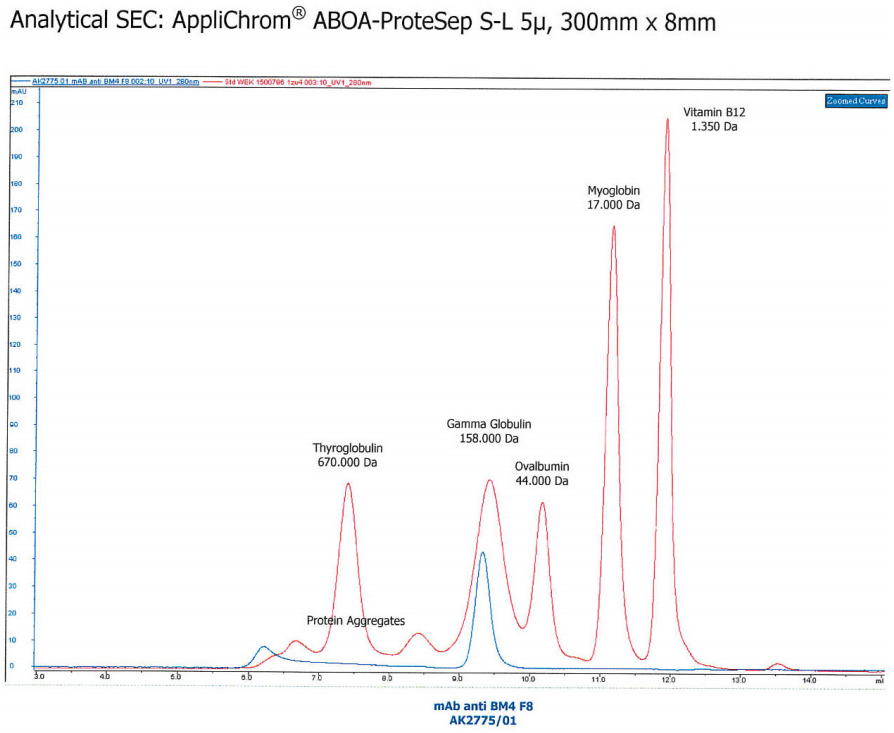


Figure 39. Analytical SEC of anti BM41 antibody provided by InVivo BioTech

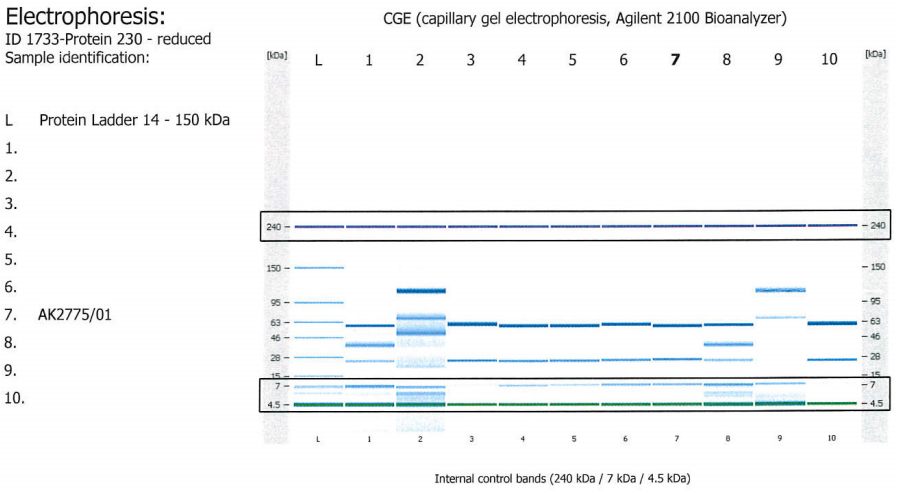


Figure 40. Electrophoresis and sample identification of anti BM41 antibody (InVivo BioTech)

The binding capacity of an antibody to the antigen can be affected by temperature, pH, buffer constituents and concentration of antibody or antigen. Therefore we made a set of titration experiments to obtain the optimal antibody concentration which will support best staining by generating a minimum of background. The titration experiment was performed by using different BM41 antibody dilutions, the unformulated BM41 (0.5µg/lane) as antigen and a specific anti mouse IgG AP conjugated secondary antibody (1:10.000). SDS Page was performed according to established protocol.

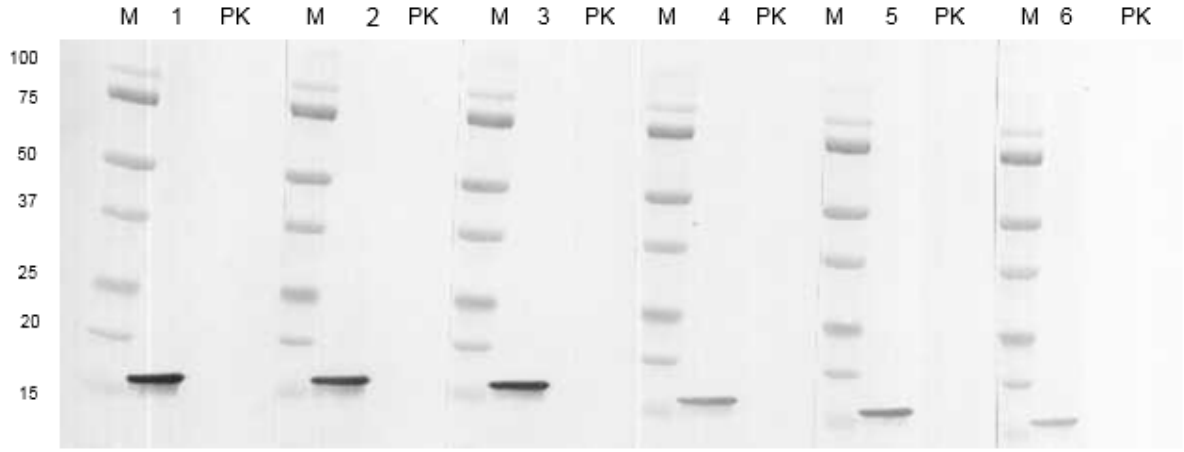


Figure 41. Westernblot Titration of monoclonal mouse anti BM41 antibody

Figure 41 shows different dilutions of the monoclonal mouse anti BM41 antibody containing dilution 1:10.000 (lane 1), negative control (PK), dilution 1:30.000 (lane 2), dilution 1:75.000 (lane 3), dilution 1:150.000 (lane 4), dilution 1:300.000 (lane 5), dilution 1:600.000 (lane 6).

All detected bands were in accordance with the expected BM41 protein band at kDa 17.4. As expected, color saturation decreased from antibody dilution 1:10.000 to dilution 1:600.000 (figure 41).

The following densitometric evaluation approved this result. The red peak showed color saturation of lane number 1 (dilution 1:10.000) and the green peak showed color saturation of lane number 6 at an BM41 antibody dilution of 1:600.000 (figure 42).

RD……relative density
Rf……. relative migration distance

The calculation of the molecular weight using Rf and following quotation

$$(-2.0742 \times Rf) + 2.8 = y$$
$$10^{y} = kDa^{70}$$

confirmed that the detected band is approximately visible at MW = 17.4 kDa:

Protein lane 1:
(-2.0742 x 0.751)+2.8 = 1.2423
$10^{1.2423}$ = 17.4703 kDa

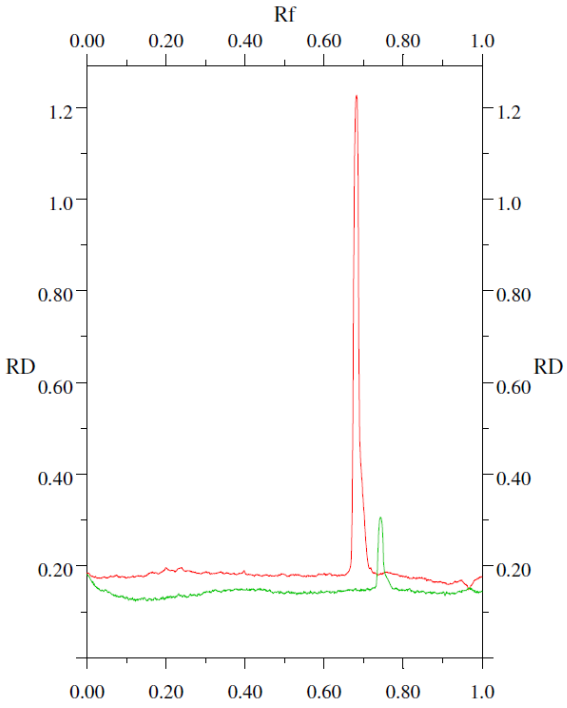


Figure 42. Densitometric evaluation

Figure 42 shows the expected decreasing color saturation from lane number 1 (mouse anti BM41 antibody diluted 1:10.000) to lane number 6 (mouse anti BM41 antibody diluted 1:60.000).

For further westernblot analysis we decided to use a 1:30.000 dilution because at this dilution step, the band was expected to be clearly visible.
To affirm the experiment we made a second antibody titration by using 3 different antibody dilutions and different concentrations of BM41 (figure 43).

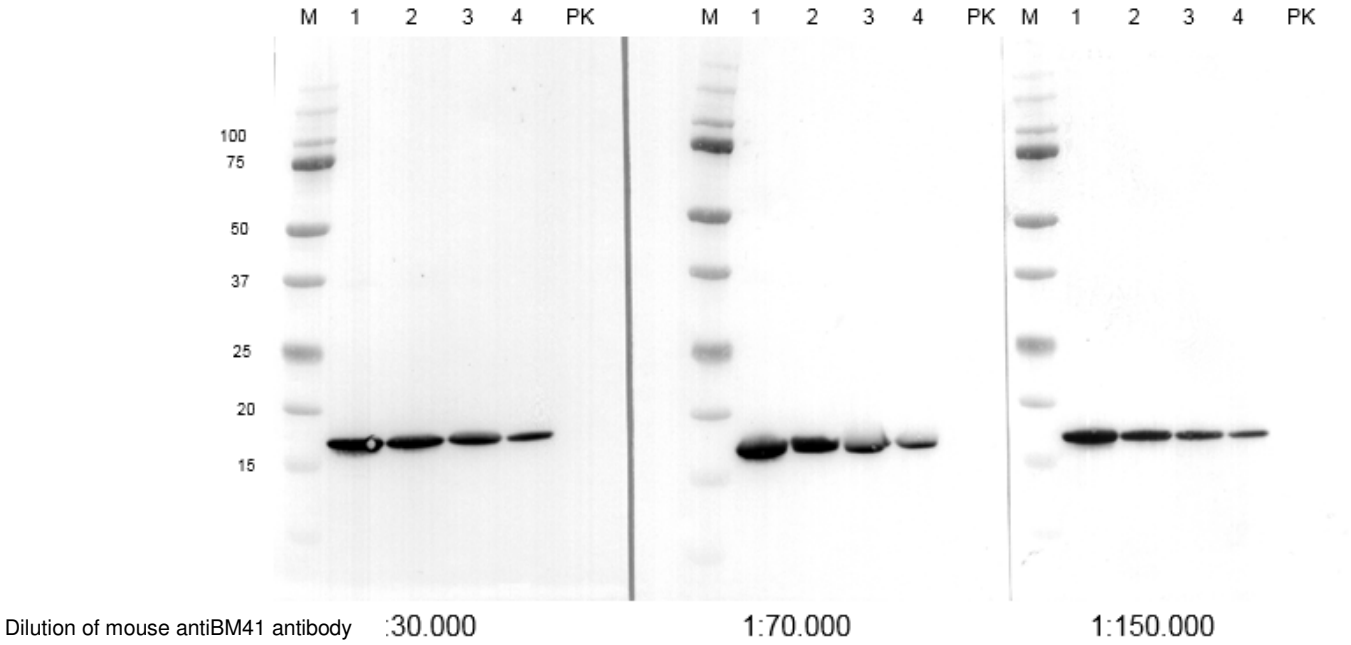


Figure 43. Westernblot Titration of monoclonal mouse anti BM41 antibody

Figure 43 shows different dilutions of the monoclonal mouse anti BM41 antibody and different dilutions of the antigen (BM41) containing 0.5 µg BM41 (lane 1), 0.25 µg BM41 (lane 2), 0.125 µg BM41 (lane 3) and 0.0625 µg BM41 (lane 4)

This titration was based on the same conditions like the titration described above and demonstrated again, that an antibody dilution of 1:30.000 resulted in clear and nice bands.

Referring to the obtained results we finally agreed for further BM41 westernblots on an antibody (mouse anti BM41 antibody) dilution of 1:30.000.

Stability Testing – Westernblot BM41 SPT Solution A, B, C, D

Time point t=0 weeks

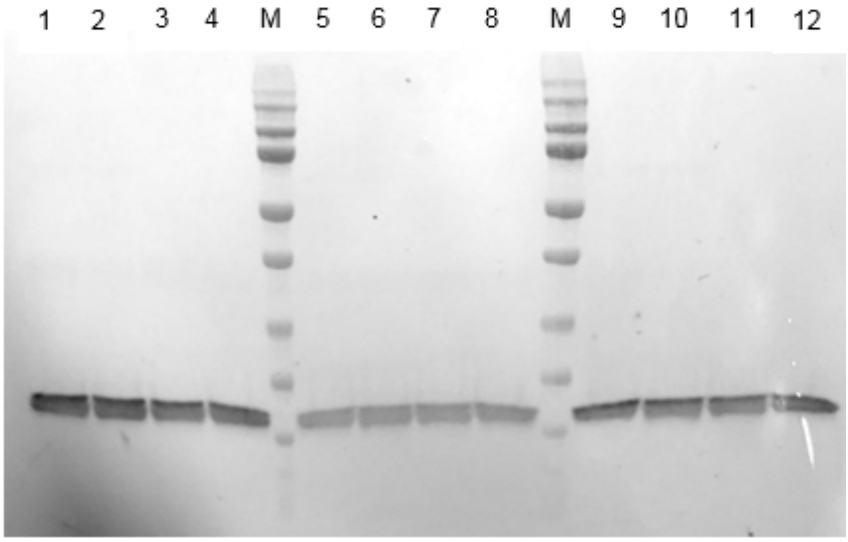


Figure 44. Western blot of Skin Prick Test Solution A, B, C, D

Figure 44 shows a western blot analysis using the predefined antibody dilution of solution A room-temperature (lane 1), solution B room-temperature (lane 2), solution C room-temperature (lane 3), solution D room-temperature (lane 4), solution A 4℃ (lane 5), solution B 4℃ (lane 6), solution C 4℃ (lane 7), solution D 4℃ (lane 8), solution A -20 ℃ (lane 9), solution B -20 ℃ (lane 10), solution C -20 ℃ (lane 11) and solution D -20 ℃ (lane 12).at time point t=0 weeks

Western blot analysis resulted in a low background signal and a clear BM41 fragment without any non-specific products. Additionally no BM41 protein degradation was visible (figure 44).

Time point t=24 weeks

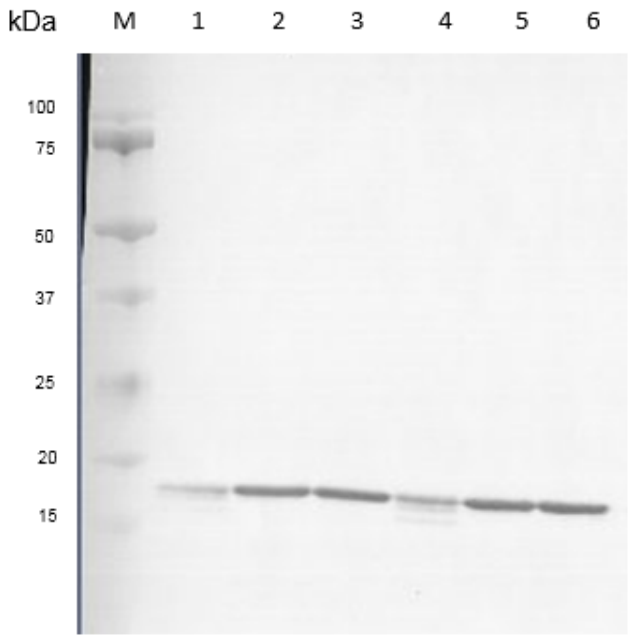


Figure 45. Westernblot of Skin Prick Test Solution D at time point t=24 weeks

Figure 45 shows a westernblot analysis using the predefined antibody dilution of solution D room-temperature (lane 1), solution D 4 ℃ (lane 2) and solution D -20 ℃ (lane 3) under reduced conditions and under non reduced condition solution D room-temperature (lane 4), solution D 4 ℃ (lane 5) and solution D -20 ℃ (lane 6) at time point t=0 weeks

After 24 weeks, the BM41 protein of the SPT solution D which was stored at +4 ℃ and -20 ℃ was still stable; a clear and visible band could be detected by the specific BM41 monoclonal antibody. As demonstrated in the SDS Page analysis before, the BM41 protein, stored at room temperature, started to degrade (figure 45).

Since degradation is usually defined in terms of loss of activity or performance, a product is considered to be degrading when any characteristic of interest (for example potency or performance) decreases. Therefore recommended storage of BM41 solution D for further human application to avoid degradation will be at 4 ℃.

f) HPLC

In order to check the quality of the BM41 protein solutions a reversed Phase chromatography was performed by an Agilent 1260 HPLC station with acetonitrile complemented with 0.1% TFA as solvent and UV detection at 280nm.

By injection of a standard solution (BM41 drug substance) under similar analytical conditions and comparing the retention factor and response of the peak in the chromatogram of the standard solution, the BM41 peak could be clearly identified (figure 46).

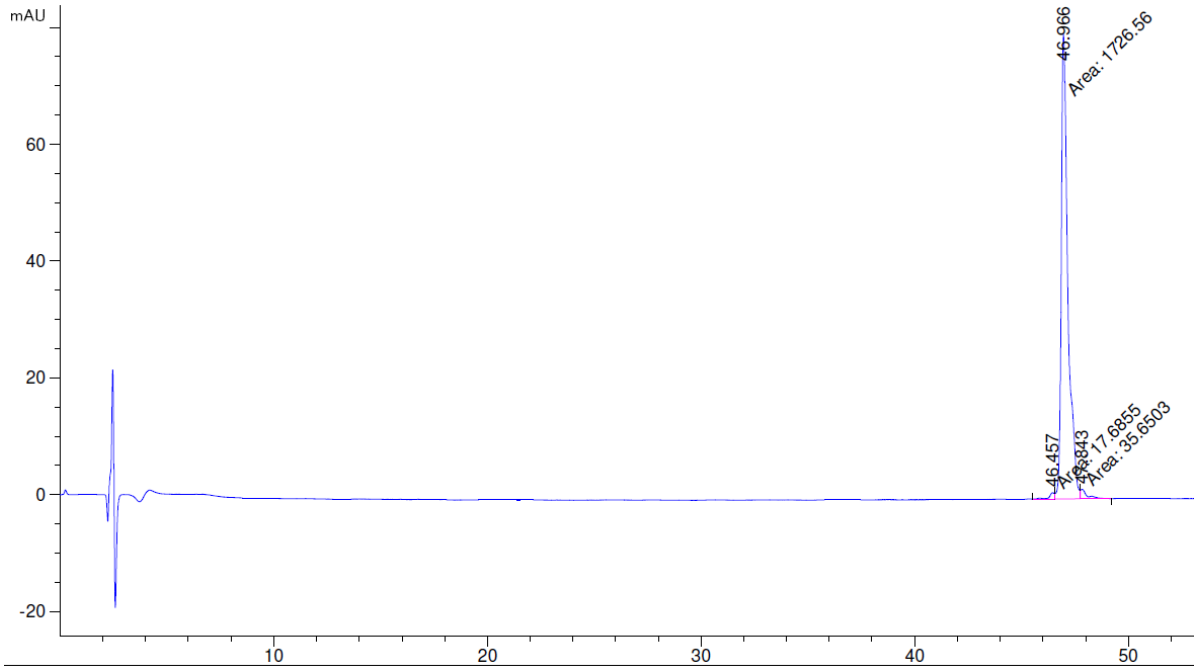
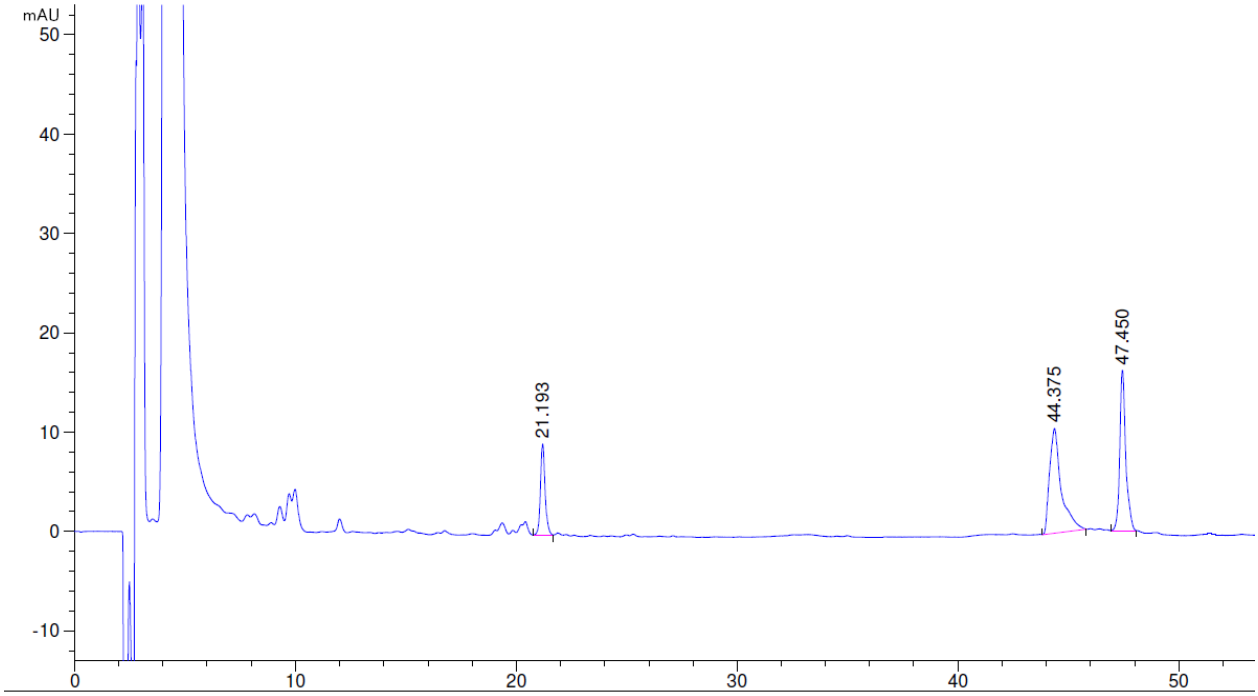


Figure 46. .Reversed Phase HPLC chromatogram of BM41 standard protein

Figure 46 shows the result of the RP-HPLC of the standard protein BM41. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes

The major protein peak at a retention time of 46.966 minutes represented the BM41 protein.

SPT solution A



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	21.193	BB	0.2085	134.08737	9.17525	16.2392
2	44.375	BB	0.4333	387.55728	10.53222	46.9368
3	47.450	BV R	0.2729	304.05563	16.13116	36.8240
Totals :				825.70029	35.83862	

Figure 47. Reversed Phase HPLC chromatogram of stability testing of SPT Solution A

Figure 47 shows the result of the RP-HPLC of Solution A. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes.

The RP-HPLC of SPT solution A resulted in three peaks at retention time of 21.193, 44.375 and 47.450 minutes (figure 47). The major protein peak at a retention time of 47.450 minutes could be identified as the BM41 protein.

The protein peak at 44.375 minutes was identified as BSA (which was added as a protein stabilizer) because every solution containing BSA showed the same peak at approximately 44 minutes.

The detected impurities (e.g. peak 21.193) were analysed by RP-HPLC; to identify the source different suspected solutions were tested:

- HEPES buffer in which the API BM41 is dissolved and
- Glycerol which was used to stabilize the droplet on human skin for skin prick testing

The source of the detected impurities (figure 46) could be identified as the glycerolic ingredient of the SPT solutions. For RP-HPLC analysis glycerol was diluted in WFI so that the concentration of glycerol was equal to the SPT samples (figure 48):

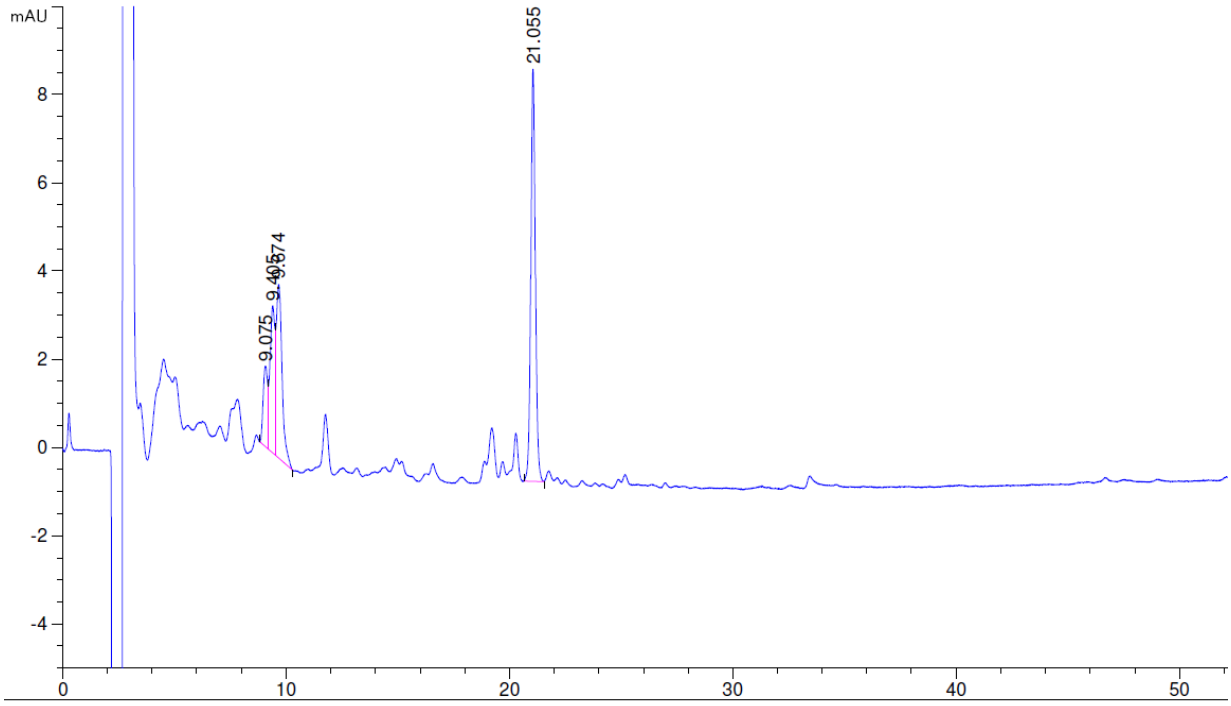
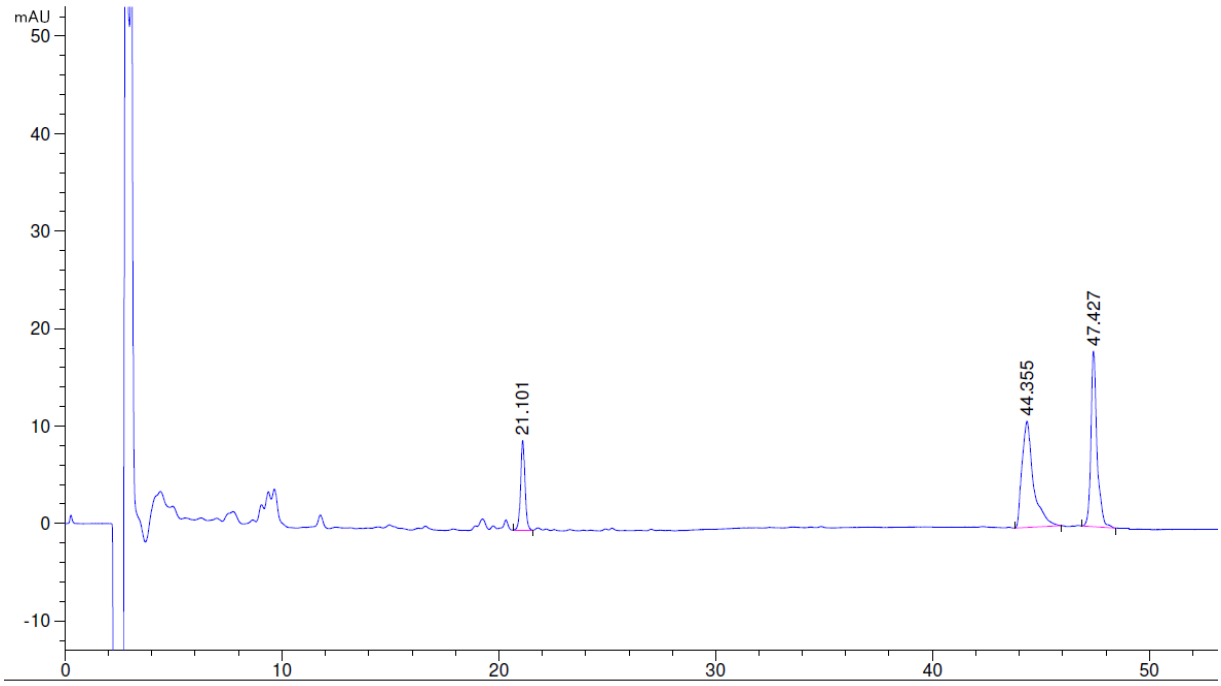


Figure 48. Reversed Phase HPLC chromatogram of glycerol (50:50)

Figure 48 shows the result of the RP-HPLC of glycerol which was used for SPT solutions. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes.

The analysis clearly demonstrated that the detected impurities in the SPT solutions were a typically result of the used contaminated glycerol.

SPT solution B



Signal 1: MWD1 A, Sig=280,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	21.101	BB	0.2100	130.01442	9.19268	14.7398
2	44.355	BV R	0.4333	399.15417	10.89701	45.2522
3	47.427	BB	0.2793	352.89749	18.03271	40.0081
Totals :				882.06609	38.12239	

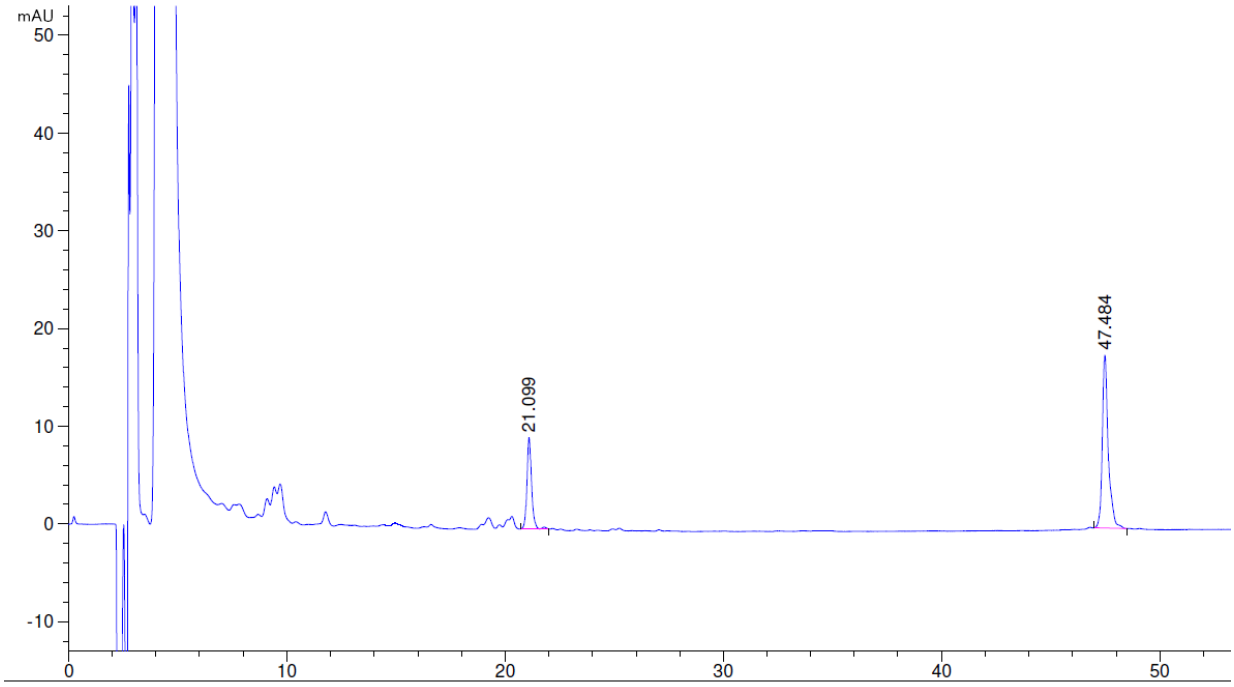
Figure 49. Reversed Phase HPLC chromatogram of stability testing

Figure 49 shows the result of the RP-HPLC of Solution B. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes.

The RP-HPLC of SPT solution B resulted in three peaks at retention time of 21.101, 44.355 and 47.427 minutes (figure 49). The major protein peak at a retention time of 47.427 minutes could be identified as the BM41 protein.

The protein peak at 44.355 minutes was identified as BSA (which was added as a protein stabilizer). The contaminated glycerol was the reason for the detected impurities (figure 48).

SPT solution C



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	21.099	BV R	0.2115	139.96233	9.35941	29.3291
2	47.484	BB	0.2779	337.25143	17.69175	70.6709
Totals :				477.21376	27.05116	

Figure 50. Reversed Phase HPLC chromatogram of stability testing

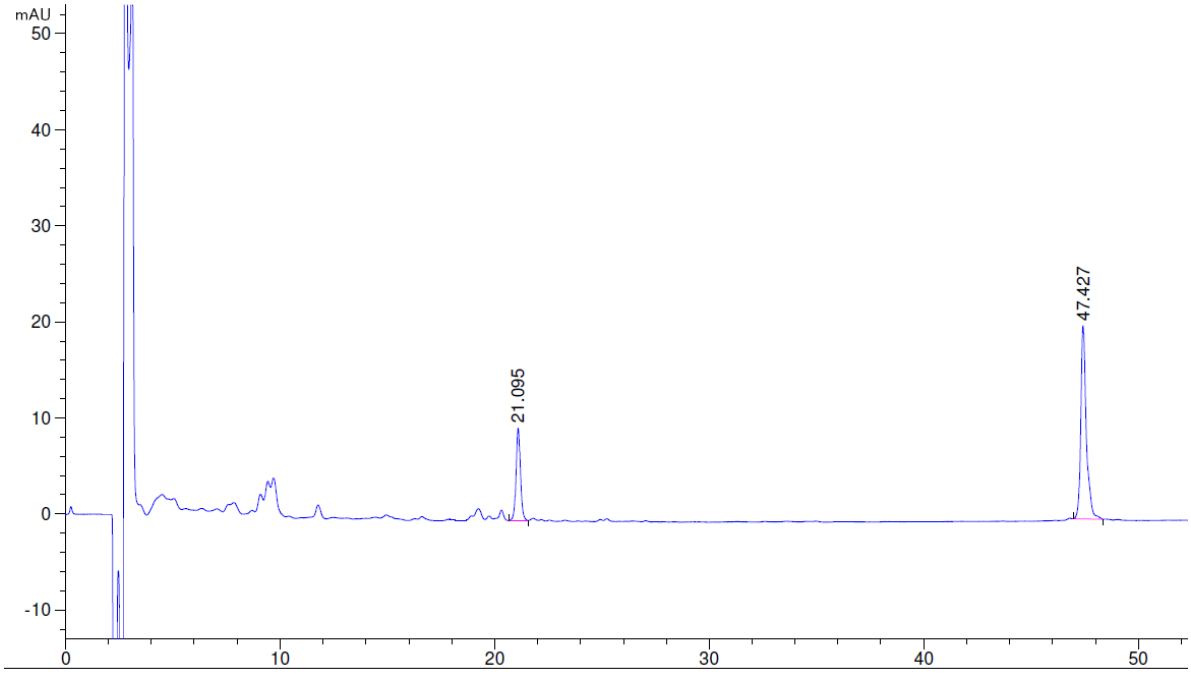
Figure 50 shows the result of the RP-HPLC of Solution C. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes.

The RP-HPLC of SPT solution C resulted in two peaks at retention time of 21.099, and 47.484 minutes (figure 50). The major protein peak at a retention time of 47.484 minutes could be identified as the BM41 protein.

This solution did not contain BSA, therefore no peak which identifies this ingerdient could be detected.

The contaminated glycerol was the reason for the detected impurities (figure 48).

SPT solution D



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	21.095	BV	0.2049	136.96741	9.60514	27.5467
2	47.427	BV R	0.2559	360.25180	20.11938	72.4533
Totals :				497.21921	29.72452	

Figure 51. Reversed Phase HPLC chromatogram of stability testing

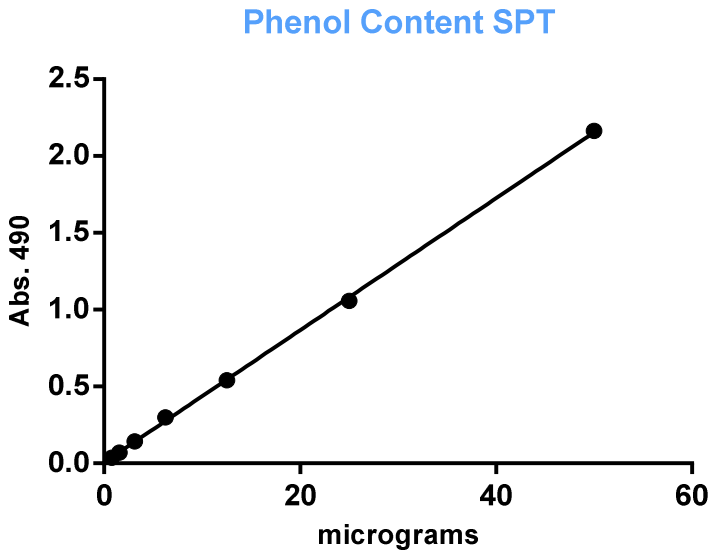
Figure 51 shows the result of the RP-HPLC of Solution D. Y axis indicates the milli absorption units per minute (mAU), X axis indicates the retention time of the protein in minutes.

The RP-HPLC of SPT solution D resulted in two peaks at retention time of 21.095, and 47.427 minutes (figure 50). The major protein peak at a retention time of 47.427 minutes could be identified as the BM41 protein.
This solution did not contain BSA, therefore no peak at 44.355 minutes could be detected. Again, the contaminated glycerol was clearly visible by detection and identification of the impurities (figure 48).

The qualitative RP-HPLC analysis of all solutions identified the active pharmaceutical ingredient as the BM41 protein.

g) Phenol Content

For stability testing at different time points all solutions containing phenol were tested in terms of phenol content determination according to test parameters and acceptance criteria, which are shown above (table 30). The obtained results of phenol analysis at time point t=8 weeks which are representative for all phenol time point measurements are shown in table 31. The mean of the results were calculated. All obtained data showed that the phenol value for all time points for every phenolic solution was within the specification range.



Solution A 25 ℃	4.187	0.187
Solution A 4℃	4.280	0.191
Solution C 25℃	4.175	0.186
Solution C 4℃	4.851	0.215

Figure 52. Phenol content of SPT Stability Testing

This figure shows the standard curve of the phenol content evaluation and the measured absorbance at 490 nm as well as the corresponding calculated micrograms of phenol in the sample of Skin Prick Test Solutions A and C (stored at 25 ℃ and 4 ℃)

All BM41-SPT solutions A and C were diluted 1:000 in WFI; so the total concentration of phenol was calculated as followed:

SPT Solution	c[µg/mL] x dilution factor	c[µg/mL]	Specification
Solution A 25 °C	4.187 x 1000	4187	4 – 5 mg/mL
Solution A 4 °C	4.280 x 1000	4280	4 – 5 mg/mL
Solution C 25 °C	4.175 x 1000	4175	4 – 5 mg/mL
Solution C 4 °C	4.851 x 1000	4851	4 – 5 mg/mL

Table 31. Results and calculation of Phenol content

Table 31 shows the phenol content of Skin Prick Test Solutions A and C (25 °C, 4 °C, t=8 weeks) and the Specification for Stability Testing

13.) Discussion

Allergy is the most prevalent chronic inflammatory disease, having a great socio-economic impact. Chronic lifelong dependency on symptomatic drugs is an expensive reality for the vast majority of allergy sufferers. Allergen-specific immunotherapy (SIT) is the only causal and effective treatment targeting the underlying immune mechanism in a more cost-effective way. Another important aspect is that only SIT is able stop the progress of the allergic march: The term “allergic march” describes the typical progression of allergic diseases often into severe airway diseases, particularly asthma. A Preventive Allergy Treatment study (PAT study) describes that the risk of developing asthma in children can be reduced by immunotherapy.[71] This effect could even be observed up to 7 years after treatment termination.[72]

Yet, long treatment duration and risk of anaphylactic side-effects have prevented this treatment from becoming the first choice. The European Project BM4SIT aims at providing an escape from its current niche position by making the treatment safer, more effective and patient friendlier, by developing a therapy with negligible side-effects but improved efficacy, achieved by less injections. If successful, BM4SIT will pave the way towards a more attractive therapy that will improve the cost-benefit ratio compared to symptomatic drugs and that will reduce the development of asthma.

The new concept of BM4SIT based on Birch pollen allergy comes along with two innovations:
(1) replacing current natural allergen extract (birch pollen extract) by a mutated recombinant Bet v 1 derivative having been designed to be hypo-allergenic but hyper-immunogenic, and
(2) adding an adjuvant (Vitamin D3) to support rapid and effective induction of an anti-inflammatory immune response.
The development of a new vaccine from bench to patient requires several steps. After development of the new active ingredient (the hypoallergenic Bet v 1 mutant) it is important to identify and study the final composition and adequate formulation of the vaccine which allows efficient and safe future human application.

The overall aim of my master thesis was to pave the way for future manufacturing, quality control, and stability testing of the BM41 drug substance and drug products (i.e BM41 vaccine, BM41 placebo and BM41 skin prick test solutions) under GMP conditions.

In particular, this included:

- Development and characterization of a BM41 solution for skin prick testing (SPT). Important target characteristics of the SPT solution were a relatively low concentration of BM41 (0.1µg/mL), glycerol content of 50% to allow formation of stable droplets on the skin, and suitability for storage at 2-8°C.
- Studies on the influence of different storage conditions over time on selected product characteristics of BM41 adsorbed on aluminium hydroxide (BM41 vaccine) or BM41 STP solutions. Aim of these studies was to collect data to establish recommended shelf life and storage conditions[73].
- For this purpose several biochemical assays were developed or adapted for quality control of BM41. Special focus was put on the development of a potency assay testing the integrity of BM41 via binding of specific antibodies using an inhibition ELISA approach. To prove the hypo-allergenic properties another ELISA tests was developed (Hypoallergenicity ELISA), including characterization of a monoclonal BM41-specific antibody.

The whole work was done in consideration of relevant GMP regulations and GCP requirements for application of new products in human clinical trials. Stability studies were carried out with the requirements defined by the International Council for Harmonization of Technical Requirements for Pharmaceutical for Human Use (ICH) in focus. The ICH has defined a stability testing package for new drug products sufficient for registration of the new product in the European Union, Japan and the United States.[73] There exist different factors which can cause a loss of a protein's biological activity:

- Alteration in pH or temperature
- Exposure to degrading substances (e.g. proteases, oxygen or heavy metals)
- Freezing or thawing[74]

Stability studies ensure the quality, safety and efficacy of a product throughout the shelf life for acceptance and approval of the pharmaceutical product and for future

human application. Stability testing is essential to assure identity, potency and purity of the active pharmaceutical ingredient (API) as well as for the formulated drug product. The results of a stability study provide information about the effects of environmental factors on the quality and stability of the product which are essential for prediction of its shelf life and for determining adequate storage conditions.[75]

Formulation development (BM41 SPT solution)

The formulation development evaluating different SPT solutions was essential to guarantee stability and structural integrity of the active molecule – BM41 – during storage at the assigned storage temperature of 2-8°C as well as during short periods at room temperature.[76]

According to the international guidelines of pharmakopöe phenol is an acceptable preserving agent for a multiple human use medicinal product to eliminate the risk of contamination from gram-positive, gram-negative, mycobacteria and viruses.[77] Therefore it is component in all SPT solutions currently on the market. We prepared different SPT solutions (with and without phenol A, B, C, D see table 22, 23, 254, 25) for stability testing. Based on the decision to produce a single use SPT solution for human application and based on the obtained Bioburden data (no microbiological or fungi growth) we decided to exclude the phenolic agent.

The BM41 concentration in a 0.1 mg/mL test formulation without addition of surplus protein did not decrease over time which indicated that BM41 was not adsorbed on the wall of the glass vial. Thus it was possible to omit also the use of BSA in the final formulation.

Like all SPT solutions the new BM41 SPT formulation contains 50 % glycerol to facilitate the controlled application of small droplets on the skin. In addition, glycerol is known to have a stabilizing effect on proteins.[77] To prevent degradation of the protein at room-temperature (25 °C) as shown in figure 36 (SDS experiment) and therefore to avoid a loss of potency due to enzymatic proteolysis BM41 SPT solutions should further be stored at 4 °C with 50 % glycerol [78]

pH measurements

For optimal performance and stability, molecules need an adequate buffer with a specific and consistent pH value. The stability of proteins is described to be pH-dependent with as a bell-shaped curve [pH]. The solubility of a protein is also strongly influenced by the pH of its environment; the lowest solubility is at the isoelectric point which means that a pH near the isoelectric point should be avoided to keep the protein in solution. In contrast, a surrounding pH value which lies over or under the pI leads to the fact, that all molecules carry the same charge (positive or negative). Molecules with same charge repel each other, thus preventing aggregation or formation of insoluble aggregates and the protein remains in solution.[79] [74]

The isoelectric point (pI) of BM4 calculated by Expasy is around 5.6. [66] In addition, the pH of pharmaceutical products such as vaccines should be in the physiological range. Therefore we defined a range from pH 6.0 – 8.0 (buffered by HEPES) as suitable for both BM41 vaccine and BM41 SPT solution.

The obtained stability date (e.g. SDS-Page analysis) for the BM41 Vaccine Batch 20150819 as well as for the SPT solutions showed that at the chosen pH the complex is surrounded by a stable buffered environment as no aggregation was observed.

Protein Concentration

Measurement of the concentration of BM41 by using different methods and choice of the most suitable method was an essential step at several stages of the project.

When putting a solute in solution, there is always the fact that proteins can attach to the surface of the vial. This may alter the protein concentration in the solution or even deplete it (especially at low concentrations). This was a particular point of consideration during the development of the SPT solution with a BM41 concentration of only 0.1 mg/ml. A change in concentration of the active pharmaceutical ingredient over time would lead to a loss of function of the effectiveness of the medical product.

The difficulties we encountered in measuring the protein concentration in the SPT solutions could be solved by diluting the standards, the positive control as well as the background (negative control) in the same molar buffer concentration than the samples. Instead of the previously used Bradford Assay we established a protein concentration measurement for the SPT Solution without phenol (solution D) based on the colorimetric shift of bicinochinic acid (standard protocol at Biomay). This test could not be used for all SPT solutions because previous experiments (in house at Biomay)

showed that the BCA for determining protein concentration was highly interfering with samples containing phenol.
After the decision to exclude the phenolic agent it was possible to establish a new and more precise protocol (Bradford Assay) to determine the protein concentration (12.8 b).

Furthermore the protein concentration had to be controlled and validated after sterile filling of the products.

Stability of the adsorption of BM41 to aluminium hydroxide:

The Degree of Adsorption is another kind of protein concentration measurement which is an index of how much of the BM41 protein is bound to the alum adjuvant. In cases that the protein detaches from the adjuvant this could result in a decreased immune response. The aluminium adjuvant is used to potentiate the immune response, unfortunately the mode of action is until today not fully clear. One hypothesis is that the adjuvant improves the uptake of the vaccine by antigen-presenting cells and act as immunostimulator to provoke the release of TH2 cytokines.[80] [81] The aluminium hydroxide might destabilize the protein on their surface so that they are accessible for proteolytic degradation.[82]
The DoA of freshly formulated BM41 vaccines was measured to be in the range of 91-100 % even at high API concentrations in the vaccine. During a 15 months stability study all measurements of the DoA met the required specification of DoA > 90% (table 10 for BM41 drug product and placebo). These positive results showed that the active pharmaceutical ingredient is strongly adsorbed to the aluminum hydroxide adjuvant and a stable immune response in further human application can be expected.

Potency Assay

In pharmaceutical products the term "potency" is used as a measure of the biological activity of a drug describing the amount necessary to produce an effect of a certain intensity. Potency is often expressed as the drug concentration which results in 50% of the maximum possible effect (IC50). It is an important parameter for both batch release and stability testing of a pharmaceutical product. The purpose of a potency assay

is to provide detailed information about the impact of time and temperature over a period of time on the biological activity by determination of IC50 values in order to evaluate or confirm its suitability for human use.

For all vaccines the primary mode of action (=the biological effect of interest) is to induce a protective immune response upon vaccination. In the field of allergy vaccines it has been suggested that a quantitative antibody binding assay testing the structural integrity of epitopes on the API, can be used as a relevant method for measuring the potency, reflecting the potential of the vaccine to induce specific IgG antibodies upon immunization.

A similar approach based on ELISA inhibition is being pursued in the BM4SIT project: It is assumed that degradation of the BM41 protein to an extent that specific antibodies can no longer bind, will result in a loss of immunogenicity.

Every new hypoallergenic vaccine has to prove its ability to induce immunological changes before used in human clinical applications. The hypoallergenic active pharmaceutical ingredient has to be biologically active in binding high levels of IgG antibodies which cross-react with the wild-type allergen and should demonstrate a low or no binding of human IgE.[77,83]

One of the challenges in the development of the potency assay was to establish a sigmodal dose-response curve suitable for quantitative analysis using the software GraphPad Prism. To achieve this goal we evaluated different sera and sample dilutions to approach the plateau phases (top and bottom) of the sigmoidal curve. The two plateau phases are the result of the different protein concentrations. High protein concentration should lead to the plateau phase at the bottom; high amounts of serum protein get bound by the hypoallergenic protein and should lead to a low OD response. Vice versa a low concentration of sample protein leads to high OD values due to the fact that only a low amount of IgG is bound. Free IgG can bind to the epitope immobilised on the ELISA plate and can be detected by a secondary labelled antibody.

The sigmoidal behavior of the curve facilitates the calculation of the turning point, which is equivalent to the IC50 (concentration of the drug that gives half-maximum response) of the vaccine.
Finally, a standard operation procedure was established to eliminate all sources of variability (like use of same materials, incubation times and reagents…) which will be used for further potency testing in the course of stability studies carried out with the BM41 vaccine.

In the past few years approaches have been developed to improve the allergen specific immunotherapy and currently it seems that there is a chance for new opportunities for the treatment of allergic diseases. Problems which occurred with SIT could be overcome with the use of recombinant vaccines.[84] These vaccines offer a new perspective to fight against allergic diseases. The overall objective of the BM4SIT project is to bring an allergy vaccine based on the hypoallergen BM41 from pre-clinical proof-of-concept to clinical proof-of-concept. The development work done in the course of this master thesis substantially contributes to the successful provision of the investigational drug product for the clinical trial planned in BM4SIT. The success of our stability and toxicity studies with BM41 also enables us to look to the future with confidence: The treatment with recombinant hypoallergens as active ingredients could form a safer and more efficient novel therapy for people suffering allergic diseases.[44]

14.) List of Figures

15.) References

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