**Universität für Bodenkultur Wien**
University of Natural Resources
and Life Sciences, Vienna

# Master Thesis

## Characterization of the repertoires of IgE specific to the major birch pollen allergen Bet v 1

Submitted by

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in the framework of the Master programme

Biotechnology

in partial fulfilment of the requirements for the academic degree

Diplom-Ingenieurin

Vienna, November 2021

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## Eidesstaatliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

Familienname, Vorname

Ort, Datum Unterschrift

# Acknowledgements

An dieser Stelle möchte ich ein großes Dankeschön an meinen Betreuer Christian Radauer aussprechen, der mich bei der praktischen Arbeit, sowie bei der Erstellung dieser Masterarbeit mit großem Engagement, hilfreichen Anregungen und konstruktiver Kritik begleitet hat. Ein besonderer Dank gilt auch Stefanie Schmalz und Vanessa Mayr, die mir im Labor immer mit viel Geduld, Interesse und Hilfsbereitschaft zur Seite standen.

Auch bei meinem Lebensgefährten Christoph, sowie bei meinen Eltern Viktoriya und Angel, möchte ich mich bedanken, weil sie während meines Studiums immer ein offenes Ohr für mich hatten und mich zu jeder Zeit unterstützt und motiviert haben. Nicht zuletzt danke ich auch meinen Großeltern Penka und Petranka, Dimitar und Nikola, die immer hinter mir standen und mit viel Liebe und großem Interesse meinen Werdegang verfolgt haben.

Alexandra Shosherova

Wien, den 23.11.2021

## Abstract

Allergies have become a common health issue in industrialized countries. One of their most frequent manifestations is spring pollinosis, which is mainly caused by birch pollen. The major birch pollen allergen, Bet v 1, is a small protein whose homologs are ubiquitously distributed among higher plants. Bet v 1-specific IgE can therefore also bind to its homologs from certain plant foods, such as apple and peach. This cross-reactivity causes the birch pollen-plant food syndrome, which often manifests as itching and swelling of the mouth, lips, tongue and face, as well as anaphylaxis in rare cases.

Bet v 1-specific IgE, whose epitope recognition may be a marker of birch pollen-associated plant food allergy, binds exclusively to conformational epitopes. Hence the aim of this study was to identify clinically relevant IgE epitopes. This was done by creating 14 chimeric proteins by grafting patches from the Bet v 1 surface to a non-IgE-binding, structurally related bacterial protein, TTHA0849 from *Thermus thermophilus*. Four of them – TB5, TB7, TB8 and TB14 – were covered in this thesis. They were expressed in *Escherichia coli* and purified by chromatography. Three chimeric proteins – TB5, TB8 and TB14 – were soluble, monomeric in solution as shown by dynamic light scattering and correctly folded, as their circular dichroism spectra were highly similar to that of the scaffold protein. Their identity was confirmed by mass spectrometry and their interaction with IgE was studied by ELISA. TB7 was insoluble and not used for further experiments. Selection of recombinant Bet v 1-specific antibody fragments by phage display resulted in the isolation of two different antibodies.

The creation of chimeric proteins by epitope grafting was a practicable method to gain insights into the repertoire of conformational epitopes of Bet v 1, which appears to be at least partly patient specific. Furthermore, phage display proved to be a method suitable for generating Bet v 1-binding antibodies.

## Zusammenfassung

Allergien sind ein häufiges Gesundheitsproblem in Industrieländern. Ein verbreitetes Krankheitsbild ist die Frühjahrspollinose, die vorwiegend von Birkenpollen verursacht wird. Das Hauptallergen der Birke, Bet v 1, ist ein kleines Protein, das man in allen höheren Pflanzen findet. Bet v 1- spezifisches IgE kann daher auch homologe Allergene in bestimmten pflanzlichen Lebensmitteln, wie z.B. Apfel oder Pfirsich, erkennen. Diese Kreuzreaktivität verursacht eine birkenpollen-assoziierte Nahrungsmittelallergie, die sich häufig als Kratzen und Schwellung von Mund, Lippen, Zunge und Gesicht zeigt, sowie selten als Anaphylaxie.

Bet v 1-spezifisches IgE, das als Marker für die birkenpollen-assoziierte Nahrungsmittelallergie dienen könnte, erkennt ausschließlich konformationsabhängige Epitope. Das Thema dieser Studie war daher, klinisch relevanten IgE-Epitope zu identifizieren. Es wurden 14 chimäre Proteine hergestellt, indem bestimmte Bereiche der Bet v 1-Oberfläche auf das nicht-IgE-bindende, strukturell verwandtes Protein TTHA0849 von *Thermus thermophilus* übertragen wurden. In dieser Arbeit wurden vier davon, TB5, TB7, TB8 und TB14, in *Escherichia coli* exprimiert und mittels Chromatographie gereinigt. TB5, TB8 und TB14 waren löslich und lagen als korrekt gefaltete Monomere vor, was durch dynamische Lichtstreuung und Circulardichroismusspektroskopie gezeigt wurde. Die Identität wurde durch Massenspektrometrie bestätigt und die Interaktion mit IgE wurde mit ELISA untersucht. TB7 war unlöslich und wurde nicht weiterverwendet. Zur weiteren Epitopencharakterisierung wurden zwei verschiedene rekombinanten, Bet v 1-spezifische Antikörperfragmente mittels Phagen-Display selektiert.

Die Entwicklung von chimären Proteinen durch „Epitope Grafting“ war eine praktikable Methode zur Charakterisierung des patientenspezifischen Repertoires an Konformationsepitopen von Bet v 1. Weiters erwies sich Phagen-Display als geeignete Methode zur Selektion von Bet v 1-spezifischen Antikörpern.

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# 1. Introduction

## 1.1. Allergies

Allergies are the result of a hypersensitivity reaction of the immune system to normally occurring environmental factors (allergens) that usually do not have an effect in healthy people. Such factors could be from an animal source (e.g. insect stings, dust mites, pet hair and dander), from the environment (e.g. mold), from foods and plants (e.g. pollen, nuts, wheat, fruits, shellfish, etc.), from medication (e.g. penicillin) and many more (Chang et al., 2018).

The hypersensitivity reactions are traditionally classified into four types, with type I – immediate hypersensitivity - being the most common one (Warrington et al., 2011). The first exposure of the organism to a specific allergen creates and establishes the sensitization phase. The allergenic particles, which enter the body by the airways, the mouth or the skin, are taken up by antigen-presenting cells (APCs), usually dendritic cells, and presented to T-cells, which stimulate B-cells to produce immunoglobulin E (IgE) antibodies, as shown in Figure 1. The IgE antibodies first bind to Fc receptors on mast cells and basophils in the sensitization phase. During later exposure to the same antigen, the free antigen molecules are also bound by IgE. This crosslinking leads to the degranulation of the cells and histamine, cytokines, prostaglandine, macrophage inflammatory proteins and proteolytic enzymes are released. They cause increased vascular permeability and vasodilation, which can show as mild symptoms such as nasal allergic rhinitis, atopic dermatitis and ocular allergic conjunctivitis but also as severe and life-threatening soft tissue swelling and acute systemic anaphylaxis (Abbas et al., 2021).

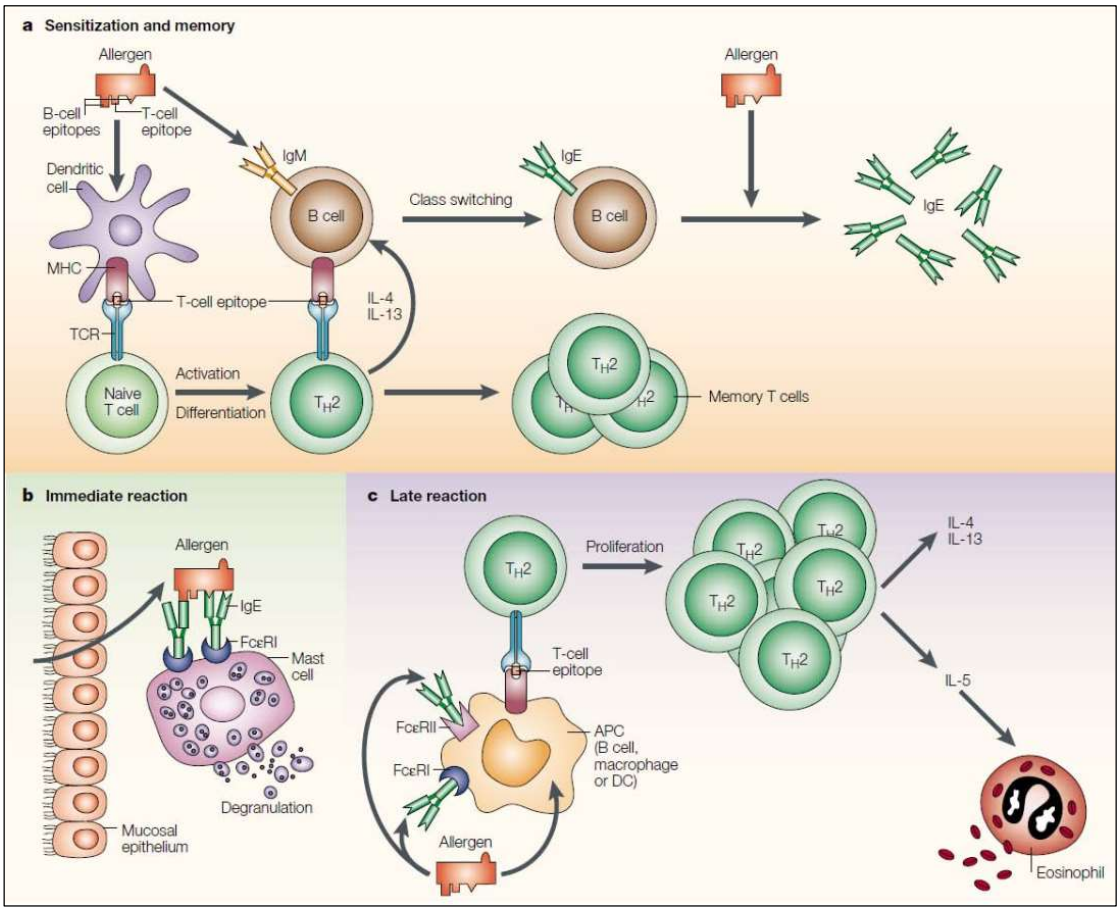


*Figure 1: Induction of type I hypersensitivity (Valenta, 2002): (a): Sensitization by presentation of antigen by dendritic cell to a naive T-cell, activation of B-cells by Th2 cells, production of IgE; (b) Immediate reaction: degranulation of mast cells after binding of antigen; (c) late reaction – proliferation of Th2 cells, leading to degranulation of eosinophils.*

In some cases, there is also a late-phase response, which occurs from to 2 to 24 hours after the initial reaction and consists of the secretion of interleukins by T-helper cells and migration of other leukocytes like neutrophils, macrophages and eosinophils to the site where the acute response took place.

The diagnosis of an allergy is made by clinical testing of allergen specific IgE antibodies. This can be done either by a blood test, showing the concentration of IgE in the blood of a patient, which usually correlates with the likelihood of showing symptoms. The second most common tests are the prick or patch test, which bring the allergen into contact with the skin, either with an adhesive or by a tiny puncture. The visible reaction of the skin after a specified time period mostly correlates with the sensitivities of the patient. This test is not suitable for patients with severe allergies and skin diseases.

The type I hypersensitivity is usually handled by avoidance of the trigger allergens. When this is not possible and especially for inhaled antigens, anti-inflammatory medication such as antihistamines is used.

Allergies have become a common health issue in industrialized countries. But this was not the case some 60 years ago – while the first case of “hay fever” can be found in 200-year-old literature, the first occurrences of more severe allergic reactions such as anaphylaxis were described for the first time in the 1960s. Around this time the culprit of a large part of the allergies – IgE - was also discovered (Campbell & Mehr, 2015).

The rise of allergies cannot be simply explained by better healthcare and thus more diagnoses nowadays – there is a growing trend in the developed and developing countries, which is based on epidemiological data (Prescott & Allen, 2011). One of the possible causes for this trend is described in the hygiene hypothesis – that the developed and urbanized lifestyle, which includes a cleaner environment in-doors and thus less exposure to infectious and parasitic diseases, leads up to the increase in the prevalence of allergies (Lambrecht & Hammad, 2017). Other theories suggest additional factors, including less close contact with animals and people due to smaller families in the cities as well as changes in the gut microbiota, play a role in the increasing cases of different allergies (Pascal et al., 2018).

## 1.2. Tree pollen allergy

Seasons, climate, geography and vegetation affect the concentration of airborne pollens in the atmosphere. This includes grass pollen as well as trees, weeds and ornamental plants. It is estimated that pollen allergy affects around 40% of older children in Europe. (D’Amato et al., 2007)

One of the first papers describing allergy to tree pollen was published in 1972 in Sweden. It described that blooming birch, alder and oak trees caused respiratory problems such as asthma or allergic rhinitis in a part of the population in the spring. They also found out that birch trees are producing a large amount of pollen and it is the dominant one in the spring, making up to 90% of all tree pollens in the air (Zetterström et al., 1972). Another study determined that birch produces the most allergenic tree pollen in central as well as in northern and eastern Europe. The frequency of positive prick tests lies between 5% (Netherlands) und 54% (Switzerland). Birch is also often used as an ornamental tree, which increases its prevalence and has led to a rise in the allergic sensitization of patients. The most common tree allergen differed in the Mediterranean regions – it was determined to be olive (*Olea europaea*) and cypress (*Cupressus*) (D’Amato et al., 2007).

The white birch (*Betula verrucosa*) is a part of the order Fagales, which includes, among others, the families Betulaceae (birch, hazelnut, alder, hornbeam etc.) and Fagacaeae (oak,

sweet chestnut and beech). The pollen of trees from both families have been identified to be a frequent cause of allergic rhinitis. There is also an idea that allergens from different sources can be put together in homologous groups due to their similar biological and physicochemical properties (Lorenz et al., 2008). This structural similarity is also the reason why components of the immune system often recognize and act cross-reactively to other members of the group, even though the organism is sensitized to only one of them. Alder and hazel bloom and shed pollen the earliest, between December and April, and can act as a booster of birch pollen sensitivity and amplify the immune response during the birch pollen season, which is between March and May, depending on the location. Birch pollen allergens are also common inducers of cross-reactivity, with Bet v 1, the major birch pollen allergen, being the most common culprit (Biedermann et al., 2019).

### 1.3. Bet v 1 – structure and classification

Bet v 1, the major birch pollen allergen, was first sequenced and cloned in Vienna, Austria (Radauer et al., 2008). It is composed of 159 amino acids and its molar mass is around 17 kilodaltons. The tertiary structure was determined using nuclear magnetic resonance and x-ray diffraction and showed that the protein is a globular monomer, composed of three alpha-helices and seven anti-parallel beta strands, as shown in Figure 2. The protein has an unusual feature – a hydrophobic cavity with the size of 30 Å, spanning the whole protein, which acts as a ligand-binding site (Gajhede et al., 1996).

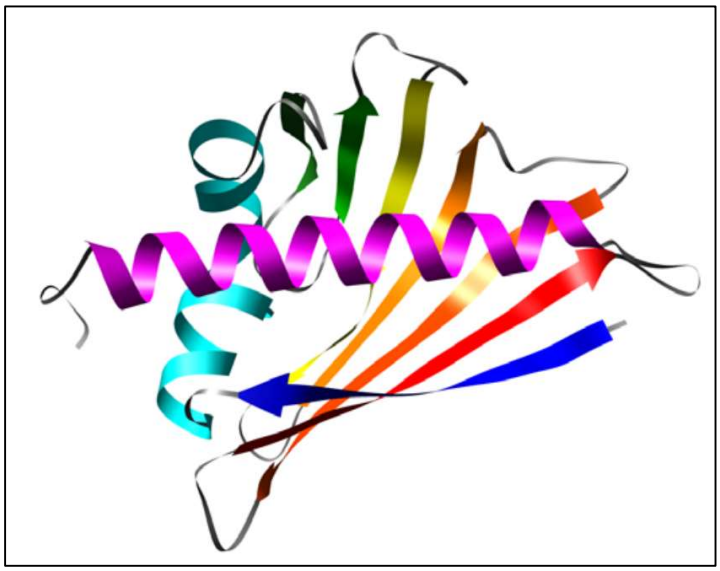

*Figure 2: The Bet v 1 structure: 7 anti-parallel beta-strands and 3 alpha-helices. (Radauer et al., 2008)*

## 1.4. Homologous proteins and cross-reactivity of Bet v 1

Allergenic proteins are classified into different groups according to similarities in the sequence, structure and biochemical properties (Lorenz et al., 2008). Bet v 1 was placed together with the already mentioned hazel, alder and hornbeam allergens into the pathogenesis-related protein class 10 (PR-10) family. Although some of members of this family show low sequence similarity, they all share conserved parts of the sequence like a glycine-rich P-loop between strands 2 and 3 (Radauer et al., 2008). The name of the family is given by the homology of Bet v 1 to a PR protein from pea and is not entirely correct as the PR proteins are only induced in pathological situations. As Bet v 1 and its homologues are expressed constitutively, the correct term would be PR-10 like proteins (Breiteneder & Kleine-Tebbe, 2016). They also share some properties like the expression in high concentrations in the reproductive tissues of the plants, usually in the pollen, fruits or seeds. The lability to digestion is also something they have in common although some of the homologous proteins are still able to activate Bet v 1-specific T cells even after digestion (Schimek et al., 2005).

The tree allergens from the group of the PR-10-like proteins are not the only ones that have sequences and structures similar to Bet v 1. The specific structure with 7 anti-parallel beta-strands and 3 alpha-helices, organized in such way that a large hydrophobic cavity is formed in the middle of the molecule, is a phenomenon seen in many different proteins. Some examples include the major latex protein from the latex of opium poppy, (S)-Norcoclaurine synthases from meadow rue (*Thalictrum flavum*) and the cytokinin-specific binding protein from mung bean. They all show low sequence similarity, but share this type of unique fold, which is also found in the most diverse species of archaea, eukarya and bacteria.

These related proteins were grouped into a superfamily, known as Bet v 1-like clan in the Pfam protein family database [PfamC:CL0209] and as Bet v 1-like superfamily in the Structural Classification of Proteins (SCOP) database (Radauer et al., 2008). According to Pfam, as of the 20$^{th}$ of March 2021, there are 83651 protein domains from 6436 species in the Bet v 1-like clan that share this specific structure (Mistry et al., 2021).

Up to 70% of patients with birch pollen allergy also often have cross-allergies against different plant-derived foods (Kleine-Tebbe et al., 2017). This most often includes apple, cherry and other stone fruits, hazelnut, carrot, celery but also nuts and legumes (Geroldinger-Simic et al., 2011). A list with the most common examples for sources of allergens, causing IgE cross-reactivity, is shown in Figure 3 below. The IgE produced against the major birch pollen allergen usually triggers mild allergic symptoms like itchiness and swelling of the face, mostly the mouth, lips, tongue and throat, where the contact with the allergen occurs. These reactions are referred to as the “oral allergy syndrome” or “pollen-associated allergy syndrome”. There are also some cases with severe symptoms like systematic anaphylactic shock after

consumption of raw foods with high concentrations of allergens (e.g. soy milk) (Kleine-Tebbe et al., 2002).

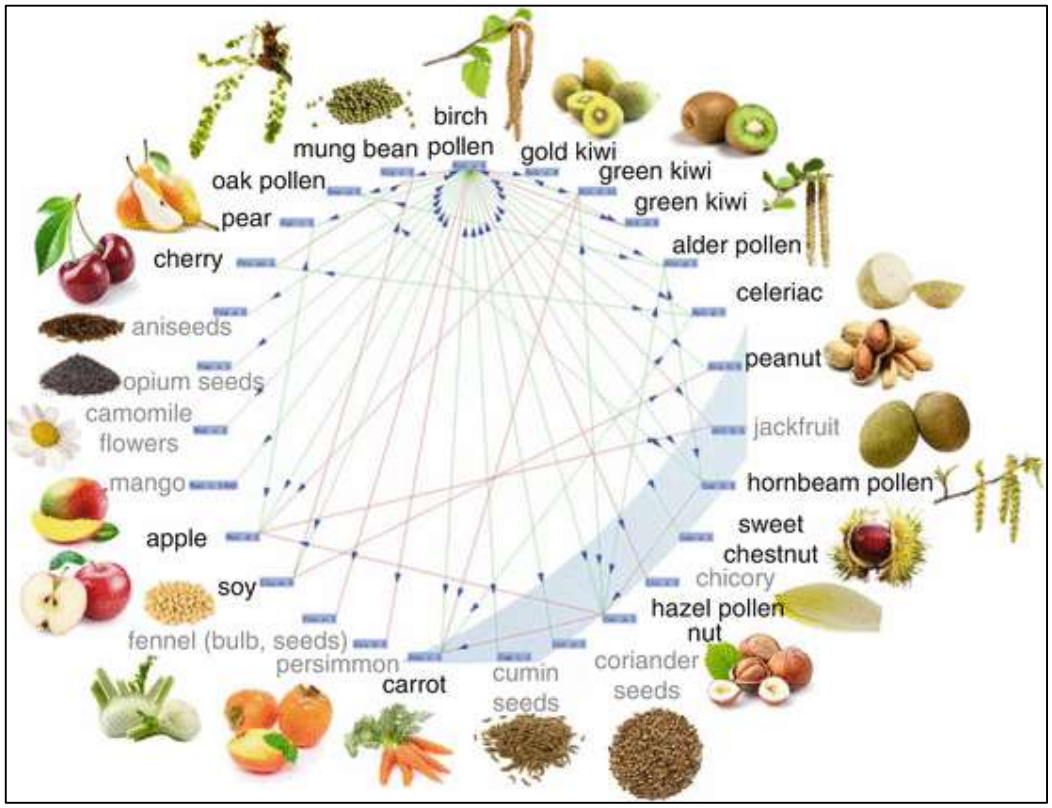


*Figure 3: Map of the sources that contain Bet v 1-related allergens, causing IgE cross-reactivity in Bet v 1-sensitized individuals. The main sensitizer, birch pollen, is shown in the upper center, surrounded by sources, cross-reactive with birch pollen - plants, fruits, nuts, legumes etc. The red arrows indicate reciprocal cross-reactivity, green arrows show unilateral cross-reactivity. Sources that do not contain known Bet v 1-related allergens listed in the International Union of Immunological Societies allergen database as of March 2015 are gray (Kleine-Tebbe et al., 2017).*

## 1.5. IgE epitopes of Bet v 1

Due to the fact that not all patients with a Bet v 1 sensitization have cross-allergies and that some patients only have allergic reactions to certain types of food, the question arises if there is a relationship between the types of IgE in the patient's body and the allergic symptoms.

It has been shown that Bet v 1 has exclusively conformational (discontinuous) epitopes of IgE – binding sites, dependent on the correct folding to expose the correct surface amino acids. IgE does not bind peptides derived from the sequence of Bet v 1, lacking secondary structure, called linear epitopes – see Figure 4 (Focke et al., 2004). The lack of linear epitopes was also

observed when the sequence of Bet v 1 was just split in two parts and none of the fragments bound IgE (Vrtala et al., 1997).

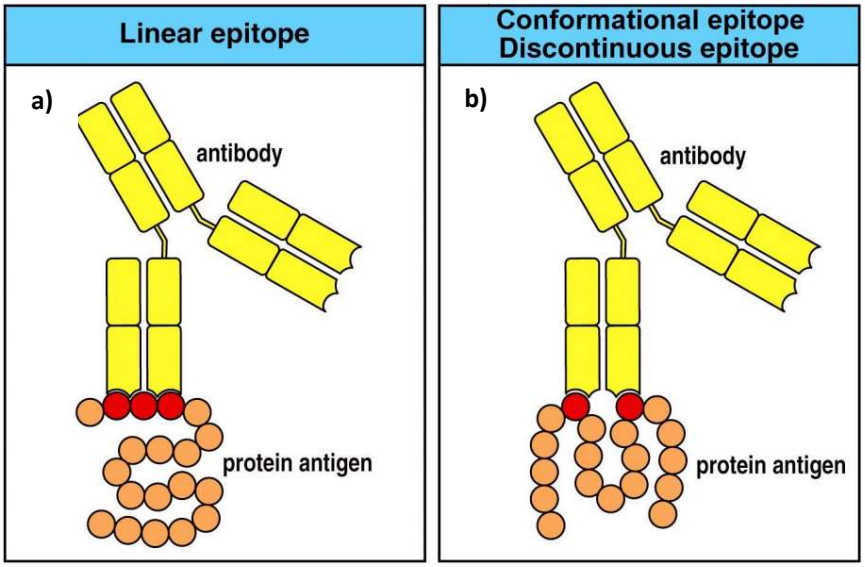


*Figure 4: Illustration of a (a) linear epitope and (b) conformational epitope, dependent on correct folding. (Sadanand, 2009)*

Different studies have already determined some of the epitopes on the surface of Bet v 1. One of them describes an IgE epitope that takes around 10% of the protein surface and is dependent on the conformation (Spangfort et al., 2003). Another two non-overlapping epitopes were defined in a study, researching Bet v 1-specific human monoclonal IgE (Levin et al., 2014).

## 1.6. Phage display

The interactions between Bet v 1 and the IgE antibodies are of interest in this master thesis. One way to study those was to create epitope-specific synthetic antibodies using the phage display technology.

Phages have been the subject of diverse studies for almost hundred years and have led to multiple innovations in the field of biomedical research. They can be used for different purposes such as exploring the interactions between proteins, vaccine development, drug delivery systems etc. The phage itself is a virus which infects bacteria and cannot replicate by itself. It has a simple structure, usually consisting of a coat, built by different proteins and sometimes specialized in a head, collar and tail, which envelops the genetic material (Aghebati-Maleki et al., 2016).

Phage display is a well-established method to select antibodies against a specific target, usually a protein, and amplify them. It starts with a gene library, which consists of diverse monoclonal antibody V genes, harvested from populations of lymphocytes or assembled in vitro using PCR (Winter et al., 1994). The fragments are usually expressed as a single chain

Fv antibody. In order to further diversify the repertoire, random residues can be inserted in the short variable complementarity-determining regions 3. These genes are then fused with the gene of a phage protein, which is expressed on the surface of the phage capsid, like the spike or coat protein. An example of a M13 phage with a spike-antibody fusion protein is shown in Figure 5.

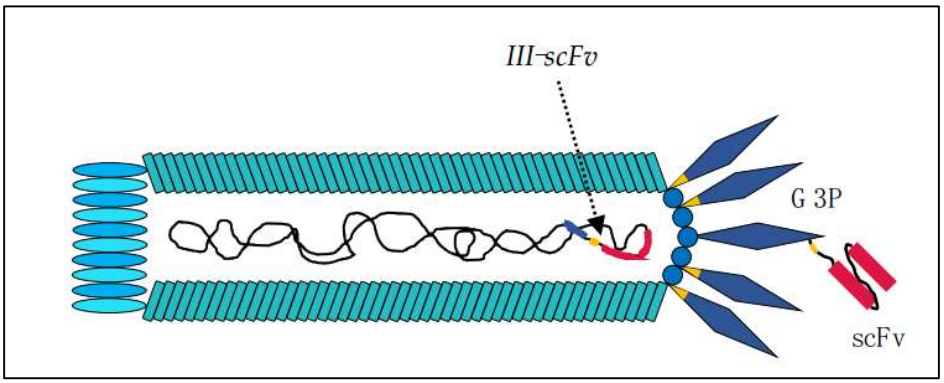


*Figure 5: M13 phage, engineered for phage display. The spike protein is fused with a human scFv. The peptide linker in between contains a trypsin cleavage site, colored in yellow.(Ledsgaard et al., 2018)*

A phage plasmid, called also a phagemid, is constructed with the fused genes, an *E. coli* and a phage origin of replication, a gene for antibiotic resistance for selection and a promoter (usually l*acZ*). *E. coli* is then transfected with the different variants of the phagemid, but no phages are produced. The production of the antibody-displaying phages begins after superinfection with a helper phage, which brings the remaining genes needed for phage production. The helper phage usually carries a second gene for antibiotic resistance, so that superinfected bacteria can be selected (e.g. by double resistance to ampicillin and kanamycin).

In order to gain antibodies with a high binding affinity, multiple rounds of panning (selection) need to be performed. The selection is done by fixing the target on a plastic surface (e.g. in a 96-well plate) or on magnetic beads and then letting it come into contact with the native library. After washing, only the binders remain, which can be eluted by chemical disruption of the target-antibody complexes using acids, bases or chaotropes, by competition with a soluble target or by digestion with trypsin. The eluted phages can then be amplified in bacteria and the selection can be repeated until highly selective and affine antibodies are attained. A scheme of a panning cycle is visualized in Figure 6.

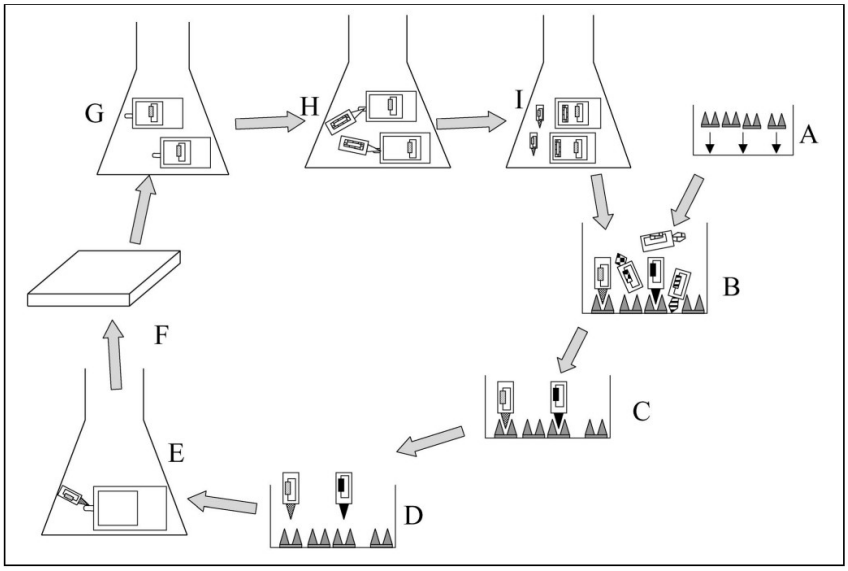


*Figure 6: Representation of the panning cycle: A) Immobilization of target protein; b) incubation with library; c) washing; d) elution of binders; e) infection and amplification in E. coli; f) selection of infected E. coli; g) amplification of selected E. coli; h) superinfection of phagemid-carrying E. coli; i) production of phages for the next round of panning (Carmen & Jermutus, 2002).*

## 2. Aim of the study

The aim of this master thesis was to determine specific epitopes on the surface of the major birch pollen allergen Bet v 1 and research the relationship between those and the cross-reactivity of patients' IgE to homologous plant-food proteins. As previously mentioned, skin tests and specific IgE measurements are used for diagnosis nowadays, but there is low specifity and low positive predictive value of those tests due the fact that patients sometimes show cross-sensitization in vitro without experiencing any symptoms (Villalta & Asero, 2010). The knowledge where different IgE antibodies bind on Bet v 1 and what role they play in cross-reactivity would enable an accurate prediction for the course of a patient's allergy, as well as the development of a more targeted therapy against it.

A special approach was needed in order to study the conformational epitopes on the surface of Bet v 1, recognized by IgE. The method of grafting a part of the sequence in a scaffold protein, creating a patch of Bet v 1 surface, has been already described in the literature (Gepp et al., 2014). For this thesis, the approach was adjusted to use a non-allergenic protein as a scaffold. The protein TTHA0849 from *Thermus thermophilus* was chosen due to its high structural similarity to Bet v 1 and similar sequence length, which should allow the correct folding of the inserted patch. Another reason was the low sequence identity to Bet v 1, so that no IgE is bound by the scaffold. 14 chimeric proteins, carrying patches with a part of the Bet v 1 surface, were planned to be produced and characterized.

Furthermore, antibodies against Bet v 1 were to be created using phage display. Their affinity for different proteins, including homologues, was tested in order to further research the relationship between specific surface epitopes and cross-reactivity.

# 3. Materials and methods

*Note: For all buffer preparation recipes, see Appendix: chapter 7.1.*

## 3.1. Expression and purification of Bet v 1-TTH chimeras

### 3.1.1. Design of chimeric proteins and plasmid construction

In order to research the conformational epitopes on the surface of Bet v 1, 14 chimeric proteins were designed by Christian Radauer by the method of epitope grafting. A part of the sequence of Bet v 1 was inserted into a non-allergenic scaffold creating a patch on the surface, corresponding to a part of the surface of Bet v 1.

The protein TTHA0849 from *Thermus thermophilus* was used as a scaffold– see Figure 7. It shows structural similarity but low sequence identity to Bet v 1, so that no specific IgE is bound. The sequence length is similar in order to allow the correct folding of the inserted patch.

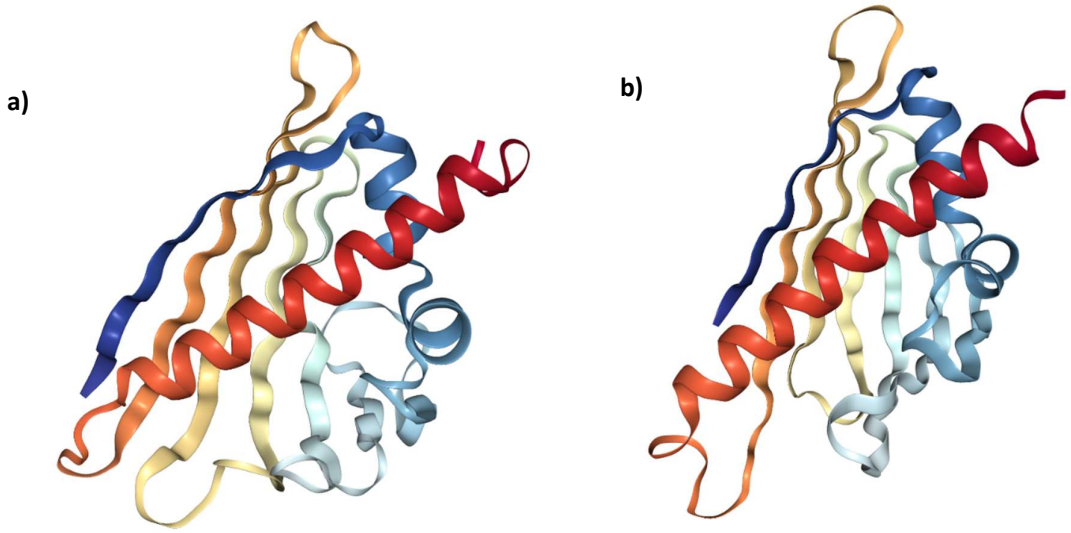


*Figure 7: Comparison of the structures of a) Bet v 1 (PDB: 4a88) and b) the scaffold protein TTHA0849 (2d4r). Source: RCSB Protein Data Bank*

The patches were designed to represent the whole surface of Bet v 1 with overlapping areas, so that the surface is covered twice. Each patch area is around 1000 $Å^2$. The concept is visualized in Figure 8.

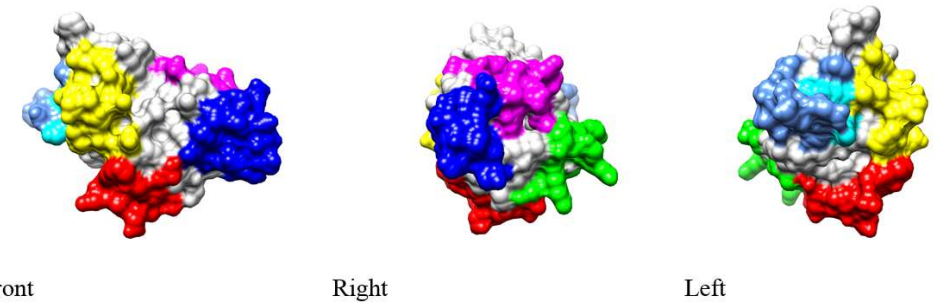


*Figure 8: Six of the patches, shown in different colors on the surface of Bet v 1. The picture was kindly provided by Christian Radauer.*

All of the chimeric proteins were designed as fusion proteins with a hexa-histidine tag at the N- or the C-terminus, which is an established method to facilitate protein purification using a nickel-nitrilotriacetic acid resin column (Hochuli et al., 1988).

This thesis covers four of the chimeric proteins – TB5, TB7, TB8 and TB14 – see Figure 9.

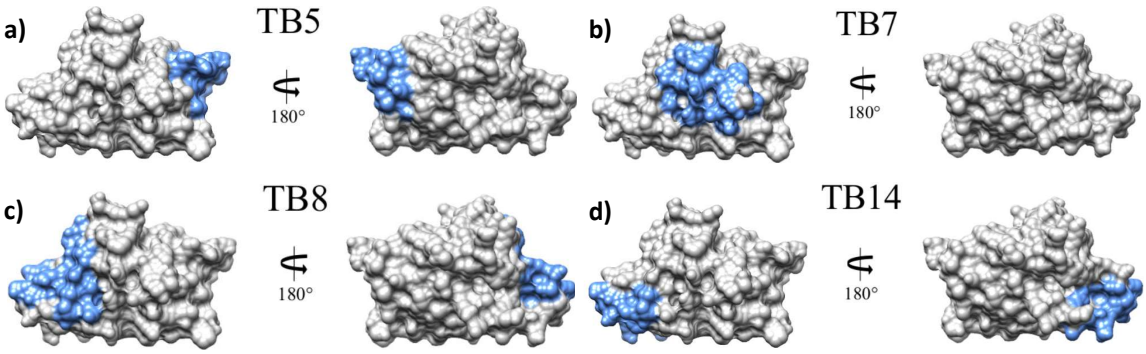


*Figure 9:The patches covered in this thesis, shown on the surface of Bet v 1: a) TB5; b) TB7; c) TB8 and d) TB14. (Schmalz et al. 2021)*

Their sequences, aligned with the sequence of TTHA0849, are shown in Figure 10.

```
TB-5          MGHHHHHHGGPEVRAERYIPAPPERVYKLAKDLEGLKPYLKEVESLEVVAREG-ARTRSR   59
TB-7          --------MGPEVRAERYIPAPPERVYRLAKDLEGLKPYLKEVESLEVVAREGGPGTRSR   52
TB-8          --------MVPEVRAERYIPAPPERVYRLAKDLEGLKPYLKEVESLEVVAREG-ARTRSR   51
TB-14         ------MGVFPEVRAERYIPAPPERVYRLAKDLEGLKPYLKEVESLEVVAREG-ARTRSR   53
TTHA0849      ----------PEVRAERYIPAPPERVYRLAKDLEGLKPYLKEVESLEVVAREG-ARTRSR   49
                        ***************** ************************   ****

TB-5          WVAVAMGKKVRWLEEE-EWDDENLRNRFFSPEGDF-----DRYEGTWVFLPEGEGTRVVL   113
TB-7          WVAVAMGKKVRWLERDEVDHENFKNNFSSPEGDF-----DRYSGEWKFLPEGEGTRVVL   107
TB-8          WVAVAMGKKVRWKERE-EWDDENLRNRFSSIEGDFIGDTLEKYSGTWVFLPEGEGTRVVL   110
TB-14         WVAVAMGKKVRWLEEE-EWDDENLRNRFFSPEGDF---TLDRYEGTWVFLPEGEGTRVVL   109
TTHA0849      WVAVAMGKKVRWLEEE-EWDDENLRNRFFSPEGDF-----DRYEGTWVFLPEGEGTRVVL   103
              ************ * * * * **  * * * ****       * * * ************

TB-5          TLTYELTIPIFGGLLRKLVQKLMQENVESLLKGLESYVLAHSDAYN----    159
TB-7          TLTYELTIPIFGGLLRKLVQKLMQENVESLLKGLEERVLAASSGHHHHHH    157
TB-8          TLKYHTKIPIFGGLLRKLVQKLMQENVESLLKGLEERVLAASSGHHHHHH    160
TB-14         TLTYHTKGD---HEVKALQQKLMQENVESLLKGLEERVLAASSGHHHHHH    156
TTHA0849      TLTYELTIPIFGGLLRKLVQKLMQENVESLLKGLEERVLAASS-------    146
              ** *             * ****************  *** *
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*Figure 10: Multiple sequence alignment of TB5, TB7, TB9, TB14 and TTH by Clustal Omega. The mutated amino acids are marked in bold; the hexa-Histidine tag is marked in bold and underlined. The positions marked with (*) underneath show conserved residues.*

The chimeric proteins were encoded by codon optimized synthetic genes in order to increase the expression rate (Chung & Lee, 2012). The genes were fused to the hexa-His Tag and were inserted into pET28b(+) expression vector (Novagen) between the restriction sites of EcoRI and NcoI. As shown in Figure 11, it has a promotor for bacteriophage T7 polymerase and provides kanamycin resistance as a selection factor.

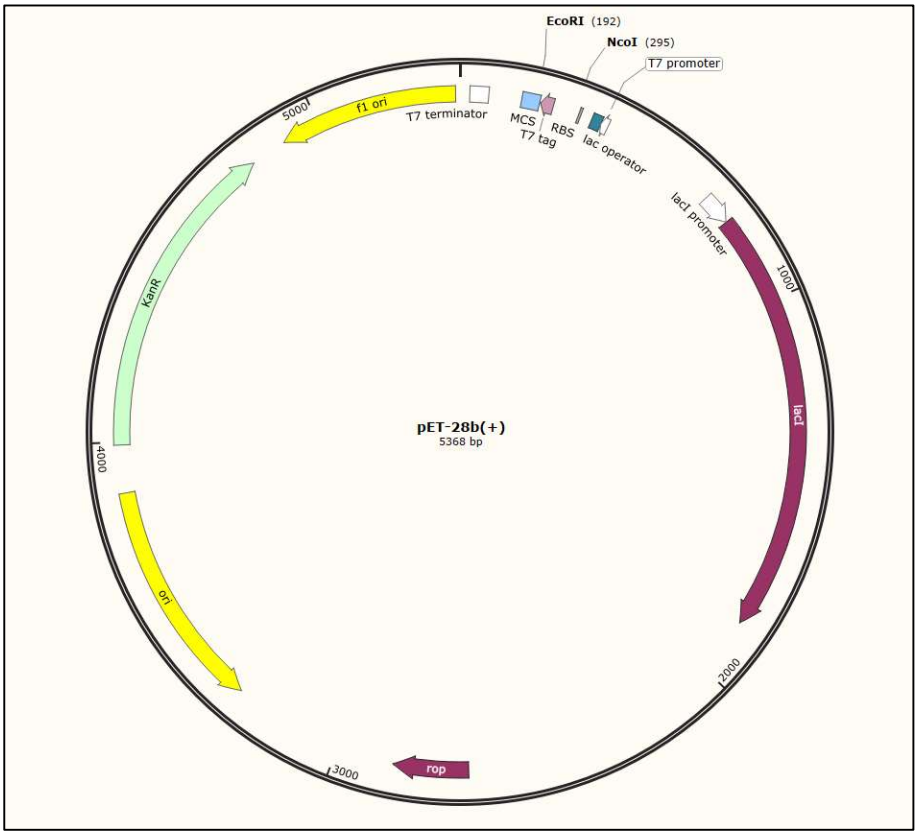



*Figure 11: pET-28-b plasmid with marked restriction sites, map created with SnapGene. T7 promoter, Kanamycin resistance and F1 ori.*

### 3.1.2. Amplification of plasmid DNA

The plasmids, carrying the coding sequence of the chimeric proteins, were inserted into NEB 5-alpha competent *E. coli* (C2987I, New England Biolabs) as described in the manufacturer's instructions. This strain is engineered for high transformation efficiency and was chosen for the production of additional plasmid DNA to be used in further experiments.

**Genotype of NEB 5-alpha Competent *E. coli***
*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

### 3.1.3. Isolation of plasmid DNA

Using a HiSpeed Plasmid Midi Kit (Prod. #12643, Qiagen), according to manufacturer's instructions.

### 3.1.4. Bacterial strain for protein expression and purification

The isolated plasmids were used to transform NiCo21 (DE3) competent *E. coli* (Prod. #C2529H, New England Biolabs). The metal-binding proteins in the NiCo-21 cells are genetically engineered to either not bind the nickel-NTA column or fused to a chitin binding

domain. The chitin binding proteins show a specific pattern in the higher molecular weight area of the SDS-PAGE gels.

The plasmid transformation was performed as described in the manufacturer's High Efficiency Transformation Protocol.

**Genotype of NiCo21 (DE3) Competent *E. coli***
*can::CBD fhuA2 [lon] ompT gal (λ DE3) [dcm] arnA::CBD slyD::CBD glmS6Ala ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*

### 3.1.5. Preparation of an overnight culture

Overnight cultures of the bacterial strains, carrying the plasmid with the chimeric protein of interest, were prepared in order to shorten the time until reaching the logarithmic phase of growth. Another advantage of the O/N culture achieving comparability between the different plasmid-carrying bacteria by always starting with the same amount of cells. 5 mL of LB medium with 25 mg/L kanamycin were put into a sterile 15 mL tube. A sterile pipette tip was dipped into the glycerol stock of NiCo21 *E. coli* and used to inoculate the medium. The tube was incubated at 37 °C, 180 rpm overnight.

### 3.1.6. Optimization of the culture medium

This test was conducted in order to determine the most suitable growth medium for each expressed protein. Bacterial cultures were incubated with different media under the same conditions and the target protein concentrations were compared using SDS-PAGE.

1 mL of a fresh overnight culture was used to inoculate 100 mL medium with 25 mg/L kanamycin, which was then incubated at 37 °C, 120 rpm until the optical density at 600 nm ($OD_{600nm}$) reached 0.6-0.8. The first sample was taken out directly before the addition of 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG). No IPTG solution was added to the autoinducing medium. The second sample was taken before harvesting by centrifugation at 7000 g for 30 minutes.

Each pellet was resuspended in 3 mL Buffer A (Lysis buffer) and Protease inhibitor cocktail (4693159001, Roche) was added in the recommended working concentration. Each pellet was lysed separately by passing three times through a microfluidizer (Model LV1 - Low Volume High Shear Benchtop Homogenizer, Microfluidics International Corporation). The lysates were centrifuged at 24 000 g for 30 minutes at 4° C. Biocryl BPA 1000 diluted 1:1000 was added to the supernatant to bind remaining DNA, which was removed by a second centrifugation at 24 000 g for 30 minutes at 4° C. The last sample was taken in order to compare the protein expression before and after induction with IPTG, as well as the protein solubility. The volume of samples 2 and 3 was chosen in a way to represent the cell density at the time of sample 1,

so that the difference in protein quantity is due to the altered expression and not due to increased cell quantity.

### 3.1.7. Optimization of expression conditions

This test was conducted to determine the best time point for harvesting the bacteria. Too short incubation time might lead to insufficient protein yield and too long times might lead to very high protein concentration and thus protein aggregation. Bacterial cultures were incubated with the same media at different temperatures (25 °C and 37 °C) and samples were taken at different time points.

The experiment was conducted in the same way as the media test and the target protein concentrations were compared using SDS-PAGE.

### 3.1.8. Fermentation

Five mL of an overnight culture were diluted to 500 mL with the medium of choice, containing 25 µg/mL Kanamycin, and further incubated at the optimal temperature determined as described above at 120 rpm. The $OD_{600nm}$ was monitored and when the value of 0.6-0.8 was reached, a sample was taken, and the protein expression was induced by the addition of 1 mM IPTG. The bacterial culture was then further incubated overnight.

### 3.1.9. Cell harvesting and lysis

The cells from the fermentation were harvested by centrifugation at 7000 g for 15 minutes at 4° C. The supernatant was removed, and the pellet was resuspended in 12 mL Buffer A (Lysis buffer). Protease inhibitor cocktail was added in the recommended working concentration. The suspension was then lysed by passing three times through a microfluidizer.

Another method for cell lysis was used in some experiments with TB7-HisC. The cells were again harvested by centrifugation and the pellet was resuspended in 12 mL Buffer A (Lysis buffer): 1 mL of ceramic beads was added to 1 mL suspension and the tube was frozen in liquid nitrogen. After 5 minutes, the tube was removed, clamped into a homogenizer (TissueLyser Mixer Mill, Retsch) without thawing and shaken at 30 Hz for 3 minutes. The freeze and grind process was repeated 3 times.

The cell debris was removed by centrifugation at 24 000 g for 30 minutes at 4° C. Biocryl BPA 1000 was added to the supernatant diluted 1:1000 and a second centrifugation followed at 24 000 g for 30 minutes at 4° C. If the sample still appeared cloudy, it was filtered through a 0.45 µm filter in order to prevent damage to the column and the chromatography equipment. The supernatant was then loaded onto a nickel-nitrilotriacetic acid (NTA) column.

### 3.1.10. Native protein purification by immobilized metal affinity chromatography (IMAC)

A glass column (d = 1 cm) was packed with 3 mL PureCube Ni-NTA Agarose (Prod. #2631105, Biozym), which has a protein binding capacity of up to 70 mg/mL. The column was stored until use in 20% ethanol at 4 °C to inhibit bacterial growth. If the resin was already used once, 500 µL 0.1 M $NiCl_2$ were flushed slowly through to reload it before use.

The column was connected to a BioRad NGC chromatography system and equilibrated with Lysis Buffer A until a stable conductivity value was reached. The lysate was loaded on the column at 0.5 mL/min. At the end, lysis buffer A was added to the sample injector in order to flush the remaining lysate out of the capillary into the column. The resin was washed with Lysis buffer A at 1 mL/min until the baseline absorbance at 280 nm was reached. Then the pump settings were switched to 8% Buffer B, which corresponds to 20 mM imidazole, to elute weakly binding proteins, until the baseline was reached again. The elution steps were repeated with 20% buffer B (50 mM imidazole), 40% buffer B (100 mM imidazole) and 100% buffer B (250 mM imidazole). At the end, the resin was stripped by flushing with 0.1 M EDTA.

Samples were collected from the flow-through during loading and washing, as well as from all elution steps.

For re-use, the column was flushed with 20% EtOH and stored at 4 °C. One portion of resin was used up to three times with reloading of nickel ions in between.

### 3.1.11. Denaturing protein purification by IMAC

This method was used for TB7-HisC due to its insolubility. The aim of the experiment was to avoid protein aggregation during protein purification.

The cells were harvested and resuspended in lysis buffer A, supplemented with protease inhibitors. They were lysed by passing three times through a microfluidizer and the lysate was centrifuged for 30 minutes at 24 000 g, 4 °C in order to remove the cell debris. The pellet was resuspended in 12 mL buffer B (8 M urea) on a stirrer for 1 h at 4 °C and then centrifuged for 45 minutes at 24 000 g, 4 °C. The supernatant was filtered through a 0.45 µm filter,

A Ni-NTA column was equilibrated with Buffer B and the filtered supernatant was loaded at 0.5 mL/min. The loaded column was washed with Buffer C until the baseline was reached. The elution of weakly bound proteins was done with Buffer D (pH 5.9) until the baseline was reached and then with Buffer E (pH 4.5).

The fractions with the highest protein concentration were identified using an SDS-PAGE. They were pooled and loaded on a Ni-NTA column. The renaturation of the protein was done while bound to the column using a linear gradient from 6 M urea (buffer R6) to 0 M urea (buffer R0)

for 1.5 h, so that the secondary and tertiary protein structure are restored correctly. At the end, the protein was eluted from the column using the elution buffer B without urea, also used in the native purification.

### 3.1.12. Further purification by chitin resin binding

Two mL chitin resin (S6651S, NEB) was washed with 2 mL Buffer B three times. The buffer was removed after every washing step by centrifugation at 1500 rpm for 10 min at 4 °C. 20 µL of the fraction that contained the target protein and the chitin-binding proteins were taken for a comparison via SDS-PAGE before and after the purification. The fraction was then mixed with the washed resin and incubated for 40 minutes on a roller at 4 °C. The mixture was then centrifuged at 4000 g for 20 min at 4 °C and the supernatant was taken for further analysis.

### 3.1.13. Protein purification with Anion Exchange Chromatography

Another method for removing impurities from the protein solution is by anion exchange chromatography. This method was used when there were still visible bands of impurities on an SDS-PAGE gel. In this method, the different proteins in the sample are eluted from the column at different times according to their charge.

The sample was dialyzed so that lysis buffer A was exchanged for IEX buffer A. After that, the lysate was loaded onto an ion exchange (IEX) column with Q Sepharose™ Fast Flow resin (Prod. # 17-0510-01, GE Healthcare). After washing, a gradient from 0% IEX buffer B to 50% IEX buffer B with high salt concentration was run for 15 column volumes to elute the bound proteins. The 50% concentration of IEX buffer B was held for 10 column volumes and the final elution was done with 100% IEX buffer B. All fractions were collected.

### 3.1.14. Desalting and buffer exchange

In order to prepare the protein solution for further experiments, the following products were used depending on the volume of the solution.

- Large volumes: (>1 mL): Dialysis O/N in 2 L buffer:
  Spectra/Por Dialysis Membrane, Molecular weight cut-off: 6 - 8000 kDa, 23 mm flat width; Prod.#132650, SpectrumLabs.com
- Small volumes: (~100 µL): Dialysis for 2 hours in 13 mL buffer:
  Slide-A-Lyzer MINI Dialysis Device, 3.5K MWCO, Prod.#88400, ThermoFisher
- Very small volumes: (~10 µL): Dialysis for 30 min in ~5 mL buffer:
  Floating dialysis: MF-Millipore membrane Filters, Prod.#VSWP02500, Millipore

## 3.2. Analysis of expressed proteins

### 3.2.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

This method was used to separate the different proteins, contained in the loaded samples, by mass and thus compare the differences in the expression of the target protein.

All gels for this method were prepared in-house – for the list of reagents see appendix 7.1. Glass plates and 0.75 mm spacers were used. The 12% analytical resolving gel was poured first, then covered with 70% isopropanol and let polymerize for 30 minutes. The isopropanol was then poured out, the gel was washed with a small volume of distilled water and dried with a paper towel. Finally, the 4.5% stacking gel was poured, the comb was inserted, and the gels were let polymerize for at least 2 hours at room temperature. For later use, the gels were wrapped in paper towels, wetted with distilled water, then wrapped in plastic foil and stored at 4 °C.

The samples taken from the different purification steps were mixed with 4x sample buffer, heated for 5 minutes at 95 °C, centrifuged for 5 minutes at 1200 g and then loaded onto a SDS-PAGE gel. A protein ladder (PageRuler™ Plus Prestained Ladder, 10-250 kDa, ThermoFisher, 26619) was used to determine the molecular masses of the bands. The gels were run at 120 V for 15 minutes and then at 160 V for around 40 minutes until the dye front migrated out of the gel.

**Staining with Coomassie Brilliant Blue**

The SDS-PAGE gels were stained in a Coomassie Brilliant Blue solution in order to visualize the separated protein bands. The gels were shaken for 120 minutes in staining solution. After that, they were washed shortly with distilled water and then incubated O/N with a destaining solution. The destained gels were then photographed using a ChemiDoc Imaging System (BioRad).

### 3.2.2. Western blot

When the results from the SDS-PAGE were inconclusive and it could not be determined whether the protein of interest was contained in the sample, a Western blot was done. This immunoblot is more specific, because the visualization of the protein is not done by staining, but by the binding of protein-specific antibodies. In this case, the primary antibody bound to the His-Tag of the chimeric proteins.

An SDS-PAGE gel (not stained) was put onto a nitrocellulose membrane (Whatman, GE Healthcare), soaked in transfer buffer. Both were then wrapped in filter paper and put into the electrophoresis equipment in order to transfer the proteins from the gel to the membrane. The transfer took place for 40 minutes at 100 V. The nitrocellulose membrane was then blocked in

MTBST 5% for 1 hour. After that, the membrane was incubated O/N at 4 °C on a shaker with the primary antibody, (Mouse anti-Penta His, 2400320, AB_1309300, 5 Prime Inc.), diluted 1:1000 in MTBST 3%. The membrane was washed 3 times for 10 minutes with 15 mL TBST in order to remove all of the unbound primary antibody. The membrane was incubated for 2 hours with the secondary antibody (Alkaline phosphatase-conjugated AffiniPure Rabbit Anti-Mouse IgG+IgM (H+L), Prod. # 315-055-048, Jackson Immunoresearch), diluted 1:10000 in TBTS. It was then washed again three times with TBST and the substrate solution was added. After 5 minutes of incubation, the membrane was washed with water to stop the reaction.

### 3.2.3. Solubility and storage conditions test

After it was determined if the protein of interest is present in the samples after fermentation and native purification, a solubility test was done to determine whether the protein solution stayed stable in the buffers needed for further experiments (eg. IEX, CD etc.) as well as to determine the most suitable storage buffer.

As the in-lab produced proteins are frozen in liquid nitrogen and stored at -80 °C until needed, an additional test was conducted to determine whether protein precipitation and aggregation occurs when freezing and thawing the protein solution

The protein concentration of the solution was determined by photometry at the start of the test. A small sample (~500 µL) was then dialyzed with MINI Slide-A-Lyzer against the buffer to test. The sample was centrifuged at 24 000 g for 10 minutes and the concentration was measured again. Additionally, a measurement of dynamic light scattering was performed to check whether protein aggregates formed.

In the last step, a 100 µL sample was frozen in liquid nitrogen for 3 minutes and stored for 1 h at -20°C. It was then thawed and centrifuged at 24 000 g, 4°C for 10 minutes. The measurements of the concentration and the dynamic light scattering were repeated, and the results were compared with those before freezing.

If most of the protein molecules were not present as oligomers and there were no major changes in the concentration, the whole sample was dialyzed and used for further experiments. Small differences in the concentration were to be expected due to inaccuracies in the measurement, as well as due to osmosis and thus dilution of the sample.

### 3.2.4. Dynamic light scattering

The measurement of dynamic light scattering was conducted in order to determine the hydrodynamic radii by measuring the diffusion coefficient of the particles in the protein solution. Both of those numbers are influenced by the size and shape of the proteins and thus a conclusion about the protein aggregation in the sample could be drawn (Stetefeld et al., 2016).

All chimeric proteins had the size of around 17-18 kDa and the expected results were around 1.4 nm radius. If, for example, the measurement showed that most particles in the solution were around 35 kDa, then protein dimers were formed.

50 µL of the sample were transferred to an UVette® (0030106300, Eppendorf) and measured using a DynaPro ® NanoStar ® cuvette-based DLS instrument (Wyatt Technology).

### 3.2.5. Determination of protein concentration

**Photometry**

The Nanodrop UV-Vis spectrophotometer (ThermoFisher) was used to measure the absorbance of protein solutions at 280 nm. The ExPasy: ProtParam server (https://web.expasy.org/protparam/) was used to calculate the protein concentration from the absorbance, as it depends on the fraction of the aromatic amino acids tryptophan and tyrosine in the protein chain, which varies between the proteins.

The sample pedestal was cleaned with distilled water before use. After that, a 2 µL water sample was loaded, followed by a 2 µL buffer sample. After blanking with the used buffer, three measurements with 2 µL protein solution were carried out. The mean of the absorbance measurements was calculated and the values from Table 1 were used to calculate the protein concentration.

This method provided quick results and was used for approximate determination of the concentration, e.g. if the sample needs to be diluted for a specific experiment or if the concentration changes when the storage conditions are changed (see solubility test, freeze thaw test).

*Table 1: Specific absorbance of 1 g/l chimeric protein*

| Chimeric protein | Abs 0.1% (=1 g/l) |
|---|---|
| **TB5** | 1.757 |
| **TB7** | 1.330 |
| **TB8** | 1.587 |
| **TB14** | 1.620 |

**Pierce BCA protein assay**

The bicinchoninic acid (BCA) protein assay is used to determine the total protein concentration via a colorimetric reaction. Copper ions chelate with the proteins under alkaline conditions and form a light blue complex. After that, two BCA molecules bind one copper ion, leading to an intense purple color, which absorbs at 562 nm and correlates linearly to the protein concentration. This method was deemed more sensitive and precise than the photometry

measurement, which can show false results due to air bubbles or other impurities. Due to the required reaction time, the BCA protein assay was only used before freezing the proteins for storage in order to provide precise information for future experiments.

The Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23227) was used with the following in-house protocol:

*Table 2: Preparation of standard solutions for Pierce BCA Assay*

| St. # | Vol. BSA (2 mg/mL) [µL] | Vol. Buffer [µL] | Final conc. [µg/mL] |
|---|---|---|---|
| **A** | 10 | 70 | 250 |
| **B** | 8 | 72 | 200 |
| **C** | 12 | 148 | 150 |
| **D** | 8 | 152 | 100 |
| **E** | 4 | 156 | 50 |
| **0** | 0 | 160 | 0 |

The BCA working solution was prepared by mixing 50 parts reagent A with 1 part reagent B. 200 µL working solution per sample was needed. The standard solutions were prepared as listed in Table 2. 25 µL of each sample were pipetted into a microtiter plate. 200 µL working solution per sample were added and the plate was gently tipped in order to mix the reagents. The plate was incubated for 30 min at 37 °C and the absorbance was measured at 562 nm.

### 3.2.6. Circular dichroism spectroscopy

This method was used to examine the secondary structure of the protein and thus determine whether the protein was folded correctly. It is based on the measurement of the difference in the extinction coefficients of the left and right circularly polarized light of optically active molecules. The spectrum in the far UV region (185-260 nm) is specific to certain secondary structures such as beta-sheets or alpha helices (Greenfield, 2007; Kelly et al., 2005).

All measurements were made with a 0.2 mg/mL protein solution in 10 mM sodium phosphate buffer, pH 7.5, optionally supplemented with 100 mM NaF. Each sample was measured three times in a 0.1 cm quartz cuvette in the region between 190 and 260 nm. The raw results were converted to molar residue ellipticity, which allows the comparison of spectra of samples with different concentrations and molar masses.

The spectrum compared with the spectrum of the TTH scaffold, which was recorded with the same protein concentration. If both spectra had the same shape and crossed the x-axis at the same wavelength (204 nm), then the target protein was considered to be folded correctly. Some noise was expected in the area between 190 and 200 nm due to the equipment (Jasco J-810 spectropolarimeter (Jasco International Co.)).

### 3.2.7. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a highly sensitive analytical method for determining the mass of single molecules. The proteins are mixed with and thus embedded into a matrix, consisting of weak organic acids. Due to the energy of the laser beam, the matrix ionizes and leads to desorption of the target molecules, which pass over into the gas phase, carrying a single, double or even triple charge. Those charges molecules travel in an electric field with a specific acceleration, which depends on their mass and charge, and can be determined by measuring the time of flight (Bonk & Humeny, 2001).

In this project, MALDI-TOF-MS was used to confirm the identity of the purified protein, to examine whether the N-terminal methionine was cleaved as well as to rule out proteolytic degradation during the fermentation or the purification process. The measured result was compared with the theoretical mass.

10 µL of the protein were dialyzed against distilled water on a Millipore floating membrane filter for 30 minutes. 1 µL of the dialyzed sample was placed on a MSP 96 polished steel target (Prod. #224989, Bruker Daltonics GmbH), mixed with 1 µL alpha-Cyano-4-hydroxycinnamic acid (HCCA) matrix dissolved in 50% acetonitrile, and left to dry for 5 minutes. The measurements were done using the following settings:

*Method: LP_12kDa*
*Linear mode, positive ion mode*
*Laser Power: 50%*
*Detection: 3000 – 35 000 mass range*
*Calibration: Sigma Proteomass Protein (MSCAL3, Sigma-Aldrich)*

### 3.2.8. IgE ELISA with sera

For this experiment, blood sera of patients were provided by the Allergieambulatorium Reumannplatz, Vienna, Austria. Their sensitization to Bet v 1 has been confirmed on site by a measurement with ImmunoCAP (ThermoFisher). The aim was to check how strong does the IgE in blood serum bind to the chimeric proteins. This signal was compared to the binding to Bet v 1 as a positive control, as all patients had a confirmed birch allergy, as well as to binding to TTH as a negative control. The blood plasma of 3 non-allergic individuals (NHS) was used to determine the lower limit of the positive signal. All values were measured in duplicates.

Calculation of threshold of positive values:

$$Positive\ value \geq Mean\ of\ all\ NHS + 3 * Standard\ Deviation$$

Three 96-well plates were coated with 100 µL/well chimeric protein at 5 µg/mL in 50 mM sodium carbonate buffer, pH 9.6 O/N at 4 °C. On the next day, all wells were blocked with 300 µL/well MTBST 3% for 2 hours at RT and washed 5 times. The sera, containing Betv1-specific IgE concentrations <100 kUa/L, were diluted to 0.5 kUa/L in dilution buffer. The sera with >100 kUa/L (over the limit of the method and not quantifiable) were diluted to 0.25 kUa/L in dilution buffer. Myeloma IgE, diluted 1:100; was used as a negative control, as it should not bind any of the coated proteins.100 µL/well diluted serum / IgE were added, and the plates were incubated O/N at 4 °C. The sera were then discarded, the plates were washed 5 times and then incubated with 100 µL/well detection antibody (1:1000 dilution in TBST 3%; AKP Mouse Anti-human IgE antibody (Prod. #555859, BD Pharmingen,)) for 1 hour at RT in the dark. Washing was again repeated 5 times, 100 µL/well substrate (SIGMAFAST™ p-Nitrophenyl phosphate Tablets (Prod. #N2770-50SET, Sigma-Aldrich)) were added and the plates were incubated in the dark at RT. The OD at 405 nm was measured at different time points. All samples were measured in duplicates.

### 3.2.9. Inhibition IgE ELISA with sera

The objective of this assay was to study how the chimeric proteins inhibit the binding of serum IgE to Bet v 1. If a chimeric protein bound Bet v 1-specific IgE, then it could be assumed that the grafted patch represented an IgE epitope on the surface of Bet v 1.

The coating (100 µL 5 µg/mL Bet v 1 without His-Tag), blocking and washing steps were carried out as in the direct ELISA experiment. The sera of two allergic patients were diluted to 5.5 kUa/L Bet v 1-specific IgE with dilution buffer. For the inhibition, the chimeric proteins TB4, TB8 und TB14, Bet v 1 without His-Tag and a mix of TTH-HisC and TTH-HisN as a negative control were diluted in a ten-fold dilution series from 20 µg/mL to 2 ng/mL (2x final concentration). 150 µL of the diluted serum were mixed with 150 µL of the diluted protein (10 µg/mL to 1 ng/mL final concentration) in a 96-well plate with a non-binding plastic surface and incubated for 30 minutes. In order to determine the value of 0% blocking, the diluted sera were mixed with dilution buffer instead of protein solution. 100 µL/well of the mixture were transferred in duplicates to the blocked and washed plate and incubated O/N at 4 °C. The experiment then proceeded as in the direct ELISA. The absorbance was measured at 405 nm after 60 minutes.

Calculation of the inhibition:

$$\%\ inhibition = 100.\left(1 - \frac{Mean\ value\ of\ serum\ inhibited\ by\ protein}{Mean\ value\ of\ uninhibited\ serum}\right)$$

## 3.3. Generation of Betv1-specific recombinant antibodies

In order to study the interactions between Bet v 1 with different antibodies, the method of phage display was used. The aim was to screen the library for epitope specific anti-Betv1 synthetic antibodies and select and amplify them. Subsequently, they were used in different experiments.

### 3.3.1. Materials

The whole experiment was based on the BioSource protocol for selection of human antibody fragments by Daniel Christ (Lee et al., 2007).

**Library:** Domain antibody library (complexity: ~3x10$^9$ ), based on a single human heavy chain variable domain (VH) framework in phagemid format - a gene III expressing plasmid in combination with a helper phage, ampicillin resistant. (Figure 12)

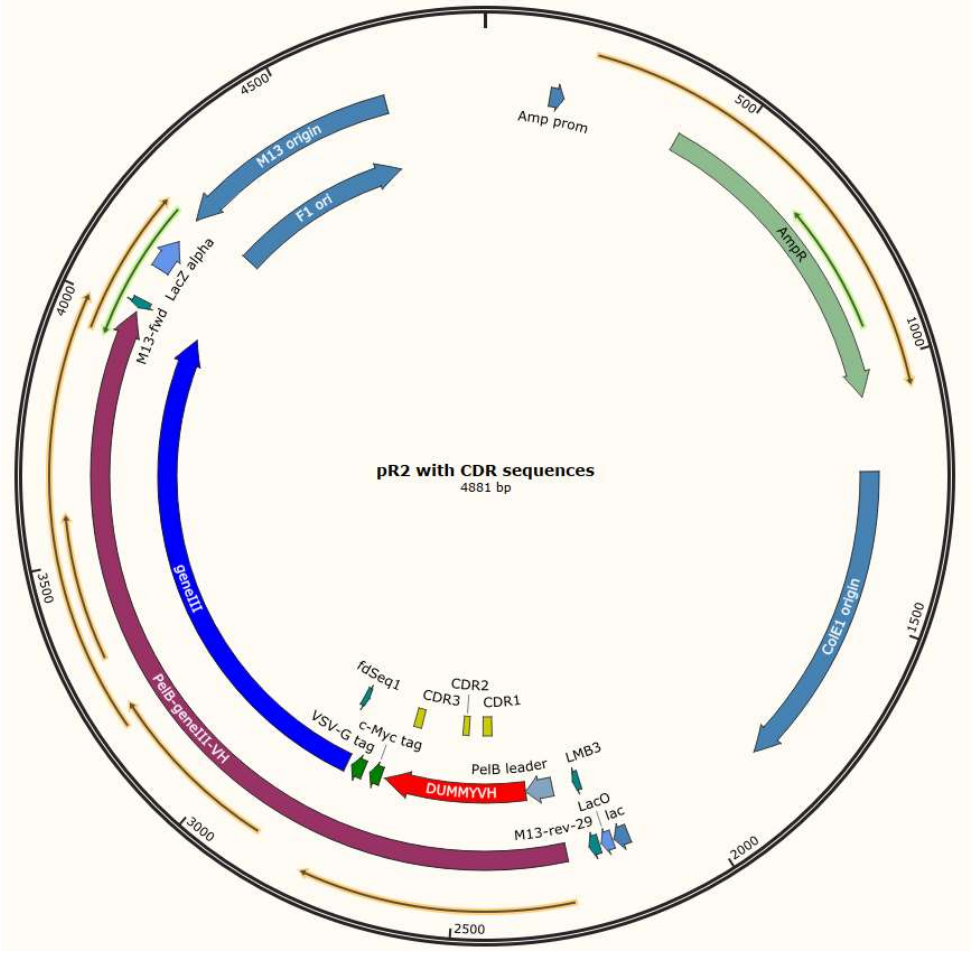



*Figure 12: Map of the used pR2 phagemid, created with SnapGene: "Dummyvh" is the placeholder for the sdAb, containing 3 CDR, fused to gene III using a c-Myc tag.*

**Genotype of T-phage resistant *E. coli* TG1 (for propagation of eluted phage):**
K12 D(lac-proAB) supE thi hsdD5/F' traD36 proA+B lacIq lacZDM15

**Helper phage:** KM13 (~$10^7$ pfu/mL), kanamycin resistant.

### 3.3.2. Production of additional stocks of the Christ domain antibody (dAb) library

750 µL of the original Christ dAb Library were diluted to 1.8 mL with 2x TY medium. The dilution was plated out on 12 245x245 mm TYE ampicillin glucose agar plates und incubated overnight at 37°C. A lot of very small colonies were observed on the next day. 12 mL of 2x TY medium were added per plate and all colonies were scraped with a sterile Drigalski cell spreader. The medium was recovered with a pipette and 1/5 volume glycerol was added in order to preserve the bacteria during freezing. The prepared suspension was then aliquoted and stored at -80 °C.

### 3.3.3. Preparation of TG1 bacteria

One M9 minimal glucose agar plate was inoculated with a sterile pipette tip, dipped into the delivered stock culture of *E. coli* TG1. The agar plate was incubated for 48 hours at 37 °C and then sealed and stored at 4 °C. For every experiment, in which TG1 were needed, 5 mL 2xTY medium were inoculated with a single colony the day before and incubated at 37 °C, 160 rpm overnight. The bacterial culture was then diluted 1:100 and incubated at 37 °C, 160 rpm until $OD_{600nm}$≈0.5.

*For high infection rates, the bacteria must be in the log phase. An $OD_{600nm}$ of slightly under 0.5 is acceptable, but if the culture is already denser than that, it should be diluted and incubated for some time under the same conditions to guarantee the log-phase of growth.*

### 3.3.4. Preparation of KM13 helper phage stock

The commercially obtained phage stock was diluted 1:100, 1:1000 and 1:10 000. 10 µL of each dilution were mixed with 200 µl TG1 bacteria in 2xTY medium at $OD_{600nm}$≈0.5 and incubated at 37 °C for 30 minutes without shaking. 3 mL aliquots of H-Top Agar were equilibrated at 42 °C in a water bath and the infected bacteria were added. Each aliquot was poured onto a pre-warmed (37 °C) TYE plate and incubated at 37 °C overnight. For further experiments, a single plaque was picked with a sterile pipette tip and transferred to 5 mL TG1 in 2x TY medium at $OD_{600nm}$≈0.5. The culture was incubated for 2 hours at 37 °C without shaking and then used to inoculate 500 mL 2x TY medium, which was incubated at 37 °C and 160 rpm. After 2 hours, 500 µL kanamycin solution (25 mg/mL) were added, the temperature was turned down to 30 °C and the bacterial culture was incubated overnight at 160 rpm.

### 3.3.5. Phage purification by PEG precipitation

The 500 mL bacterial culture was divided into 10x 50 mL tubes and centrifuged at 2500 g for 40 minutes. 10 mL aliquots of PEG solution were prepared in 12 50 mL tubes, which were then filled with 40 mL of the supernatant. The content of the tubes was well mixed by vortexing and incubated for 1 hour on ice to induce precipitation. The tubes were centrifuged at 2500 g at 4 °C for 40 minutes. The supernatant was removed, and the pellets of precipitated phages were resuspended and pooled in a total of 5 mL of sterile PBS. 1 mL PEG solution was added, and the suspension was incubated for 10 minutes on ice. The tube was then centrifuged for 40 minutes at 2500 g at 4 °C. The supernatant was removed, and the pellet was resuspended in 1 mL sterile PBS. The filtration step through a 0.45 µm filter as described in the Christ dAb protocol was skipped, because the suspension was too thick and caused filter blockage and subsequent loss of phage particles.

The phage suspension was stored at 4 °C after addition of 50 µL 20x proteolysis reducing solution.

### 3.3.6. Determination of the concentration of phage suspensions

**Titration**

10 µl phages were diluted in a ten-fold dilution series 13 times. 10 µL of each phage dilution were added to 90 µL TG1 suspension prepared according to section 3.3.1.2. The bacterial cultures were incubated for 30 minutes at 37 °C without shaking. 10 µL of each dilution were placed as a single drop without spreading onto a 2x TY agar plate with 50 µg/mL kanamycin for the helper phages or additionally 100 µg/mL ampicillin for the phage library. The plate was incubated for 24 hours at 37 °C. Uninfected bacteria and the 1:$10^3$ diluted phage suspension (10 µL each) were used as negative controls.

$$\text{phage/mL} = \text{number of colonies} * \text{dilution factor} * 1000$$

**Optical measurement**

The concentration of the phage suspension was roughly determined by measuring the $OD_{260nm}$ and calculating it with the following formula:

$$\text{phage/mL} = OD_{260nm} * 22.14 * 10^{12}$$

### 3.3.7. Preparation of phages for affinity enrichment

An aliquot of the frozen antibody library was thawed on ice, diluted into 500 mL 2x TY medium with 4% glucose and 100 µg/mL ampicillin, and incubated at 37 °C, 160 rpm. When an $OD_{600nm} \approx 0.5$ was reached, 2x$10^{12}$ pfu KM13 helper phages were added. The bacterial culture was incubated for 1 hour at 37 °C in a water bath and then the medium was switched – the suspension was centrifuged in 10 50 mL tubes, 50 mL each, for 15 minutes at 2500 g. The supernatant was discarded and the pellets were resuspended in 500 mL 2xTY medium with

0.1% glucose, 100 µg/mL ampicillin and 50 µg/mL kanamycin. Incubation for 20 hours at 25 °C, 160 rpm followed and the phages were then purified by PEG precipitation.

### 3.3.8. Affinity enrichment (Biopanning)

1 mL Bet v 1 solution (10 µg/mL, no tag) in 10 mM sodium phosphate buffer was used to coat a 5 mL immunotube (444202, Nunc-Thermo Fisher) overnight at 4 °C. The protein solution was removed and the tube was incubated with 4.5 mL blocking solution for 2 hours at room temperature. After that, the tube was washed three times with 5 mL sodium phosphate buffer each and incubated with the phage solution, containing $6x10^{12}$ phages in 4 mL blocking solution, for 1 hour at room temperature with rotation. The phage solution was then discarded and the immunotube was first washed 10 times with washing buffer and then 2 times with sodium phosphate buffer. The bound phages were then eluted by incubation with 4 mL freshly prepared trypsin solution for 1 hour at room temperature with rotation. The trypsin phage eluate was added to 30 mL TG-1 *E. coli* at $OD_{600nm} \approx 0.4$ and the mixture was incubated for 1 hour at 37 °C in a water bath. It was then centrifuged for 5 minutes at 3200 g, the supernatant was discarded and the pellet was resuspended in 1 mL 2xTY medium. To determine the concentration of the eluted phages, 100 µL of the 1:100 and 1:1000 dilutions each were spread onto 2 TYE ampicillin glucose agar plates. The rest of the infected cell suspension was divided and spread onto 6 large TYE ampicillin glucose agar plates (245 mm x 245 mm). All plates were incubated overnight at 37 °C.

On the next day, the large plates were scraped with a sterile Drigalski spatula, using 5 mL 2xTY medium per plate. The recovered bacterial suspension (≈20 mL) was diluted to 500 mL with 2xTY medium, supplemented with 4% glucose and 100 µg/mL ampicillin, and incubated for 2 hours at 37 °C. $2x10^{12}$ KM13 phages were added and the infection took place at 37 °C for 1 hour in a water bath. The medium of the infected bacterial culture was then switched as described on p.20 to 500 mL 2xTY medium with 0.1% glucose, 100 µg/mL ampicillin and 50 µg/mL kanamycin. Incubation for 18 hours at 25 °C, 160 rpm followed, and the phages were then purified by PEG precipitation in order to proceed to the next round of selection with Bet v 1. Three rounds of selection were performed.

### 3.3.9. Polyclonal phage ELISA with phages from all rounds of selection

This assay was performed to verify that Bet v 1-specific phages were enriched in the selection process.

A 96-well plate (Maxisorp, Thermofisher) was coated with 0.5 µg/well recombinant Bet v 1 (no tag; 100 µL/well, 5 µg/mL) in sodium phosphate buffer overnight at 4 °C. As a positive control, 4 wells were coated with Anti-M13 coat protein g8P antibody (Prod. #MA1-06604, Affinity Bioreagents), 100 µL/well, diluted 1:200 in PBS, which would bind all phages,

independent of their affinity for Bet v 1. On the next day, each well was blocked with 200 µL MTBS-T for 2 h at RT. The wells were then washed 3 times with TBS-T and incubated with the phage dilutions ($8.5x10^{10}$ phages/mL) in MTBS-T for 2 h at RT. The wells, coated with Anti-M13 coat protein g8P antibody, were incubated with samples from the third round of selection. As a negative control, 1:100 and 1:1000 dilutions of the KM13 helper phages were used, because they should not bind the coated Bet v 1. As a second negative control, the phages from the original library were taken, because the number of the Bet v 1-specific phages should be extremely low. After that, the plate was washed 5 times with TBS-T (0.5% Tween 20) and incubated for 1 hour at RT in the darkness with 100 µL/well HRP/Anti-M13 Monoclonal conjugate (Prod. #27-9421-01, Amersham Pharmacia Biotech), diluted 1:5000 in TBS-T. Subsequently, the plate was washed another 5 times and 100 µL/well o-phenylenediamine dihydrochloride substrate solution (OPD substrate tablets, SIGMAFAST™, Prod. #P9187, Sigma-Aldrich) were added. The absorbance at 450 nm was measured after 30 minutes.

### 3.3.10. Screening of single clones by ELISA

This method was used to test individual colonies for Bet v 1-specific phage binding. Colonies from round 2 and round 3 were picked from the dilution plates and transferred into a 96-deep-well plate containing 200 µL/well 2xTY medium with 100 µg/mL Amp and 4% glucose. Before inoculation of the wells, the tip was pressed onto a replica agar plate (TYE, 100 µg/mL Amp, 4% glucose) for later recovery of Bet v 1-specific clones. The 96-deep-well plate was covered with a breathable seal and incubated O/N at 37 °C, 160 rpm.

On the next day, 5 µL of each well were used to inoculate a new 96 well plate with 200 µL/well 2xTY medium with 100 µg/mL Amp and 4% glucose. The new plate was incubated for 3 hours at 37 °C, 160 rpm and 50 µL 2xTY medium, containing $4x10^{8}$ helper phages, were added to each well. The plate was incubated for 1 hour without shaking in order to permit infection and was then centrifuged for 10 minutes at 3200xg. The supernatant was removed and the pellets were resuspended in 200 µL 2xTY, 0.1% glucose, 100 µg/mL Amp, 50 µg/mL Kan. The plate was incubated for 18 hours at 25 °C and 160 rpm.

The 96-deep-well plate with the superinfected bacteria was centrifuged for 10 minutes at 3200xg. 50 µL supernatant/well were mixed with 150 µL/well MPBS in a separate 96-deep-well plate. 100 µL/well of the diluted supernatant were transferred to a Maxisorp (ThermoFisher) plate, coated O/N with 100 µL/well 5 µg/mL Bet v 1 (no tag) and then blocked O/N with 200 µL/well MPBS (3% milk) at 4 °C.

The ELISA plate was incubated for 1 hour at room temperature and then washed 3x with PBST (0.05% Tween 20). 100 µL/well detection antibody Anti-M13-HRP monoclonal conjugate (Prod. #27-9421-01, Amersham Pharmacia Biotech), diluted 1:1000, were added to each well

and the plate was incubated for 1 hour at room temperature in the dark. The plate was again washed 3x with PBST (0.05% Tween 20) and 1x with PBS. 100 µL/well OPD substrate solution were added and the plate was incubated at room temperature in the dark for 10 minutes. The absorbance at 450 nm wavelength was measured and the clones of interest were identified.

### 3.3.11. Production of phage clones of interest

2 µL of each clone of interest were taken from the 96-deep-well plate, preserved during screening, and were used to produce an O/N culture in 5 mL 2xTY medium with 4% glucose and 100 µg/mL Amp each, which was incubated at 37 °C, 160 rpm overnight. 20 µL of each culture were transferred to a 50 mL-tube with 20 mL 2xTY medium with 4% glucose and 100 µg/mL Amp on the next day and incubated for 4 hours at 37 °C, 160 rpm. After that, KM13 phages were added with a final concentration of $4.10^9$ phages/mL. The mixture was incubated for 1 hour without shaking and then centrifuged for 10 minutes at 3200xg. The supernatant was removed, each pellet was resuspended in 20 mL 2xTY with 0.1% glucose, 100 µg/mL Amp and 50 µg/mL Kan and incubated at 25 °C, 160 rpm for 18 hours. The phage precipitation was done as described on p.19. All reagent volumes were reduced by a factor of 25. The final pellet was resuspended in 400 µL PBS and centrifuged for 10 minutes at 3200xg. The supernatant was transferred to new tubes and 15 µL protease inhibitor cocktail (Roche) and 20 µL 20x proteolysis reducing solution were added to each. The tubes were stored at 4 °C until further use.

### 3.3.12. Single clone ELISA with Bet v 1 chimeric and homologue proteins

The objective of this assay was to test if the phages of interest, which were selected for their binding to Bet v 1, also bind to chimeric proteins and Bet v 1 homologues from plant foods. The test was done with TTHA0849 from *Thermus thermophilus* (mix of C-terminal and N-terminal hexa-Histidine-tags) as a negative control and Bet v 1 (no tag) as a positive control.

*Table 3: List of proteins used in single clone ELISA*

| Protein | Purpose |
|---|---|
| Bet v 1 | Positive control (birch) |
| TTH | Negative control *(Thermus thermophilus)* |
| TB1 | Betv1-TTH chimera |
| TB2 | Betv1-TTH chimera |
| TB5 | Betv1-TTH chimera |
| TB6 | Betv1-TTH chimera |
| TB8 | Betv1-TTH chimera |
| TB13 | Betv1-TTH chimera |
| TB14 | Betv1-TTH chimera |
| Cor a 1.04 mut | Homologous protein (hazelnut) |
| Api g 1.01 | Homologous protein (celery) |
| Mal d 1 | Homologous protein (apple) |
| Gly m 4 | Homologous protein (soybean) |
| Lysozyme | Non-homologous negative control |
| Bet v 2 | Non-homologous negative control |
| BSA | Non-homologous negative control |

The previously purified TB5, TB8, and TB14 chimeric proteins were used, as well as TB1, TB2, TB6 and TB13, which were provided by Stefanie Schmalz and Vanessa Mayr. The homologous proteins and the negative controls, listed in Table 3, were produced in-house and to assure equal concentration, the tubes were thawed on ice, centrifuged for 10 minutes at 24 000 g and the protein concentration was determined by photometry.

96-well plates were coated with 100 µL/well protein in PBS at 5 µg/mL and incubated O/N at 4 °C in PBS. On the next day, all plates were blocked with 200 µL/well MPBS (3% milk) and incubated O/N at 4 °C. The plates were then washed 3x with PBST (0.05% Tween 20) and 100 µL/well phage dilution containing $8.5x10^{10}$ phages/mL in MPBS were added. As a positive control for the detection, an in-house rabbit anti-Bet v 1 antibody (dilution 1:1000) was used. The phages from the original library (before selection) were used as a negative control. The plates were incubated for 2 hours at RT and then washed again 3x with PBST (0.05% Tween 20). 100 µL/well anti-rabbit IgG, HRP-marked antibody (Prod. #7074S, Cell signaling) were used for the wells containing the rabbit antibody. The rest of the wells was incubated with 100 µL/well Anti-M13-HRP 1:1000 detection antibody for 1 hour at room temperature in the dark. The plate was again washed 3x with PBST (0.05% Tween 20) and 1x with PBS. 100 µL/well OPD substrate solution were added and the plate was incubated at room temperature in the dark for 10 minutes. The absorbance at 450 nm was measured after 10 and 30 minutes.

### 3.3.13. Single-clone sandwich ELISA

The aim of this assay was to confirm the specificities of the selected antibodies by an independent experiment using a different ELISA format.

The protein content of the phage suspensions was determined by measuring the $OD_{280nm}$ in a 1:100 dilution in PBS in a black quarz cuvette. For the calculation, the assumption was made that 1 mg/mL protein has an absorbance of 1. Plates were then coated with 20 µg/mL of selected clones or the original library at 100 µL/well. The blocking and washing steps were carried out the same way as in the previously described ELISAs. The plates were then incubated with 100 µL/well protein of the same chimeric and homologue proteins as in the previous method at 3 µg/mL. The following non-homologous proteins were used as additional negative controls: Hev b 11 from *Hevea brasiliensis* (Para rubber tree (latex)), Phl p 5 from *Phleum pratense* (Timothy grass) and Lyc e 2 from *Lycopersicon esculentum* (tomato). The plates were incubated for 2 hours, washed and then 100 µL/well detection antibody were added – rabbit-anti-Bet v 1 (in-house production, dilution 1:1000) to the wells with non-tagged proteins, and anti-His-HRP, Mouse IgG1κ (Prod. #652504, BioLegend) (dilution 1:5000) to the wells with proteins with a hexa-histidin tag. The plates were incubated O/N at 4 °C. On the next day, the plate with His-tagged proteins was washed and incubated with 100 µL/well OPD substrate. The plate with non-tagged proteins was incubated with 100 µL/well detection antibody – goat anti-rabbit-HRP (dilution 1:5000, Prod. #97051, Abcam,), incubated for 2 hours at RT, washed and finally incubated with OPD substrate. The absorbance at 450 nm was measured after one hour.

### 3.3.14. Preparation of plasmid DNA for sequencing

The clones of interest were grown as an overnight culture in 2x TY, supplemented with 4% glucose and 100 µg/mL ampicilling. 4 mL of each bacterial culture were used to isolate plasmid DNA for sequencing. The kit PureYield Plasmid Miniprep System (Prod. #A1222, Promega) was used according to the manufacturer's instructions.

### 3.3.15. DNA Sequencing

15 µL (100 ng/µL) of each of the plasmid solutions were submitted for sequencing to Eurofins Genomics, Ebersberg, Germany. The following primers were chosen from the Eurofins standard primer list:

- Forward: "M13-rev (-29)": CAGGAAACAGCTATGACC

- Reverse: "-96gIII (2886-2867)": CCCTCATAGTTAGCGTAACG

### 3.3.16. Sequence alignment

An alignment was performed in order to compare the sequences of the three clones. First, the forward and reverse sequences of each clone were aligned with BLAST2 to ensure that there are no mismatches Small differences at the beginning and end of the sequence were found due to sequencing errors, but those are not critical as they were located outside of the sdAb sequence. After that, the sequences of all clones were aligned with Clustal Omega for the antibody region. Additional pairwise alignment with Emboss needle was used to confirm that some of the sequences were identical.

# 4. Results

## 4.1. Purification and analysis of Bet v 1-TTH chimeras

### 4.1.1. TB5-HisN

#### 4.1.1.1. Optimization of the expression

**- Optimization of the culture medium**

This test was conducted in order to determine the most suitable medium for the expression of TB5-HisN. It was performed with RICH, LB, SOC, SB and auto-inducing media, as seen in Figure 13. There was a visible darker band at around 17 kDa in all samples after induction, which mostly disappeared in the lysed samples. This was caused either by insoluble protein or by incomplete lysis. The LB medium was chosen for further experiments.

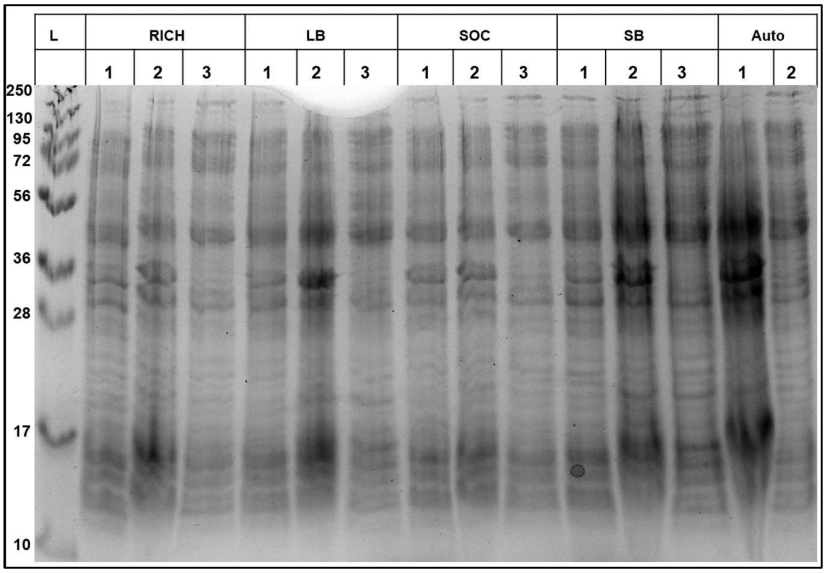



*Figure 13: CBB-stained SDS-PAGE gel of the TB5 media test with samples from bacterial cultures in RICH, LB, SOC, SB and auto-inducing media.(L – protein ladder [kDa]; 1 – Cells before induction; 2 – Cells after induction; 3 – Lysate.)*

### 4.1.1.2. Purification

#### - Purification by IMAC

After the expression of TB5 in *E. coli*, the target protein was purified by immobilized metal affinity chromatography, using a nickel-nitrilotriacetic acid column. The chromatogram of the Ni-NTA protein purification (Figure 14), showed very high absorbance when eluting with 20 mM imidazole, which was probably caused by the removal of a large amount of weakly bound bacterial proteins from the column. The peak at 50 mM imidazole reached around 1000 mAU and the peak at 100 mM was almost double in size and it was suspected that the target protein was eluted at this imidazole concentration. There were no peaks at 250 mM imidazole and after regenerating the column with EDTA solution.

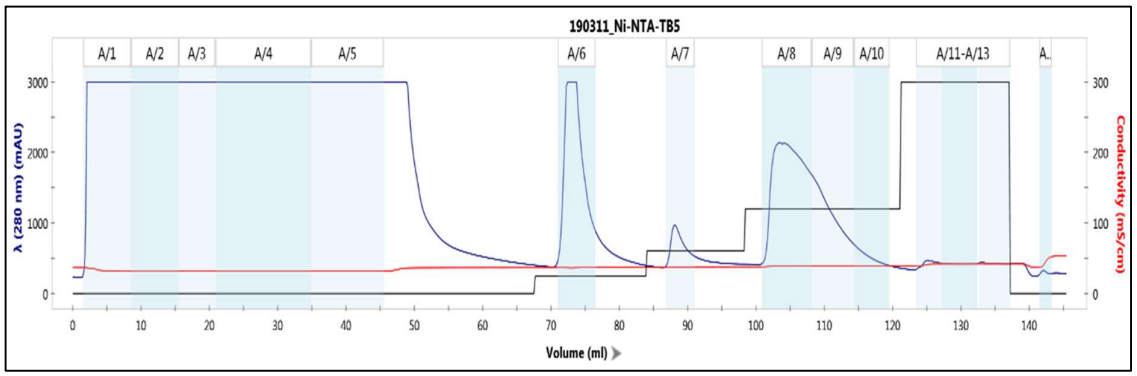



*Figure 14: Chromatogram of the purification of TB5 by IMAC (blue – absorbance at 280 nm, red – conductivity, black - % buffer B)*

Samples were taken during the fermentation, as well as after lysis and purification of the protein. They were analyzed using SDS-PAGE (Figure 15). There were strong bands at around 17 kDa in the pooled samples from fractions A8 – A11, taken during the stepwise elution with 100 mM imidazole. The highest protein concentration was found in the lane with fractions A8+A9 but there were visible impurities with higher molecular weights present. Fractions A10+A11 and A12 were purer but contained less protein. The pooled fraction A10+A11 was used for further experiments due to its purity.

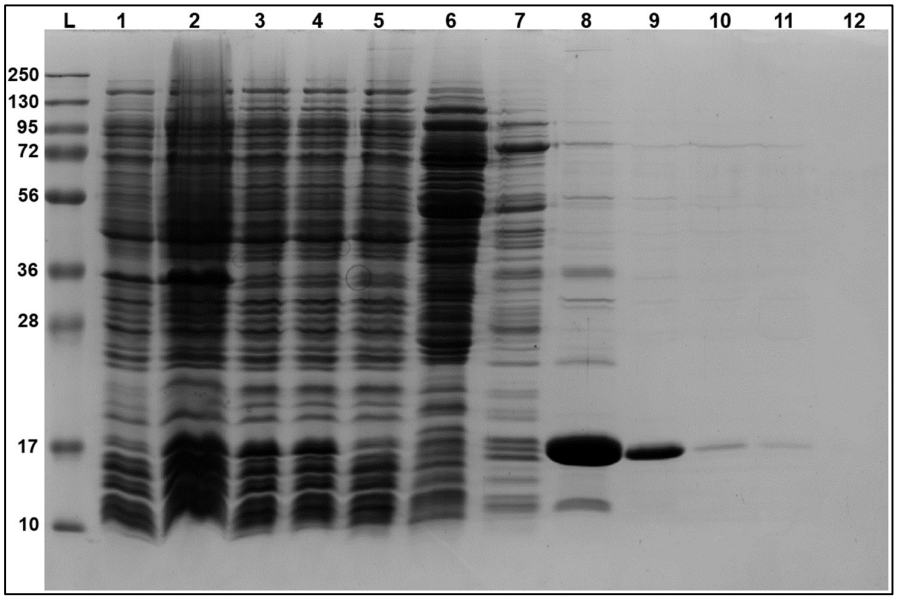



*Figure 15: CBB-stained SDS-PAGE of samples from the purification of TB5 by IMAC. ( L – protein ladder [kDa]; 1 – Cells before induction; 2 – Cells after induction; 3 – Lysate; 4 – Lysate after Biocryl treatment; 5 – Fraction A5 Flow-Through; 6 – Fraction A6 20 mM imidazole; 7 – Fraction A7 50 mM imidazole, 8 – fractions A8+A9 100 mM imidazole; 9 – fractions A10 100 mM imidazole+A11 250 mM imidazole; 10 – fraction A12 250 mM imidazole; 11 – fraction A13 250 mM imidazole; 12 – fraction A14 EDTA eluate.)*

### - Purification by ion exchange chromatography

Fractions A8 and A9 from the Ni-NTA protein purification contained the most target protein. In order to remove the remaining impurities, a purification by ion exchange chromatography was performed. Both fractions were pooled and dialyzed against IEX buffer A. Some precipitation occurred, so the protein solution was filtered through a 0.45 µm filter. The yield after filtering was around 13 mL, which was loaded on a 1.5 mL column. As shown in Figure 16, the protein started eluting at around 20% IEX buffer B and the absorbance reached its peak at around 30%. Fractions from before, during and after the peak were loaded onto an SDS-PAGE (Figure 17).

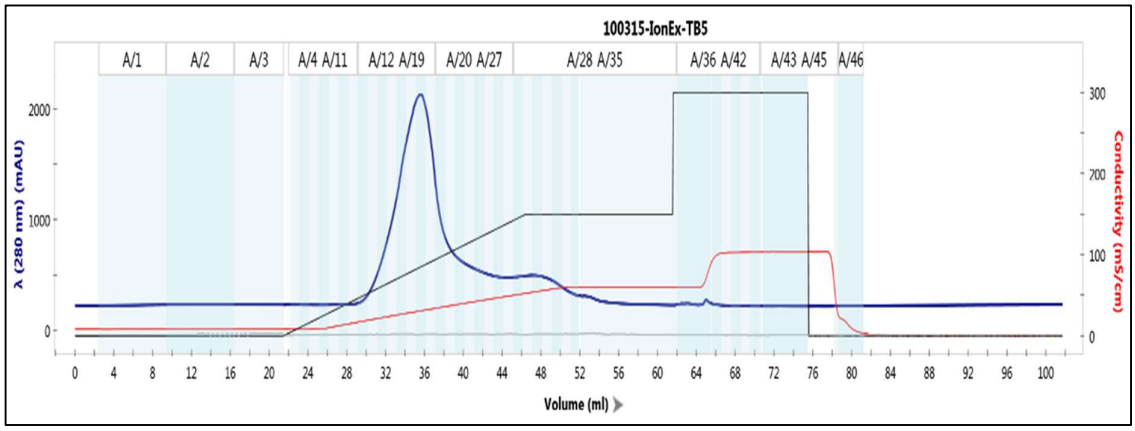



*Figure 16: Chromatogram of the purification of TB5 by ion exchange chromatography. (blue – absorbance at 280 nm; red – conductivity; black - % buffer B; gray – sample pump pressure)*

The expected band at 17 kDa was visible in multiple samples, but there were still impurities between 36 kDa and 72 kDa. The probable cause was that the target protein formed dimers or tetramers, because the concentration of the impurities increased with higher target protein concentrations. Due to this, the purification was considered done, the fractions from A15 to A20 were pooled and used for characterization.

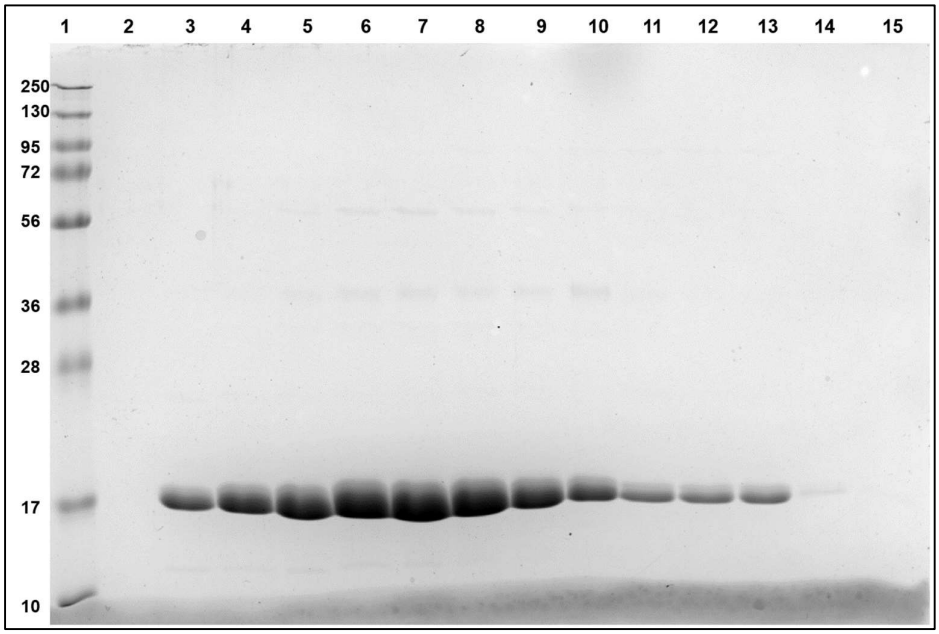



*Figure 17: CBB-stained SDS-PAGE of samples from purification of TB5 by IEX. Legend: See Table 4.*

*Table 4: Samples loaded onto the SDS-PAGE gel shown in Figure 19*

| Gel lane | Fraction | Gel lane | Fraction |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **9** | A20 |
| **2** | A3 | **10** | A21 |
| **3** | A14 | **11** | A29 |
| **4** | A15 | **12** | A30 |
| **5** | A16 | **13** | A31 |
| **6** | A17 | **14** | A36 |
| **7** | A18 | **15** | A39 |
| **8** | A19 | | |

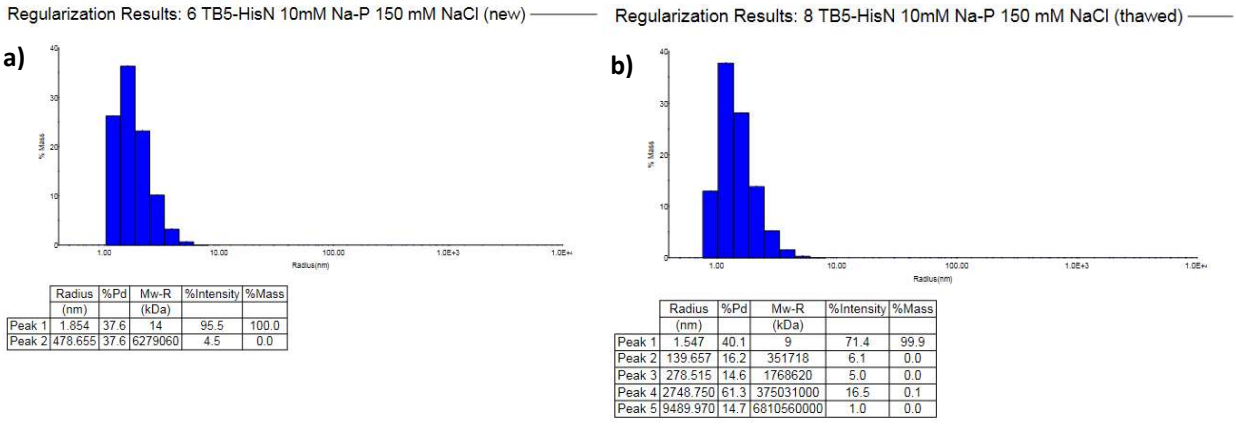
### - Dynamic light scattering and storage conditions test

At the end of the purification, a storage conditions test was done in order to determine in which buffer would the protein remain stable during storage at -80 °C. The regularization results from the DLS measurement in two different buffers before and after freezing are shown in Figure 18. The protein in 10 mM sodium phosphate, 150 mM NaCl, pH 8 showed a lower amount of aggregates than the one without salt. Peak 1, corresponding to the monomeric protein, accounted for 99.9% of the total mass and its molar mass was in the order of magnitude of the theoretical one.

The protein concentration in the pool of fractions A15 to A20 from the ion exchange chromatography was measured before and after dialysis against the two different buffers and the results were compared in Table 5. They show that TB5 is stable in all buffers. The slight fluctuations in the concentration (less than 10%) might have been caused by the increased volume and thus dilution during the dialysis. The buffer with salt was thus chosen as a storage buffer.

**The final concentration was determined by BCA assay to be 0.975 mg/mL.**

**The final yield was 2.14 mg protein/L bacterial culture.**




| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 1.854 | 37.6 | 14 | 95.5 | 100.0 |
| Peak 2 | 478.655 | 37.6 | 6279060 | 4.5 | 0.0 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 1.547 | 40.1 | 9 | 71.4 | 99.9 |
| Peak 2 | 139.657 | 16.2 | 351718 | 6.1 | 0.0 |
| Peak 3 | 278.515 | 14.6 | 1768620 | 5.0 | 0.0 |
| Peak 4 | 2748.750 | 61.3 | 375031000 | 16.5 | 0.1 |
| Peak 5 | 9489.970 | 14.7 | 6810560000 | 1.0 | 0.0 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 2.152 | 54.8 | 20 | 67.0 | 99.7 |
| Peak 2 | 137.632 | 16.5 | 339902 | 17.1 | 0.1 |
| Peak 3 | 282.350 | 14.7 | 1826130 | 6.6 | 0.0 |
| Peak 4 | 501.435 | 14.8 | 7000690 | 3.8 | 0.0 |
| Peak 5 | 2609.150 | 49.3 | 331973000 | 5.6 | 0.2 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 2.462 | 19.8 | 28 | 15.6 | 97.1 |
| Peak 2 | 10.860 | 72.0 | 893 | 68.6 | 2.6 |
| Peak 3 | 125.702 | 13.1 | 274928 | 12.2 | 0.1 |
| Peak 4 | 1845.090 | 27.2 | 147573000 | 3.6 | 0.2 |

*Figure 18: Regularization results from DLS measurement of TB5: a) in 10 mM sodium phosphate, 150 mM NaCl, pH 8 before freezing; b) as in a), after freezing; c) TB5 in 10 mM sodium phosphate buffer, pH8; d) as in c), after freezing.*

*Table 5: Concentration of pooled fractions from A15 to A20 from the IEX protein purification, measured by photometry: A) Before dialysis; b) After dialysis against 10 mM sodium phosphate buffer, pH 8; c) After dialysis against 10 mM sodium phosphate, 150 mM NaCl buffer, pH 8.*

| ***a)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | 0.52 | 2.03 | 1.16 | 1.16 |
| | 0.52 | 2.04 | 1.16 | |
| | 0.52 | 2.05 | 1.17 | |

| ***b)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | 0.52 | 1.91 | 1.08 | 1.09 |
| | 0.54 | 1.95 | 1.11 | |
| | 0.56 | 1.88 | 1.07 | |

| ***c)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | 0.53 | 1.88 | 1.07 | 1.06 |
| | 0.53 | 1.87 | 1.06 | |
| | 0.53 | 1.86 | 1.06 | |

### 4.1.1.3. Physico-chemical characterization

#### -MALDI-TOF-MS

The purified protein was analyzed by mass spectrometry in order to confirm its identity. The results of MALDI-TOF-MS are shown in Figure 19 and present double peaks for each ionization form. The data, evaluated in Table 6, shows that the determined molecular mass of the protein without methionine is almost identical to the theoretical mass. The difference between the determined and the theoretical mass of the protein with methionine is 44 Daltons. One possible explanation for this difference might be a formyl group at the N-terminus, accompanied by oxidation of the N-terminal formylmethionine (fMet) to fMet sulfoxide.

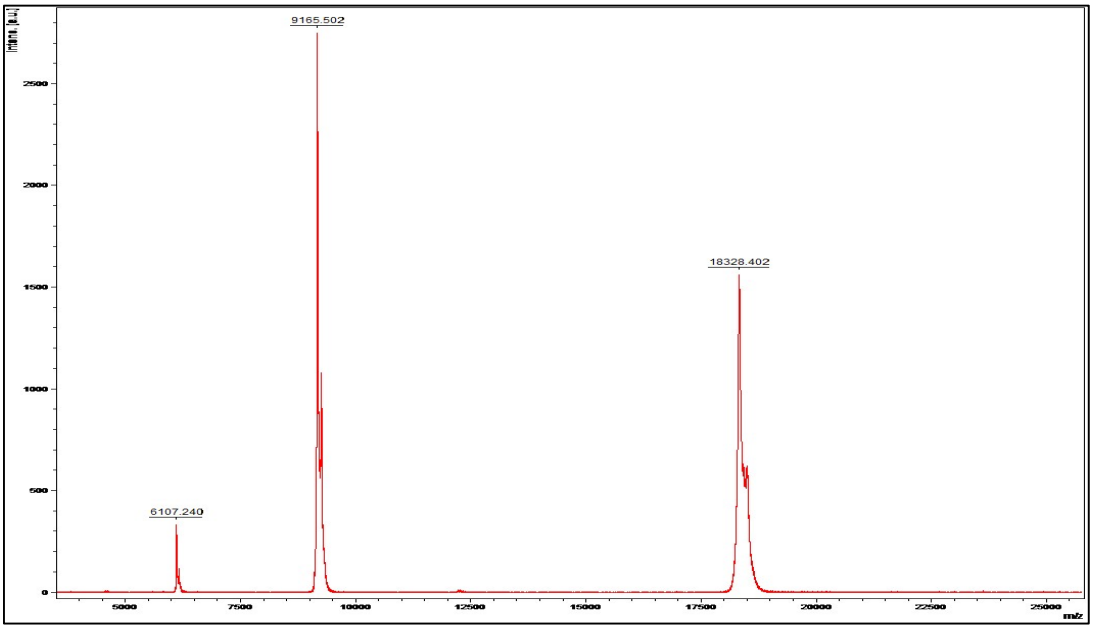



*Figure 19: MALDI-TOF-MS spectrum of purified TB5*

*Table 6: Evaluation of results of MALDI-TOF-MS of TB5.*

| **Minor peak:** | | | |
|---|---|---|---|
| **With Methionine at N-Terminus** | | | |
| **Ion** | **m/z** | **z** | **M** |
| [M+3H]3+ | 6166,3 | 3 | 18495,9 |
| [M+2H]2+ | 9253,9 | 2 | 18505,8 |
| [M+H]+ | 18504,1 | 1 | 18503,1 |
| | | **Mean** | **18501,6** |
| **Theoretical mass:** | | | **18458** |

| **Major peak:** | | | |
|---|---|---|---|
| **Without Methionine at N-Terminus** | | | |
| **Ion** | **m/z** | **z** | **M** |
| [M+3H]3+ | 6107,2 | 3 | 18318,7 |
| [M+2H]2+ | 9165,5 | 2 | 18329,0 |
| [M+H]+ | 18328,4 | 1 | 18327,4 |
| | | **Mean** | **18325,0** |
| | | | **18327** |

### - Circular dichroism spectroscopy

To confirm the correct folding of TB5, its secondary structure was compared with the secondary structure of the scaffold protein TTH in Figure 20. There is high similarity between 200 nm and 240 nm and both graphs cross the x-axis at the same positions, so it is assumed that the chimeric protein was folded correctly. The rugged line between 190 nm and 200 nm was probably caused by noise during the measurement. No protein denaturation was visible.

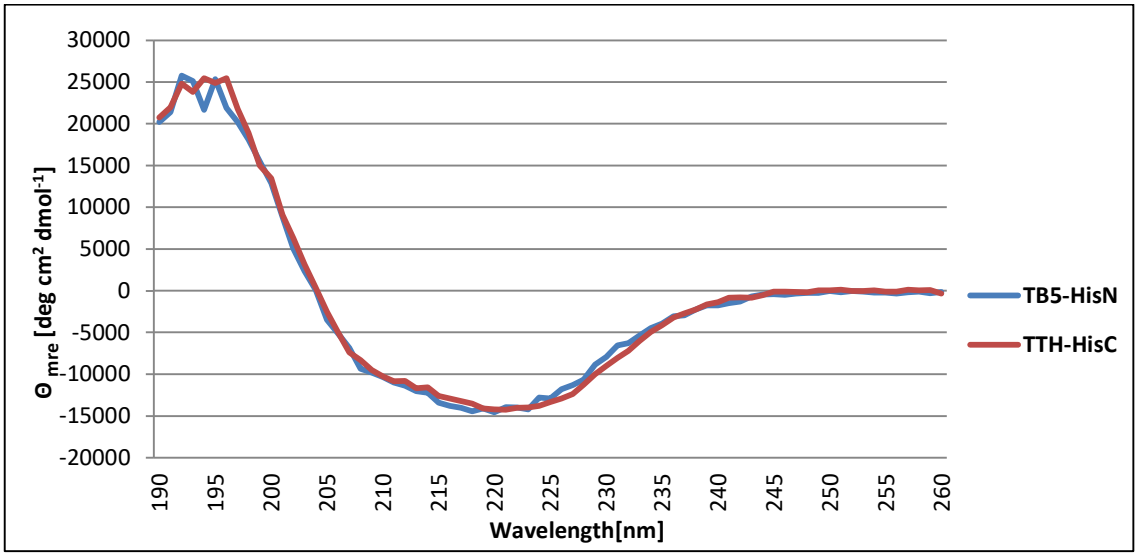



*Figure 20: CD spectra of TB5 and TTH*

### 4.1.2. TB7-HisC

#### 4.1.2.1. Optimization of the expression

##### - Optimization of the culture medium

The first step of the preparation for the purification of TB7-HisC was to determine the most suitable medium. The media test was performed with LB, TB, SB, Rich and SOC (Figure 21). TB7-HisC showed good protein expression as visible by the bands at around 17 kDa in the lanes showing cells after induction on the SDS-PAGE gels. The bacterial cells were lysed by freezing in liquid nitrogen and milling in a Retch homogenizer. It appears that the lysis was incomplete, because the amount of protein was very low in the lysed column. Another possibility is that the protein was insoluble due to aggregation, which might have been caused by its high concentration in the cells but then the amounts of most *E. coli* protein would have been comparable with the amounts in the total cell extract.

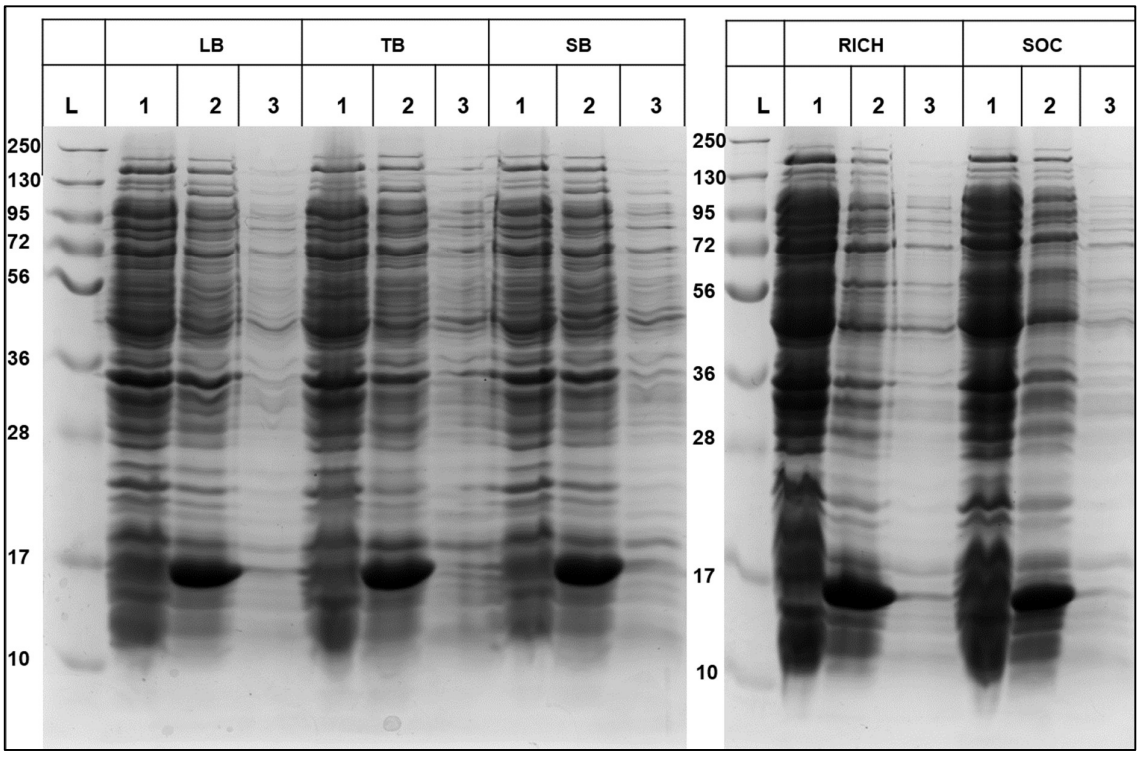



*Figure 21: CBB-stained SDS-PAGE gels of the TB7 media test with samples from bacterial culture in LB, TB SB, RICH and SOC media. L – protein ladder; 1 – cells before induction; 2 – cells after induction; 3 – lysate.*

### - Optimization of expression temperature and duration

In this test, we compared different durations and different temperatures of expression in order to find the most suitable conditions to produce soluble TB7. All samples were loaded onto an SDS-PAGE gel (Figure 22). TB7 was present in all samples after induction. The highest amount of supposedly TB7-HisC at around 17 kDa was present in the sample incubated at 30 °C for 4 hours. The protein was still insoluble, because the band at around 17 kDa was not present in the lysed samples. For further experiments, 30 °C were chosen as expression temperature and the expression was done overnight.

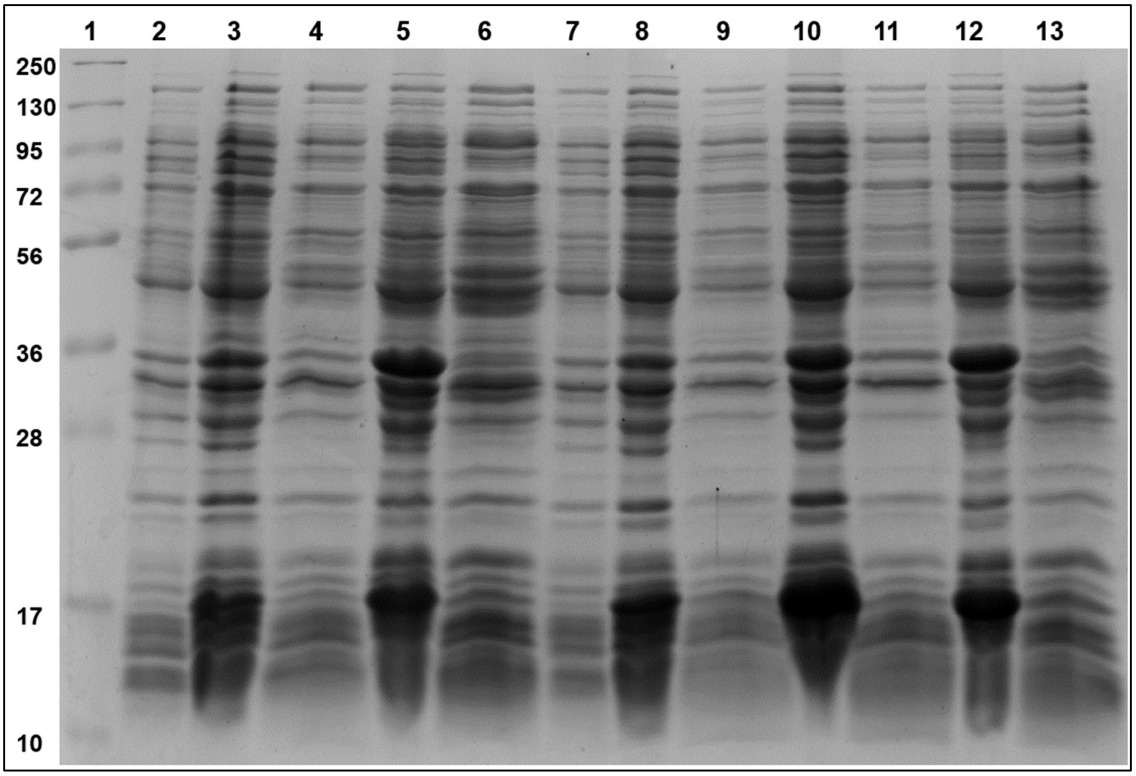



*Figure 22: CBB-stained SDS-PAGE gel of testing expression conditions of TB7: samples of expressing bacterial cultures in RICH medium at 25 °C and 30 °C, taken at different time points. (Legend: see Table 7).*

*Table 7: Samples loaded onto the SDS-PAGE gel shown in Figure 22:*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **9** | 30 °C, before induction |
| **2** | 25 °C, before induction | **10** | 30 °C, 3h after induction |
| **3** | 25 °C, 3h after induction | **11** | 30 °C, 3h after induction - lysate |
| **4** | 25 °C, 3h after induction - lysate | **12** | 30 °C, overnight after induction |
| **5** | 25 °C, overnight after induction | **13** | 30 °C, overnight after induction - lysate |
| **6** | 25 °C, overnight after induction - lysate | | |

### 4.1.2.2. Purification

#### - Purification by IMAC

The lysate of the bacterial culture was loaded on a Ni-NTA column. The chromatogram, displayed in Figure 23, showed a high protein amount being eluted at 20 mM imidazole. It was supposed that these were proteins with low affinity for the column. There were also small peaks after elution with higher imidazole concentrations and a high peak after stripping with an EDTA solution. It is possible that TB7 was eluting slowly and all protein was removed from the column before adding the EDTA solution. The samples, taken from different stages of the purification, were loaded onto an SDS-PAGE gel, shown in Figure 24. The lane containing the 250 mM imidazole sample, showed a band at around 17 kDa, which could be a small amount of the target protein. The EDTA fraction contained only impurities with high molecular weights.

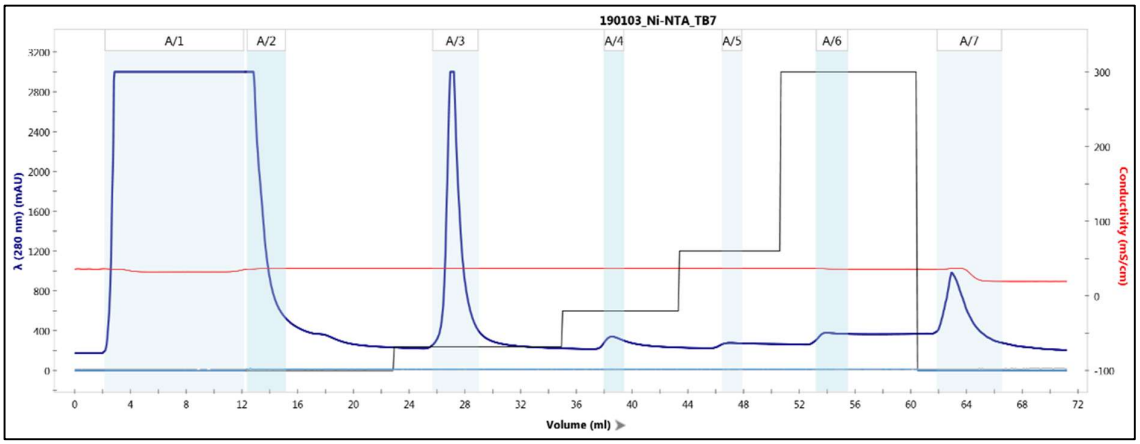



*Figure 23: Chromatogram of the purification of TB7 by IMAC (blue – absorbance at 280 nm; red – conductivity; black - % buffer B; light blue – system pressure; gray – sample pump pressure)*

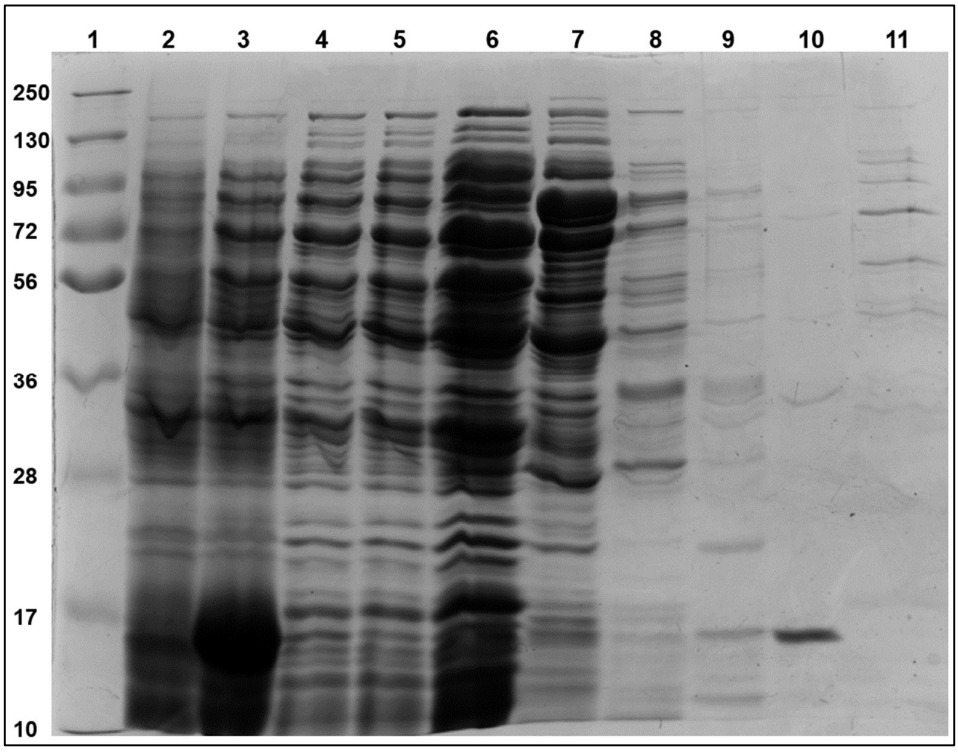



*Figure 24: CBB-stained SDS-PAGE gel of the samples from the native IMAC purification of TB7. (Legend: see Table 8)*

*Table 8: Samples loaded onto the SDS-PAGE gel shown in Figure 24.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **7** | 20 mM imidazole |
| **2** | Cells before induction | **8** | 50 mM imidazole |
| **3** | Cells after induction | **9** | 100 mM imidazole |
| **4** | Lysate | **10** | 250 mM imidazole |
| **5** | Lysate after biocryl treatment | **11** | EDTA eluate |
| **6** | Flow-through | | |

### - Western blot

A western blot was done in order to confirm the identity of the band in the 250 mM imidazole eluate - the same SDS-PAGE gel from was used for immunoblotting (Figure 25). The 17 kDa-band was bound by the anti-penta His antibody and thus confirmed to be TB7. It is also visible that a high amount of protein was expressed after induction, which was then lost due to incomplete lysis or insolubility.

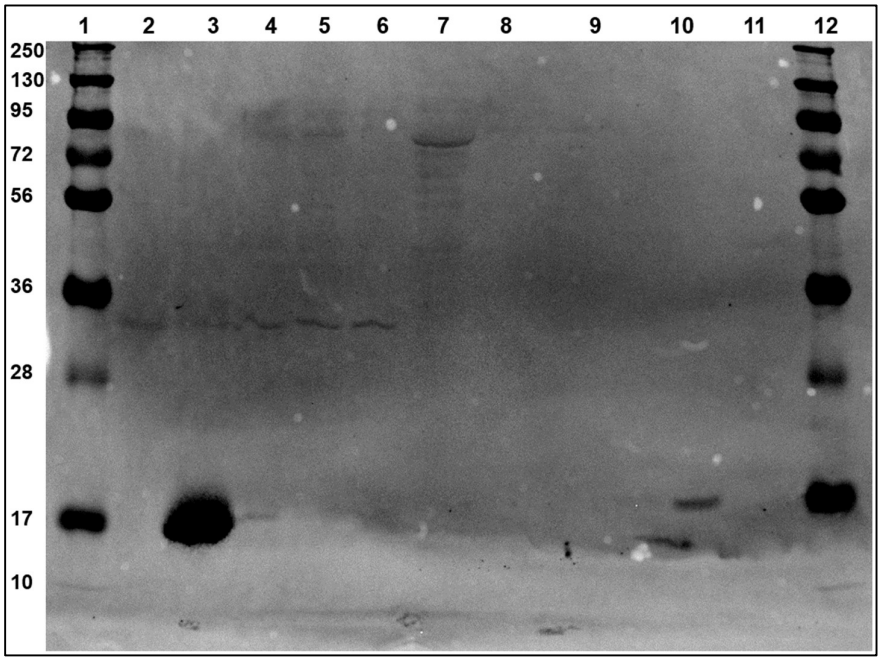



*Figure 25: Western blot of samples from purification of TB7 by IMAC (Legend: See Table 9).*

*Table 9: Samples loaded onto the SDS-PAGE for the Western blot shown in Figure 25.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **7** | 20 mM imidazole |
| **2** | Cells before induction | **8** | 50 mM imidazole |
| **3** | Cells after induction | **9** | 100 mM imidazole |
| **4** | Lysate | **10** | 250 mM imidazole |
| **5** | Lysate after biocryl treatment | **11** | EDTA eluate |
| **6** | Flow-through | **12** | Protein ladder [kDa] |

### - Purification by ion exchange chromatography

In order to try to remove the impurities, an ion exchange chromatography was performed. The fraction containing TB7 was dialyzed and then loaded onto an ion exchange column. As seen in Figure 26, the signal during the whole purification was very low with a peak of around 250 mAU at 100% IEX buffer B. Samples from some fractions before the peak as well as all fractions during the peak were loaded onto an SDS-PAGE gel. After staining, no protein bands were visible. This was caused by the low concentration of TB7, which was then further diluted by spreading into different fractions. The gel was repeated in order to exclude a mistake during the gel electrophoresis, but it provided the same result – all lanes were empty.

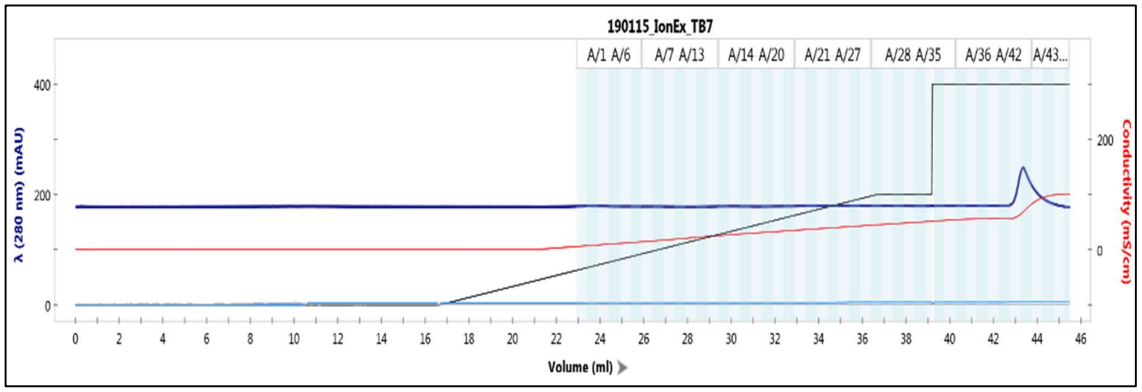



*Figure 26: Chromatogram of the purification of TB7 by IEX. (dark blue – absorbance at 280 nm; red – conductivity; black - % buffer B; blue – system pressure; gray – sample pump pressure)*

### - Purification under denaturing conditions

The last attempt to purify the large amount of TB7, which is being expressed, was done under denaturing conditions. The fermentation and the lysis were done under the same conditions as for the native purification. Before the purification, buffers with high concentrations of urea were used to denature the insoluble target protein, found in the pellet after lysis and centrifugation. The chromatogram of this run is shown in Figure 27. After each buffer change to a lower pH, a peak was visible. The highest signal was reached with buffer E with pH 4.5, where most of protein was eluted. Samples from different fractions were taken and loaded on an SDS-PAGE gel (Figure 28).

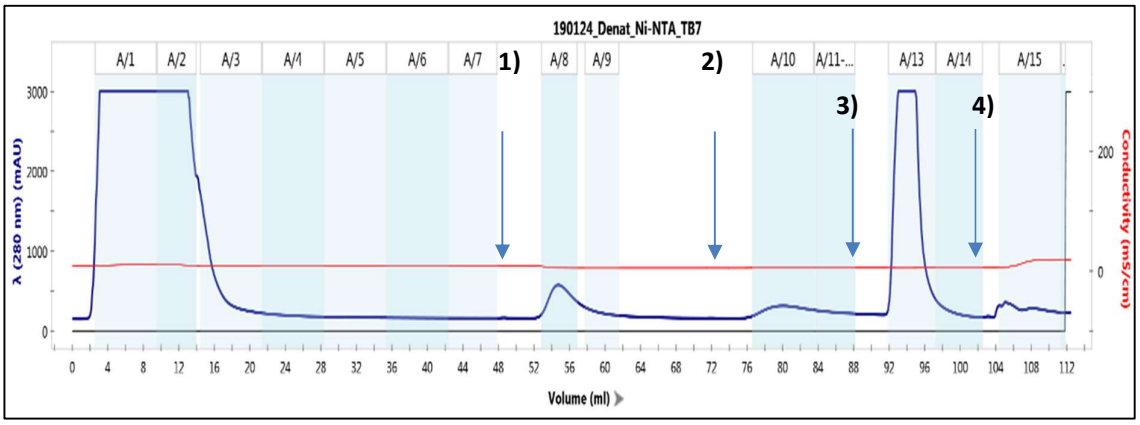



*Figure 27: Chromatogram of the purification of TB7 under denaturing conditions by IMAC. The approximate time points of buffer change are marked with arrows: 1) elution with buffer C, pH 6.3; 2) elution with buffer D, pH 5.9; 3) elution with buffer E, pH 4.5; 4) elution with 0.1 M EDTA. (dark blue – absorbance at 280 nm; red – conductivity.)*

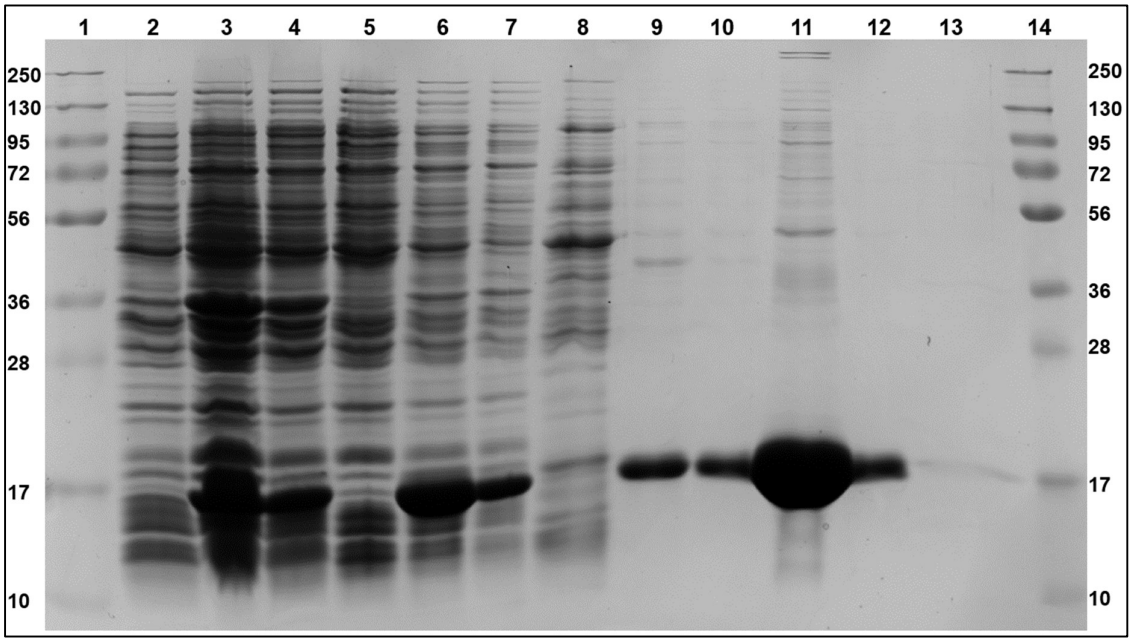



*Figure 28: CBB-stained SDS-PAGE gel of samples from purification of TB7 under denaturing conditions by IMAC (Legend: see Table 10)*

*Table 10: Samples loaded onto the SDS-PAGE gel shown in Figure 28.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **8** | A8 washing of column |
| **2** | Cells before induction | **9** | A10 pH 5.9 |
| **3** | Cells after induction | **10** | A11 pH 5.9 |
| **4** | Lysate - pellet | **11** | A13 pH 4.5 |
| **5** | Lysate - supernatant | **12** | A14 pH 4.5 |
| **6** | Supernatant after adding urea to the lysis pellet | **13** | A15 EDTA eluate |
| **7** | A1 flow-through | **14** | Protein ladder [kDa] |

There was a visible difference between the target protein content in the supernatant after native and denaturing lysis. There was a strong band at around 17 kDa, which corresponds to the molecular weight of TB7. It was even visible in the flow-through, as not all of the protein was bound in the column due to the large amount. Small amounts were eluted with buffer C and buffer D, but the largest protein content was found in the fractions with buffer E, especially A13. This fraction also contained the highest concentration of impurities. Fractions A10, A11, A13 and A14 were pooled and used for re-folding. They were loaded on an Ni-NTA column and the renaturing was done using a linear gradient from 6 M to 0 M urea for 90 minutes. At the end, the protein was eluted using buffer B for native purification. The overall signal during refolding, including elution, was very low. The chromatogram was lost due to software problems. The samples from all stages of refolding were examined by an SDS-PAGE (Figure 29).

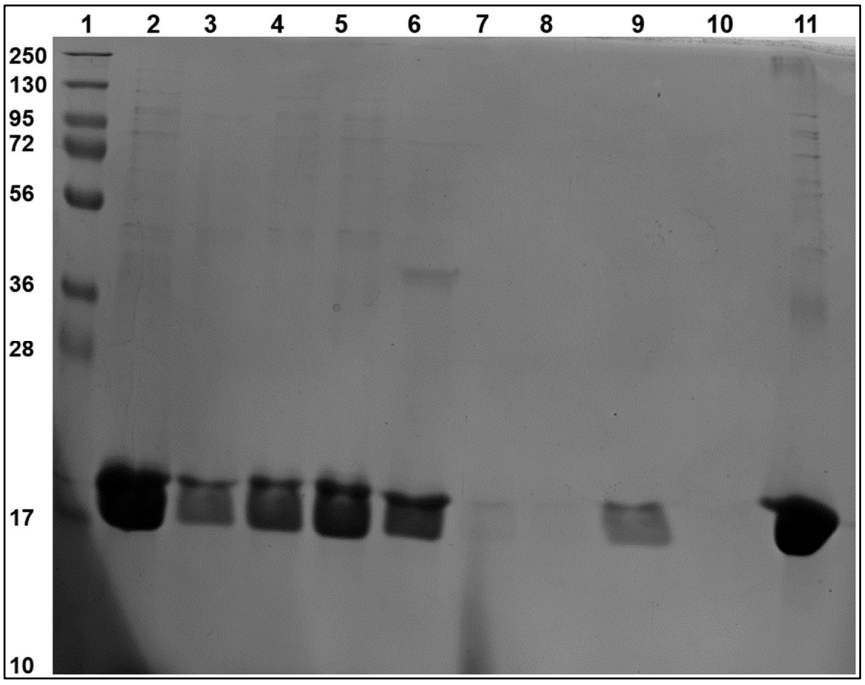



*Figure 29: CBB-stained SDS-PAGE gel of samples from protein refolding after purification of TB7 under denaturing conditions. (Legend: see Table 11)*

*Table 11: Samples loaded onto the SDS-PAGE gel shown in Figure 29. (RE – refolding)*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **7** | RE6 Elution |
| **2** | Pooled fractions before refolding | **8** | RE7 Elution |
| **3** | RE1 Flow-through | **9** | RE8 Elution |
| **4** | RE2 Flow-through | **10** | RE9 EDTA |
| **5** | RE3 Flow-through | **11** | Resin |
| **6** | RE5 Elution | | |

A lot of protein was not bound by the column and was washed out during loading. Moreover, there was a high amount of target protein that was not eluted and stayed in the resin. The fractions RE1, RE2 and RE3 from the flow-through as well as RE5 from the elution were pooled and loaded onto a freshly prepared Ni-NTA column in order to repeat the refolding.

The chromatogram of the repeated refolding of TB7 is displayed in Figure 30. The absorbance at 280 nm showed a very small peak during the loading of the sample and stayed stable during the whole gradient. There was also a small peak during elution (only around 400 mAU), which indicated a low amount of eluted protein. All samples were loaded onto an SDS-PAGE gel (Figure 31).

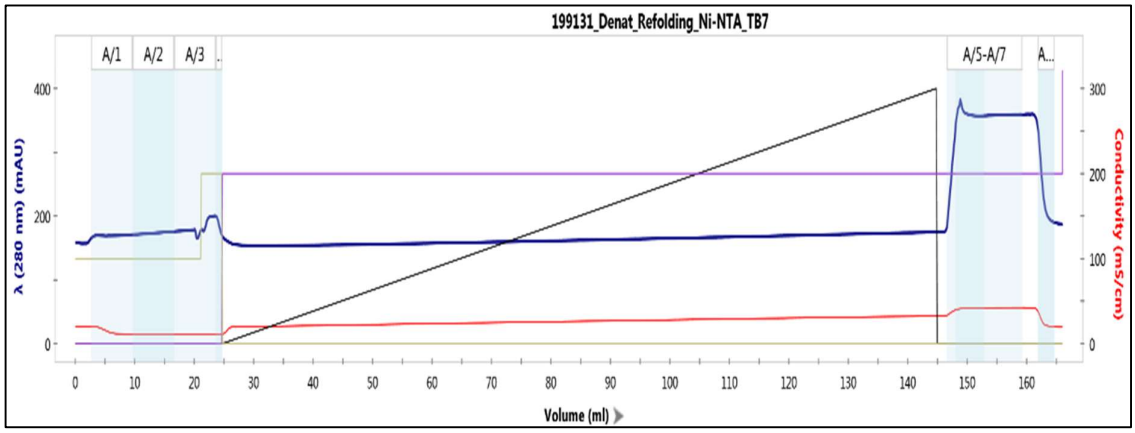



*Figure 30: Chromatogram of repeated refolding of TB7 after purification under denaturing conditions ( blue – absorbance at 280 nm; red – conductivity; black - % buffer B; purple – flow rate; green– sample pump pressure)*

The SDS-PAGE showed that there was a band around 17 kDa in the sample that was loaded onto the column. The protein was then diluted into all fractions and could not be purified further due to the large volume of the protein-containing solution.

All the purified, but not correctly folded protein was lost. The experiments with TB7-HisC were postponed until a new approach for hardly soluble proteins was designed. These experiments are not in the scope of this master's thesis.

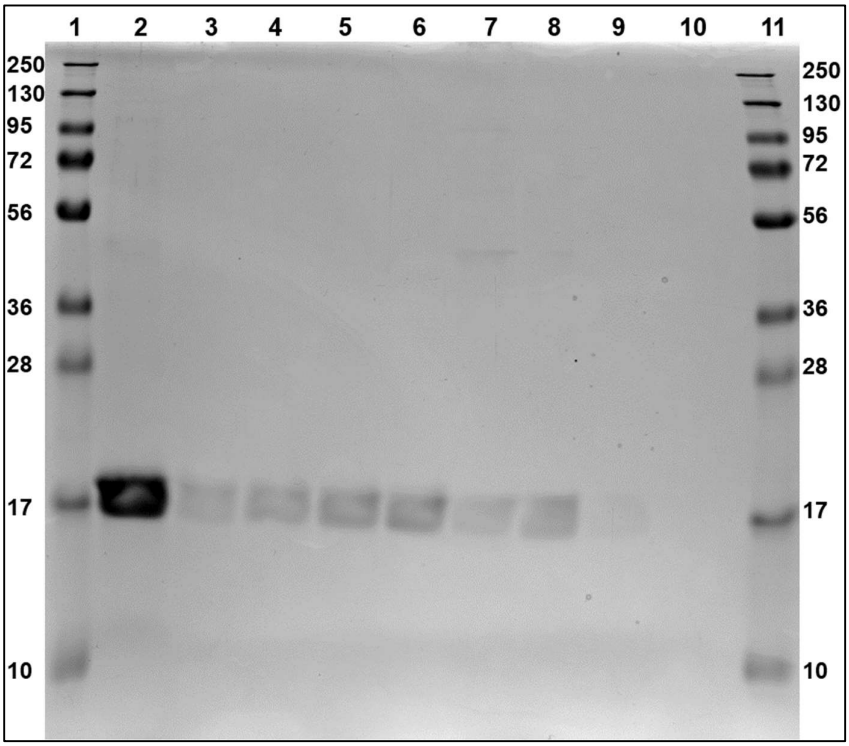



*Figure 31: CBB-stained SDS-PAGE gel of samples from repeated protein refolding after purification of TB7 under denaturing conditions. (Legend: see Table 12)*

*Table 12: Samples loaded onto the SDS-PAGE gel shown in Figure 31.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **7** | A5 Elution |
| **2** | Before loading on column for refolding | **8** | A6 Elution |
| **3** | A1 Flow-through | **9** | A7 Elution |
| **4** | A2 Flow-through | **10** | A8 EDTA |
| **5** | A3 Flow-through | **11** | Protein ladder [kDa] |
| **6** | A4 Flow-through | | |

### 4.1.3. TB8-HisC

#### 4.1.3.1. Optimization of the expression

**- Optimization of the culture medium**

A media test was performed in order to determine the most suitable medium for the expression of TB8. The mediah RICH, SB, SOC, Auto-inducing medium and LB were tested (Figure 32). The results show that TB8-HisC did not have high expression levels after induction – a band around 17 kDa was visible, but it was relatively faint and not present in the lysate samples. This was probably caused low solubility of the protein.

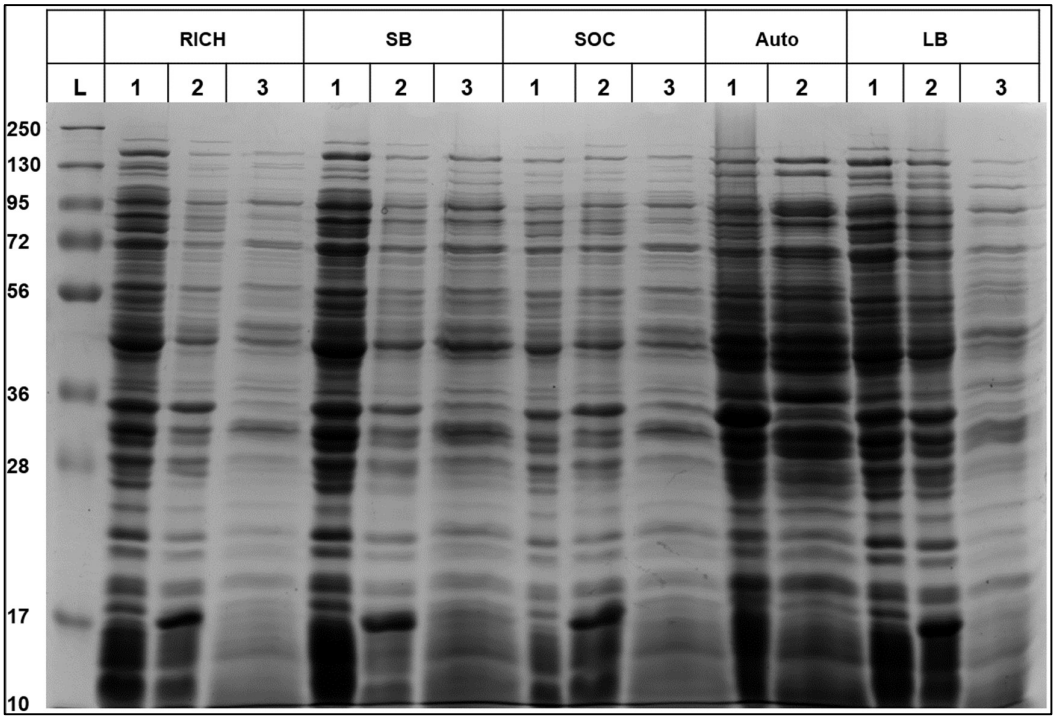



*Figure 32: CBB-stained SDS-PAGE gel of the TB8 media test with samples from bacterial culture in RICH, SB, SOC, auto-inducing and LB media. (L – protein ladder [kDa]; 1 – cells before induction; 2 – cells after induction; 3 – lysate)*

### - Optimization of the expression temperature and duration

In order to optimize the expression, different expression temperatures and times were tested in LB medium. The SDS-PAGE gel in Figure 33 showed that the expression levels were generally low. The highest amount of protein was obtained after expression at 25 °C overnight. There were no bands around 17 kDa in the lysed samples, which again suggests that the protein was insoluble as the lysis worked properly.

It was decided to run the fermentation of TB8 in LB at 25 °C.

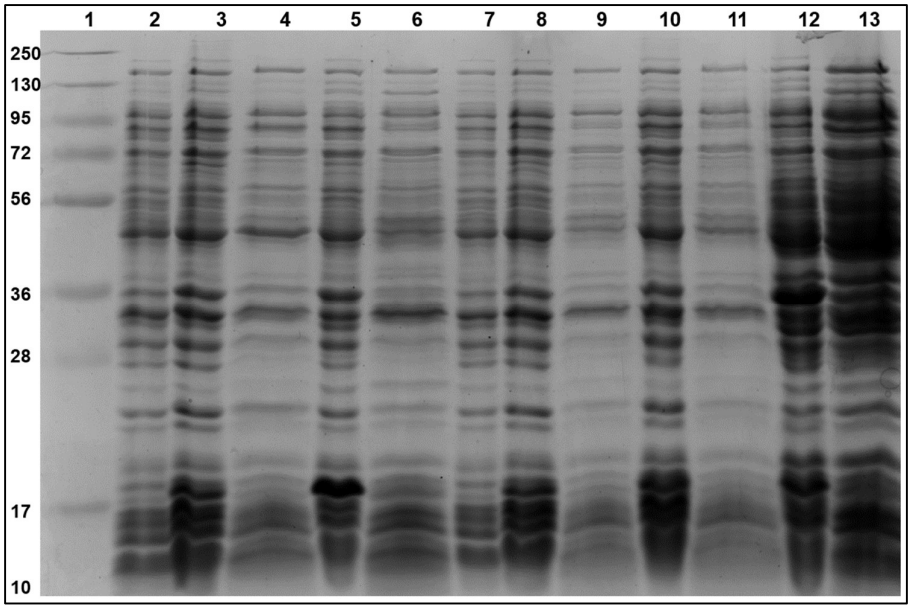



*Figure 33: CBB-stained SDS-PAGE of time interval test: samples of TB8 cell culture at different temperatures and timepoints. (Legend: see Table 13),*

*Table 13: Samples loaded onto the SDS-PAGE gel shown in Figure 33.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **9** | 30 °C, before induction |
| **2** | 25 °C, before induction | **10** | 30 °C, 3h after induction |
| **3** | 25 °C, 3h after induction | **11** | 30 °C, 3h after induction - lysate |
| **4** | 25 °C, 3h after induction - lysate | **12** | 30 °C, overnight after induction |
| **5** | 25 °C, overnight after induction | **13** | 30 °C, overnight after induction - lysate |
| **6** | 25 °C, overnight after induction - lysate | | |

### 4.1.3.2. Purification

#### - Purification by IMAC

TB8 was purified using a Ni-NTA column. The chromatogram of the purification, pictured in Figure 34, showed a peak after loading the column, but before elution with the lowest imidazole concentration. This was caused by an equipment problem – the flow stopped for a short time and due to the longer dwell time of the wash buffer in the column, a large amount of proteins was eluted at once. There were small peaks at each imidazole concentration, but the highest of them - with 250 mM imidazole – reached just below 2000 mAU for a very short time. Hence, it was suspected that the amount of purified target protein was low.

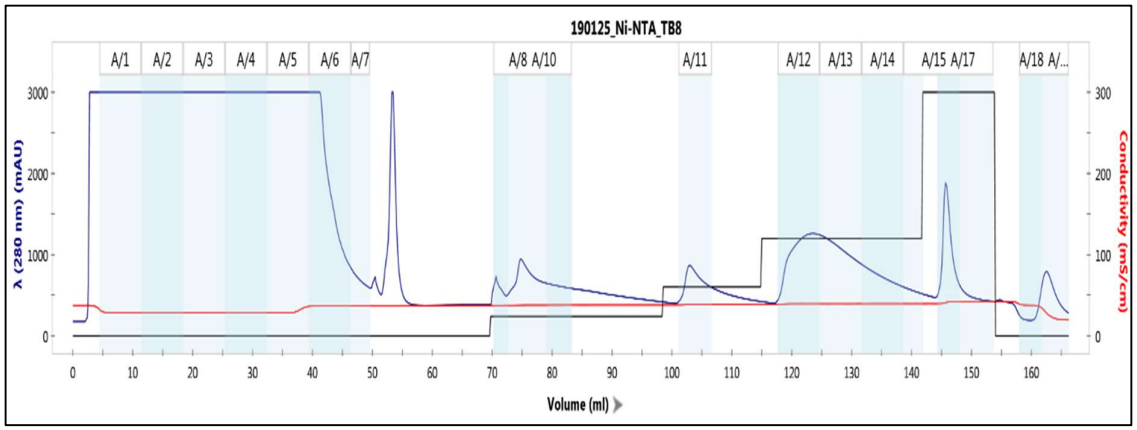



*Figure 34: Chromatogram of the purification of TB8 by IMAC. (blue – absorbance at 280 nm; red – conductivity; black - % buffer B).*

Samples were taken from each phase of the purification and analyzed on an SDS-PAGE gel, displayed in Figure 35. It showed that TB8 started eluting at 50 mM imidazole and was also present in the 100 mM and the 250 mM fractions. The 50 mM and the 250 mM fractions contained a lot of impurities, seen by the various bands present in the lanes. The 100 mM fraction (A14) was the purest as there were only very faint bands in the higher molecular mass range and was therefore used for further experiments.

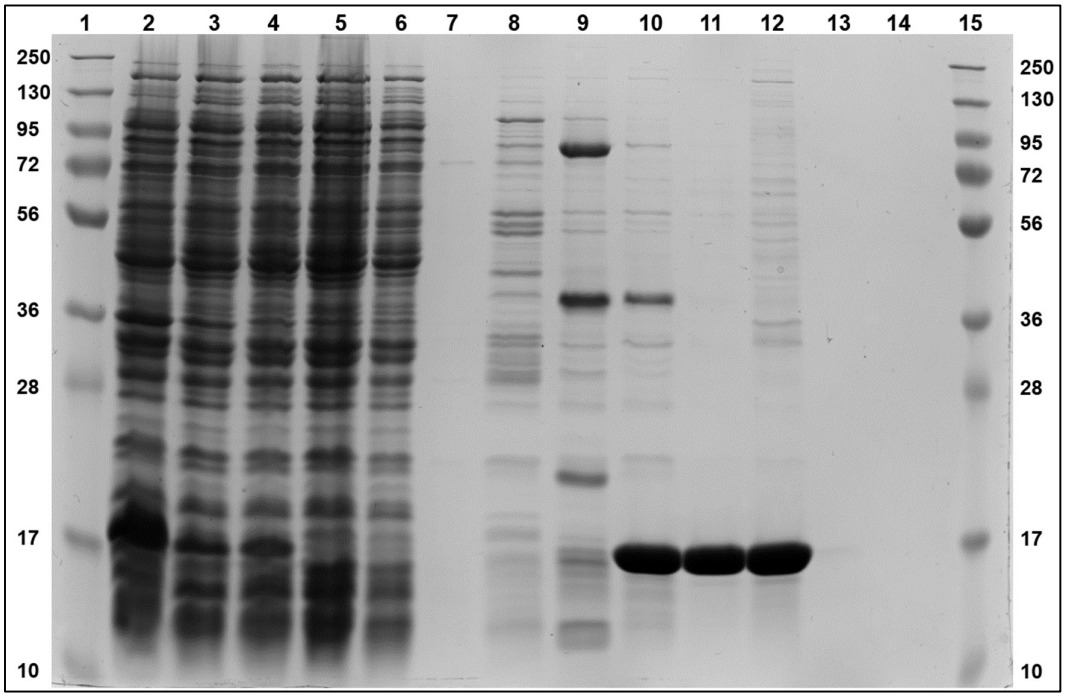



*Figure 35: CBB-stained SDS-PAGE of samples from purification of TB8 by IMAC. (Legend: see Table 15)*

*Table 14: Samples loaded onto the SDS-PAGE gel shown in Figure 36.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | **9** | A11 50 mM imidazole |
| **2** | Cells after induction | **10** | A12 50 mM Imidazole |
| **3** | Lysate | **11** | A14 100 mM imidazole |
| **4** | Lysate after biocryl treatment | **12** | A16 250 mM imidazole |
| **5** | A1 flow-through | **13** | A18 EDTA |
| **6** | A5 flow-through | **14** | A19 EDTA |
| **7** | A8 flow-through | **15** | Protein ladder [kDa] |
| **8** | A9 20 mM imidazole | | |

### - Dynamic light scattering, storage conditions test and preparation for analysis of circular dichroism

The purified protein was dialysed against 10 mM sodium phosphate, 100 mM NaF buffer for the circular dichroism analysis in order to keep it stable in a solution without NaCl. The concentration was measured, and a freeze-thaw test in 10 mM sodium phosphate, 150 mM NaCl buffer was also completed in order to determine the stability of the protein. The results are shown in Table 15. The concentration did not change after freezing and thawing and the protein was deemed stable for storage at -80 °C.

*Table 15: Measurement of concentration of TB8 by photometry: a) in 10 mM sodium phosphate, 100 mM NaF buffer for circular dichroism; b) in 10 mM sodium phosphate, 150 mM NaCl buffer after freezing and thawing.*

| ***a)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | 0.68 | 0.34 | 0.22 | 0.22 |
| | 0.60 | 0.36 | 0.23 | |
| | 0.62 | 0.34 | 0.22 | |

| ***b)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | 0.54 | 0.33 | 0.21 | 0.23 |
| | 0.58 | 0.37 | 0.24 | |
| | 0.65 | 0.37 | 0.24 | |

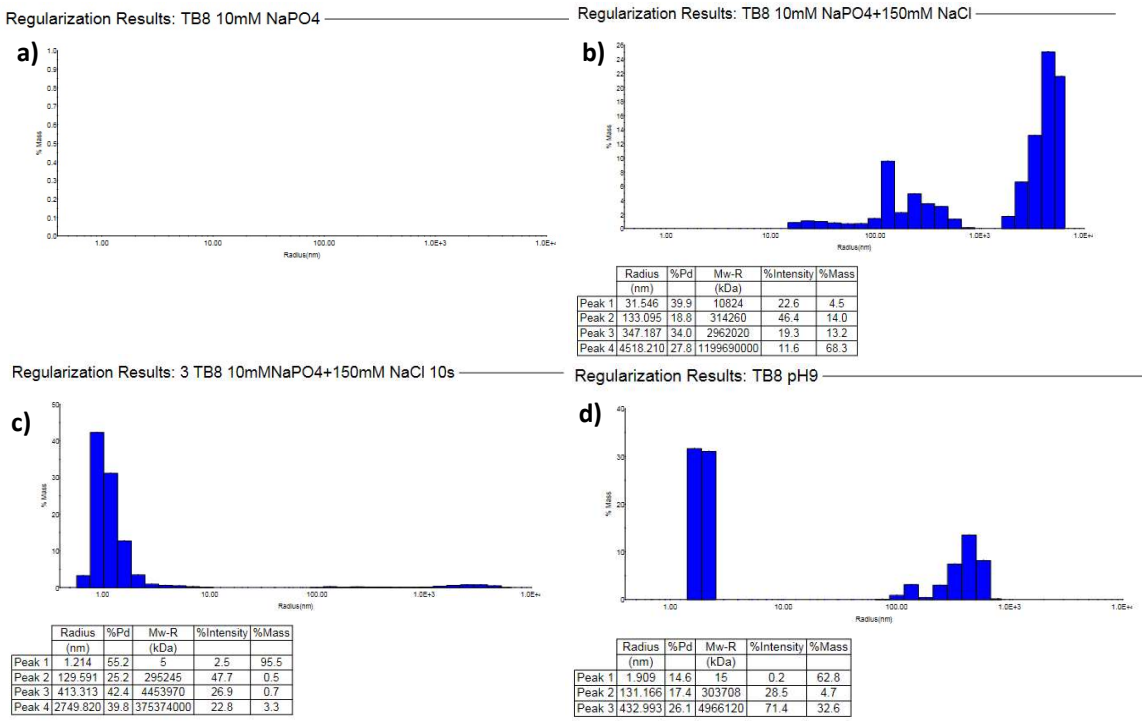
After fraction A14 was dialysed O/N against 10 mM sodium phosphate buffer without NaCl in order to remove the high concentration of imidazole and salt, which would affect further experiments, the solution turned milky. It was centrifuged and the supernatant was used for further experiments.

In order to find under which conditions would the protein be stable, dynamic light scattering of the protein in different buffers was measured. The results are shown in Figure 36. In 36a), TB8 in 10 mM sodium phosphate buffer with pH 7 showed an empty measurement - the sample has probably precipitated on the dialysis membrane. TB8 in 10 mM sodium phosphate buffer with 150 mM NaCl, pH 7, pictured in 36b), showed aggregation of the protein as the peaks were in the range from 133 nm to 4518 nm. The measurement of TB8 in 10 mM sodium phosphate buffer with 150 mM NaCl, pH 7 was repeated with 10 seconds acquisition time (see Figure 36c)), which yielded better results. Some protein aggregates with a very large molar mass were still present. The measurement of TB8 in 10 mM sodium carbonate buffer, pH9 is pictured in Figure 36d) and showed that around 63% of the mass comprised particles with ~15 kDa, which is approximately the size of the chimeric proteins. Partial aggregation of the protein was visible as a second peak at around 100 nm.

10 mM sodium phosphate buffer with 150 mM NaCl, pH 7 was chosen as a storage buffer.

**The final concentration was determined by BCA assay to be 0.22 mg/mL.**

**The final yield was 0.86 mg protein/L bacterial culture.**




| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 31.546 | 39.9 | 10824 | 22.6 | 4.5 |
| Peak 2 | 133.095 | 18.8 | 314260 | 46.4 | 14.0 |
| Peak 3 | 347.187 | 34.0 | 2962020 | 19.3 | 13.2 |
| Peak 4 | 4518.210 | 27.8 | 1199690000 | 11.6 | 68.3 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 1.214 | 55.2 | 5 | 2.5 | 95.5 |
| Peak 2 | 129.591 | 25.2 | 295245 | 47.7 | 0.5 |
| Peak 3 | 413.313 | 42.4 | 4453970 | 26.9 | 0.7 |
| Peak 4 | 2749.820 | 39.8 | 375374000 | 22.8 | 3.3 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 1.909 | 14.6 | 15 | 0.2 | 62.8 |
| Peak 2 | 131.166 | 17.4 | 303708 | 28.5 | 4.7 |
| Peak 3 | 432.993 | 26.1 | 4966120 | 71.4 | 32.6 |

*Figure 36: Results of dynamic light scattering measurement of purified TB8 in:a) 10 mM sodium phosphate buffer, pH 7; b) 10 mM sodium phosphate, 150 mM NaCl buffer, pH7; c) repeated as in b) with longer measuring time of 10 s; d) in 10 mM sodium carbonate buffer, pH9.*

### 4.1.3.3. Physico-chemical characterization

#### - MALDI-TOF-MS

MALDI-TOF mass spectrometry was performed to confirm the identity of the purified protein. The results, plotted in Figure 37, showed four single peaks. The molecules of TB8 show 4 different ionization forms – from single to quadruple ionizations. The mean of the molar mass of the four forms (Table 16) was almost the same as the theoretical molecular mass of the protein with the N-terminal Methionine cleaved off. The identity of the protein was therefore confirmed.

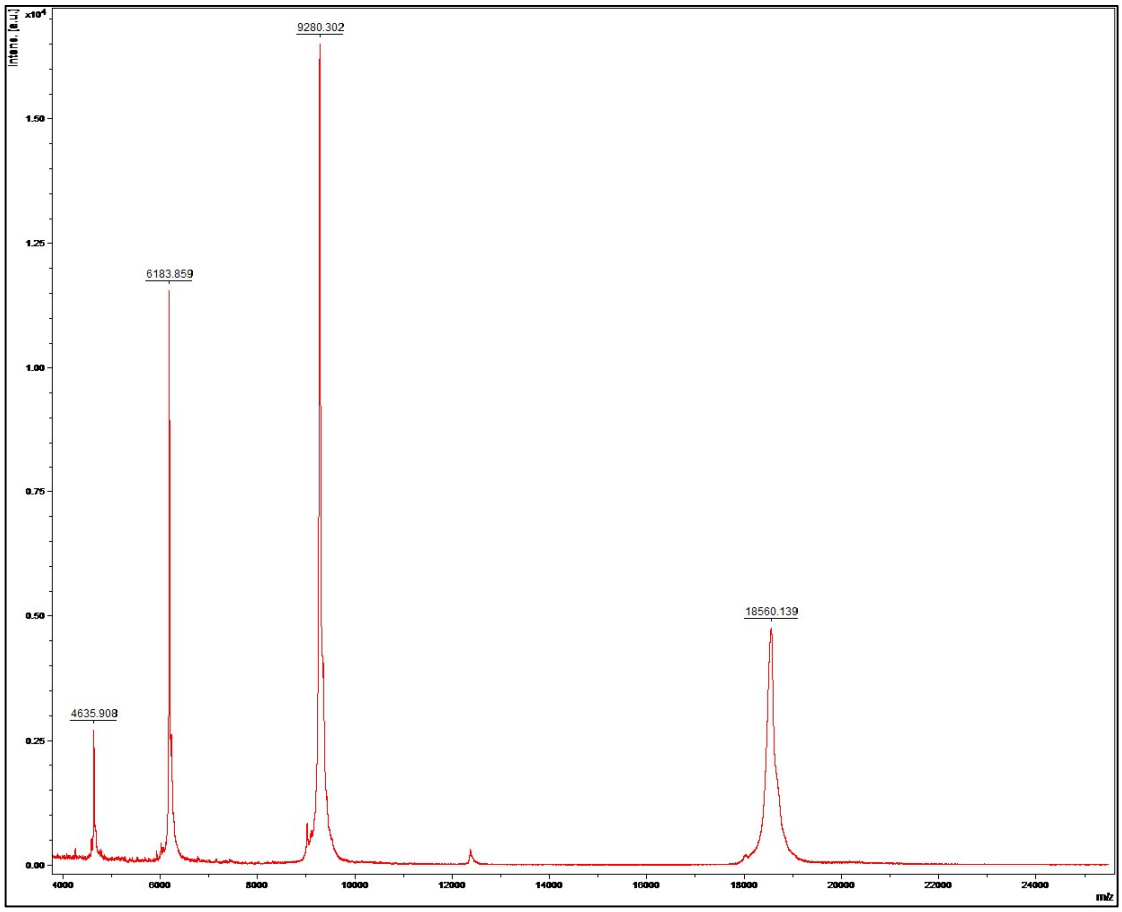



*Figure 37: MALDI-TOF-MS spectrum of purified TB8*

*Table 16: Evaluation of results of MALDI-TOF-MS of TB8.*

| Ion | m/z | z | M |
|---|---|---|---|
| [M+4H]4+ | 4635,9 | 4 | 18539,6 |
| [M+3H]3+ | 6183,9 | 3 | 18548,6 |
| [M+2H]2+ | 9280,3 | 2 | 18558,6 |
| [M+H]+ | 18560,1 | 1 | 18559,1 |
| | | **Mean** | **18551,5** |
| **Theoretical mass: 18555** | | | |

### - Circular dichroism spectroscopy

The CD spectrum of TB8 was measured at 0.22 mg/mL in 10 mM sodium phosphate, 100 mM NaF in order to confirm the correct folding. It is shown in Figure 38 and is compared with the spectrum of the scaffold protein TTH. Both curves are almost identical between 200 and 260 nm. They cross the x-axis at the same wavelength. The differences in the range between 190 and 200 nm are probably caused by noise in the measurement. These results indicate that the protein is not denatured and is correctly folded.

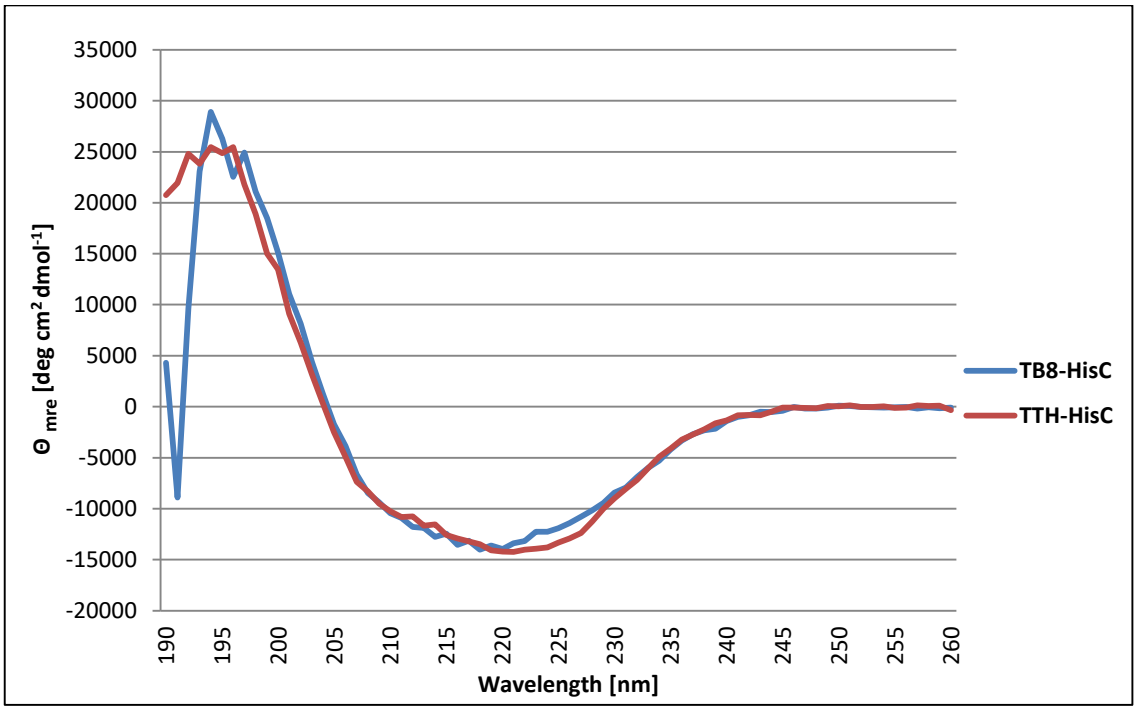



*Figure 38: CD spectra of TB8 and TTH*

## 4.1.4. TB14-HisC

### 4.1.4.1. Optimization of the expression

#### - Optimization of the culture medium

The media test for TB14 was performed with SB, LB, RICH, SOC and autoinducing medium. The used gel, pictured in Figure 39, was poured 2 hours before use and turned out blurry in the bottom half. There was a large band at and below 17 kDa, indicating good protein expression. The difference between the amounts of protein in the cells after induction and the lysates showed that TB14 was not completely soluble, but there was still a large amount of

target protein found in the lysates. The results in RICH media were deemed to be the best and it was used for the fermentation.

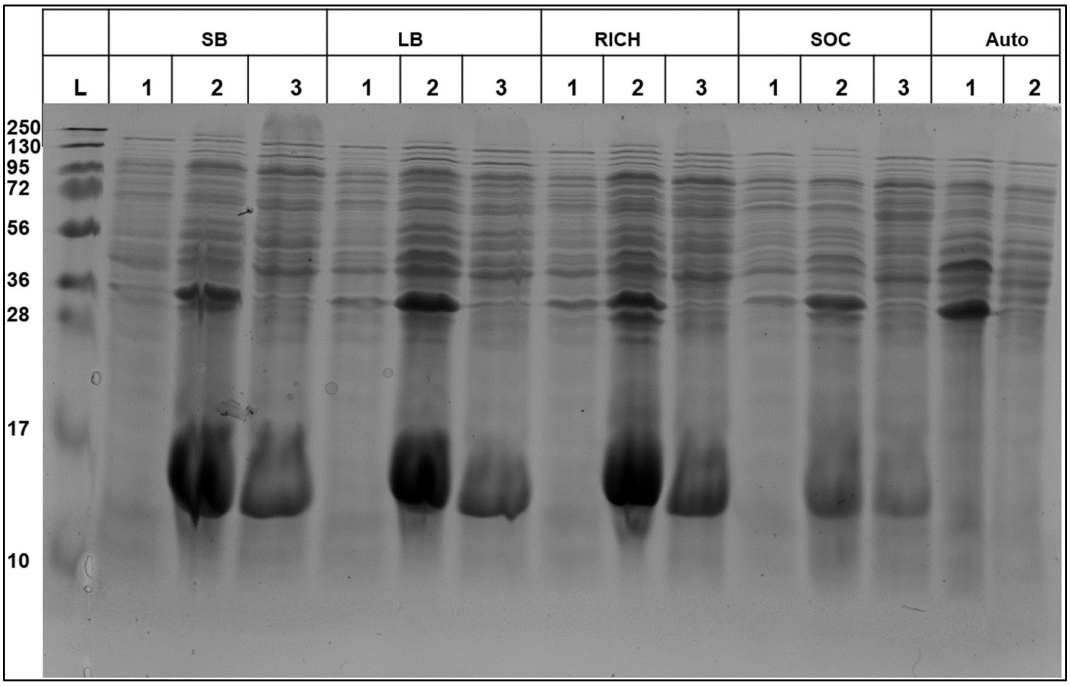



*Figure 39: CBB-stained SDS-PAGE gel of the TB14 media test with samples from bacterial cultures in SB, LB, RICH, SOC and auto-inducing media. (L – protein ladder; 1 – cells before induction; 2 – cells after induction; 3 – lysate; Auto: 1 - before lysis, 2 – lysate).*

### 4.1.4.2. Purification

#### - Purification by IMAC

The native protein purification by IMAC, pictured in Figure 40, showed peaks between 2000 and 3000 mAU after elution with each concentration of imidazole. The samples were analyzed on an SDS-PAGE gel (Figure 41), which showed that a protein with a molecular mass around 17 kDa was present in every sample. All analyzed fractions contained impurities and further purification was needed.

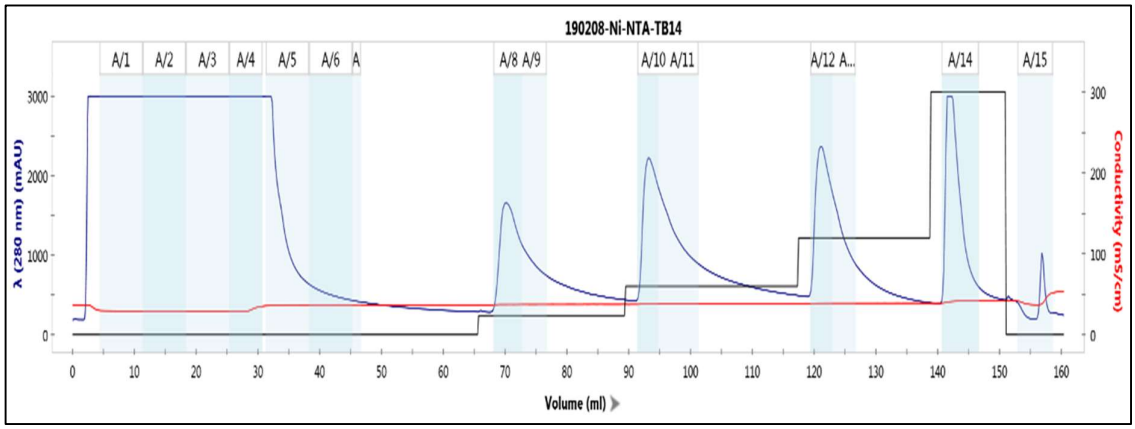



*Figure 40: Chromatogram of the purification of TB14 by IMAC. (blue – absorbance at 280 nm; red – conductivity; black - % buffer B).*

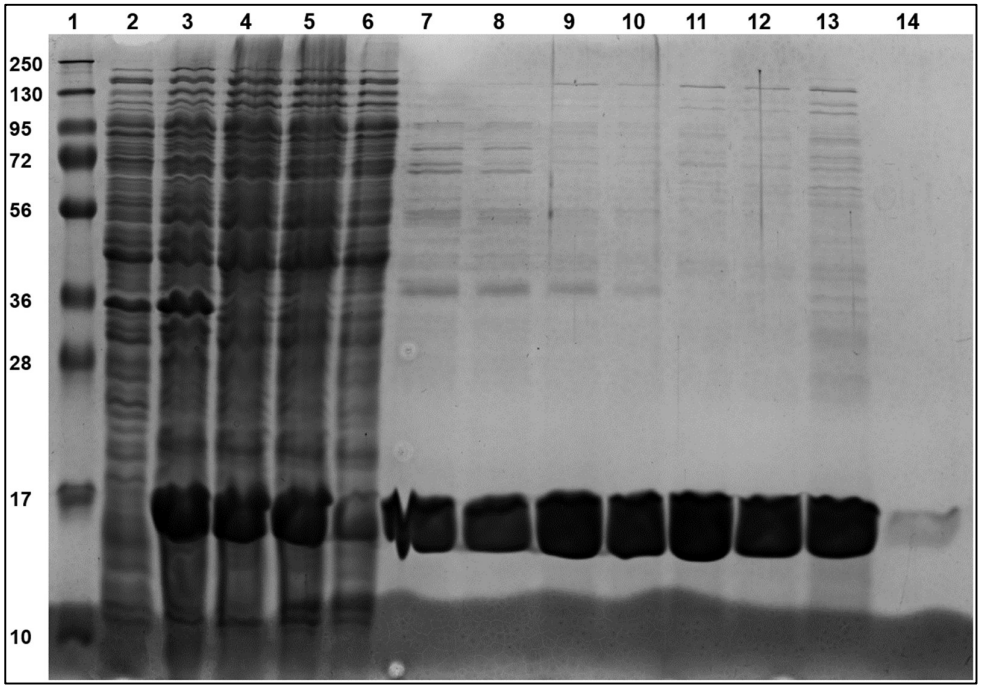



*Figure 41: CBB-stained SDS-PAGE of samples from the purification of TB14 by IMAC using a Ni-NTA column (Legend: see Table 17).*

*Table 17: Samples loaded onto the SDS-PAGE gel shown in Figure 41.*

| Lane | Sample | Lane | Sample |
|---|---|---|---|
| **1** | Protein ladder [kDa] | 8 | A9 20 mM imidazole |
| **2** | Cells before induction | **9** | A10 50 mM imidazole |
| **3** | Cells after induction | **10** | A11 100 mM imidazole |
| **4** | Lysate | **11** | A12 100 mM imidazole |
| **5** | Lysate after biocryl treatment | **12** | A13 100 mM imidazole |
| **6** | A3 flow-through | **13** | A14 250 mM imidazole |
| **7** | A8 20 mM imidazole | **14** | A15 EDTA eluate |

### - Purification with a chitin resin

The purest fractions A11 to A13 were pooled and purified using chitin resin. Samples were loaded onto an SDS-PAGE gel (see Figure 42). It did not show a difference between the two samples and it seemed that the impurities in the sample before purification were diluted due to the pooling and not visible anymore. The eluate was used for further experiments.

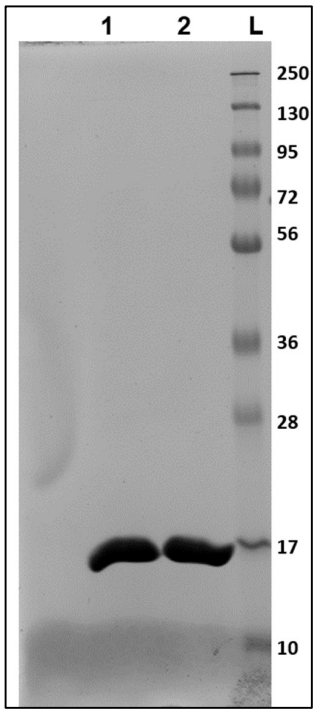



*Figure 42: CBB-stained SDS-PAGE of samples from chitin resin purification of TB14 (1 – before purification; 2 – after purification; L – protein ladder [kDa]).*

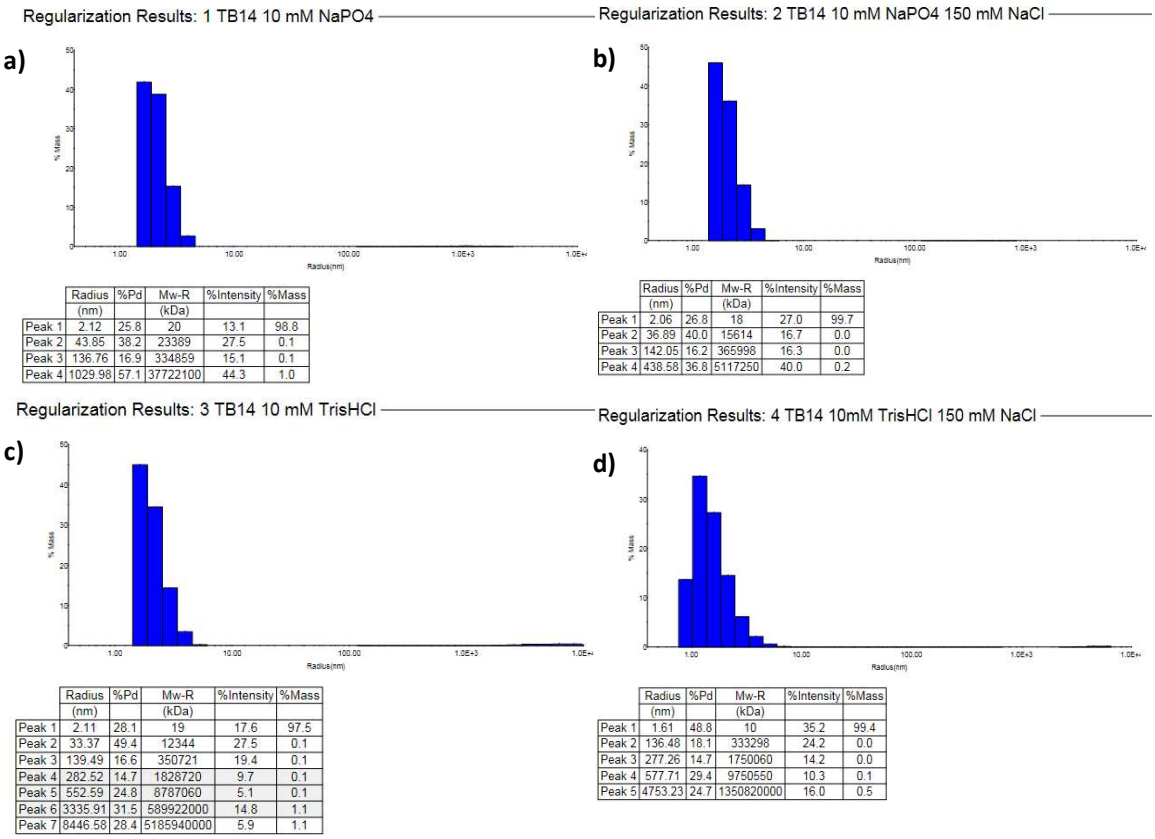
### - Dynamic light scattering and storage conditions test

In order to determine the most suitable storage buffer for TB14, a solubility test with different buffers was conducted. The measurements, seen in figure 44 a) to d), provided similar results. Less than 3% of the mass consisted of aggregated proteins, which is a satisfactory result. Buffer 3 – 10mM Tris.HCl, pH8 was chosen as a storage buffer.

An additional test of the stability when freezing and thawing was carried out. It was performed in 10 mM Tris.HCl buffer, pH 8.0, in order to check if the proteins remains stable when frozen and thawed. The results are summarized in Table 18. The mean concentration before freezing was determined to be 0.80 mg/mL. The concentration after freezing was measured at 1.17 mg/mL. The supposed rise in the concentration might be caused by incorrect measurement of the “before” samples. This argument is also supported by the higher 260/280 ratio in the “before” measurement. As the concentration in the “after” sample did not drop significantly, the protein was considered stable for storage at -80 °C.

**The final concentration was determined by BCA assay to be 0.968 mg/mL.**

**The final yield was 17.5 mg protein/L bacterial culture.**




| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 2.12 | 25.8 | 20 | 13.1 | 98.8 |
| Peak 2 | 43.85 | 38.2 | 23389 | 27.5 | 0.1 |
| Peak 3 | 136.76 | 16.9 | 334859 | 15.1 | 0.1 |
| Peak 4 | 1029.98 | 57.1 | 37722100 | 44.3 | 1.0 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 2.06 | 26.8 | 18 | 27.0 | 99.7 |
| Peak 2 | 36.89 | 40.0 | 15614 | 16.7 | 0.0 |
| Peak 3 | 142.05 | 16.2 | 365998 | 16.3 | 0.0 |
| Peak 4 | 438.58 | 36.8 | 5117250 | 40.0 | 0.2 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 2.11 | 28.1 | 19 | 17.6 | 97.5 |
| Peak 2 | 33.37 | 49.4 | 12344 | 27.5 | 0.1 |
| Peak 3 | 139.49 | 16.6 | 350721 | 19.4 | 0.1 |
| Peak 4 | 282.52 | 14.7 | 1828720 | 9.7 | 0.1 |
| Peak 5 | 552.59 | 24.8 | 8787060 | 5.1 | 0.1 |
| Peak 6 | 3335.91 | 31.5 | 589922000 | 14.8 | 1.1 |
| Peak 7 | 8446.58 | 28.4 | 5185940000 | 5.9 | 1.1 |

| | Radius (nm) | %Pd | Mw-R (kDa) | %Intensity | %Mass |
|---|---|---|---|---|---|
| Peak 1 | 1.61 | 48.8 | 10 | 35.2 | 99.4 |
| Peak 2 | 136.48 | 18.1 | 333298 | 24.2 | 0.0 |
| Peak 3 | 277.26 | 14.7 | 1750060 | 14.2 | 0.0 |
| Peak 4 | 577.71 | 29.4 | 9750550 | 10.3 | 0.1 |
| Peak 5 | 4753.23 | 24.7 | 1350820000 | 16.0 | 0.5 |

*Figure 43: DLS measurement of TB14: a) in 10 mM sodium phosphate buffer, pH 7.5; b) in 10 mM sodium phosphate, 150 mM NaCl buffer, pH 7.5; c) in 10 mM Tris.HCl buffer, pH 8.0; d) in 10 mM Tris.HCl, 150 mM NaCl buffer, pH 8.0*

*Table 18: Concentration measurement with photometry in the course of the freeze-thaw test of TB14. a) concentration before freezing; b) concentration after freezing.*

| ***a)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | **0.88** | 1.36 | 0.84 | 0.80 |
| | **0.69** | 1.24 | 0.76 | |

| ***b)*** | **260/280** | **Abs.** | **Conc.** | **Mean [mg/mL]** |
|---|---|---|---|---|
| | **0.56** | 1.895 | 1.17 | 1.17 |
| | **0.59** | 1.935 | 1.18 | |
| | **0.56** | 1.892 | 1.17 | |

### 4.1.4.3. Physico-chemical characterization

#### - MALDI-TOF-MS

The measurement, performed to confirm that the purified protein in TB14, showed three peaks, corresponding to the single, double and triple ionizated forms. The results are shown in Figure 44. Each peak was a doublet and the lower molar mass peak was higher, which means that the majority of the TB14 molecules had the N-terminal methionine cleaved off. Both determined molar masses (Table 19) were almost identical to the theoretical ones. The purified protein was identified to be TB14.

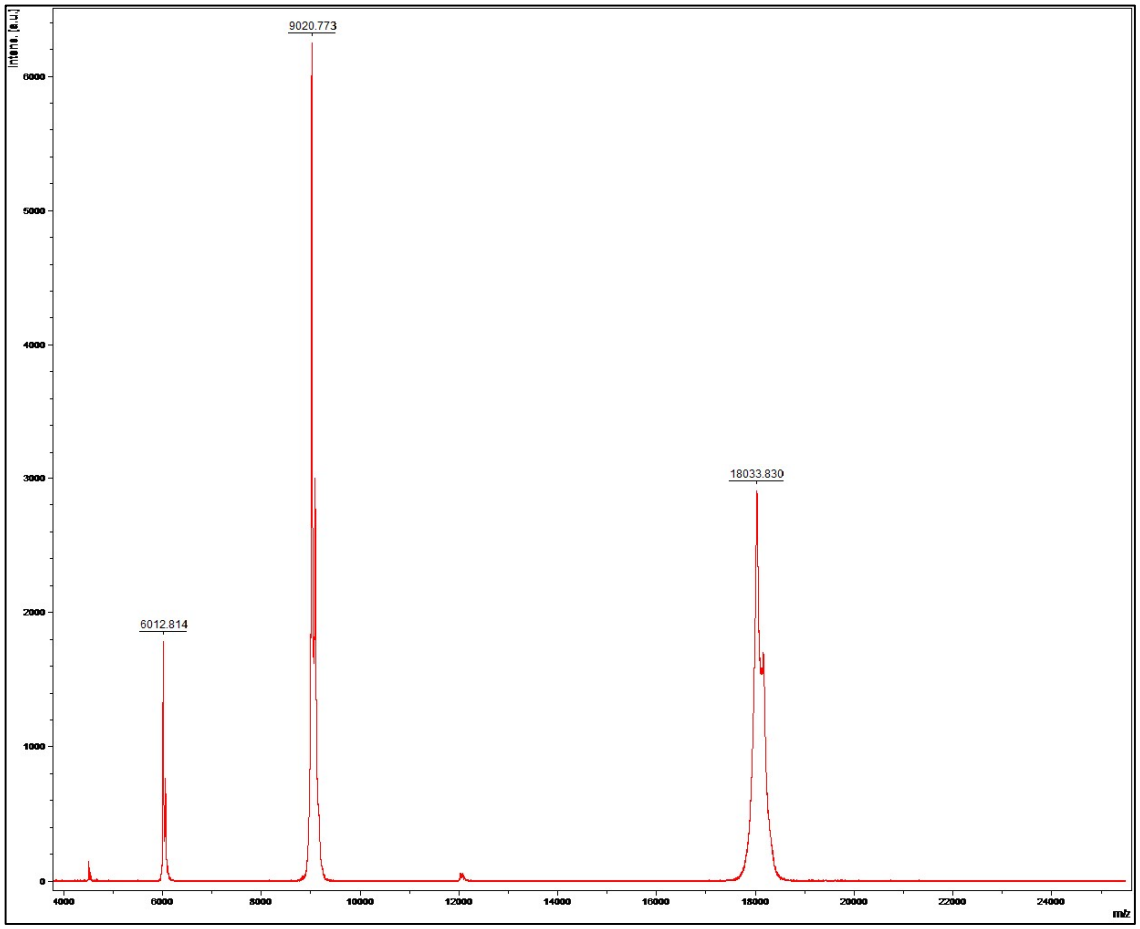



*Figure 44: MALDI-TOF-MS spectrum of purified TB14*

*Table 19: Evaluation of results of MALDI-TOF-MS of TB14.*

| **With Methionine at N-Terminus** | | | | **Without Methionine at N-Terminus** | | | |
|---|---|---|---|---|---|---|---|
| **Ion** | **m/z** | **z** | **M** | **Ion** | **m/z** | **z** | **M** |
| [M+3H]3+ | 6057,1 | 3 | 18168,2 | [M+3H]3+ | 6012,8 | 3 | 18035,4 |
| [M+2H]2+ | 9087,6 | 2 | 18173,2 | [M+2H]2+ | 9020,8 | 2 | 18039,6 |
| [M+H]+ | 18161,9 | 1 | 18160,9 | [M+H]+ | 18033,8 | 1 | 18032,8 |
| | | **Mean** | **18167,4** | | | **Mean** | **18035,9** |
| **Theoretical mass:** | | | **18175,5** | | | | **18044,4** |

### - Circular dichroism spectroscopy

The overlay of the CD-spectrum of TB14 with the one of TTH, shown in Figure 45, was not complete in some regions (205-230 nm), but their overall shapes were identical. The shift is usually caused by a difference in the protein concentration in both samples, because the results of the BCA assay can vary by 10%. The x-axis was crossed at the same wavelength. TB14-HisC was therefore not denatured and was correctly folded.

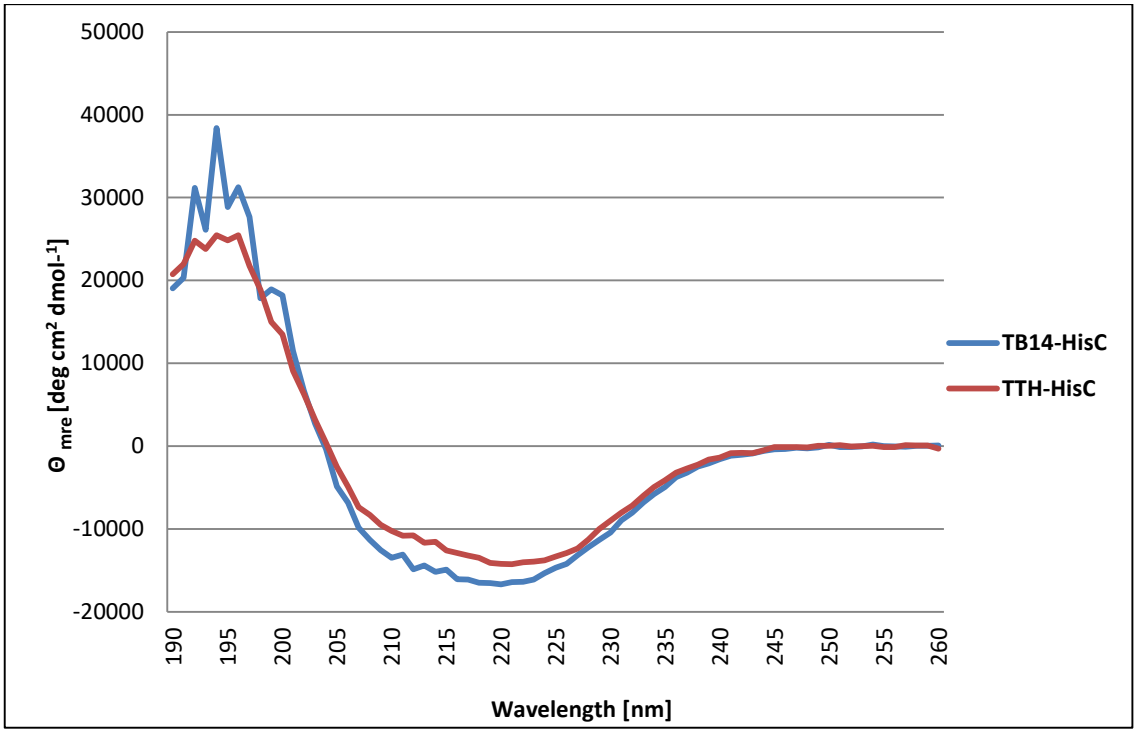



*Figure 45: CD spectra of TB14 and TTH*

## 4.2. Immunoassays with chimeric proteins

### 4.2.1. IgE ELISA with sera of allergic patients

This test was performed in order to study whether IgE from birch pollen allergic patients recognizes the chimeric proteins in the same way as Bet v 1 itself. The plates were coated with the proteins and then incubated with the sera. Myeloma IgE was used as a negative control as it should not bind any of the coated proteins.

*Table 20: Results of an IgE ELISA with sera of 20 birch pollen allergic patients and Bet v 1, TTH, TB5, TB8 and TB14.*

| | Bet v 1 | TTH | TB5 | TB8 | TB14 | | |
|---|---|---|---|---|---|---|---|
| **Patient 1** | 1.452 | 0.027 | 0.035 | 0.026 | 0.039 | | |
| **Patient 2** | 1.889 | 0.009 | 0.161 | -0.076 | 0.018 | | |
| **Patient 3** | OVER | 0.017 | 0.160 | 0.047 | 0.024 | | |
| **Patient 4** | 1.026 | 0.004 | 0.000 | -0.034 | 0.002 | | |
| **Patient 5** | 1.068 | 0.004 | 0.000 | 0.015 | -0.003 | | |
| **Patient 6** | 0.992 | 0.000 | 0.004 | -0.013 | -0.007 | Pos. value | |
| **Patient 7** | 1.215 | 0.004 | 0.003 | 0.014 | -0.002 | over: | 0.020 |
| **Patient 8** | 1.475 | 0.010 | 0.012 | 0.080 | 0.011 | | |
| **Patient 9** | 2.169 | 0.003 | 0.008 | 0.091 | 0.011 | | |
| **Patient 10** | 1.633 | 0.006 | 0.022 | 0.080 | 0.016 | | |
| **Patient 11** | 2.381 | 0.013 | 0.025 | 0.057 | 0.016 | | |
| **Patient 12** | 2.278 | 0.008 | 0.013 | 0.031 | 0.043 | | |
| **Patient 13** | 1.799 | 0.004 | 0.011 | 0.039 | 0.007 | Pos. value | |
| **Patient 14** | 1.443 | 0.003 | 0.024 | 0.001 | 0.003 | over: | 0.091 |
| **Patient 15** | 1.745 | 0.017 | 0.018 | 0.051 | 0.019 | | |
| **Patient 16** | 1.777 | 0.004 | 0.009 | 0.019 | 0.021 | | |
| **Patient 17** | 1.407 | 0.003 | 0.013 | -0.028 | 0.017 | | |
| **Patient 18** | 1.633 | 0.006 | 0.011 | 0.014 | 0.017 | | |
| **Patient 19** | 1.355 | 0.002 | 0.009 | -0.022 | 0.019 | | |
| **Patient 20** | 1.311 | 0.009 | 0.025 | 0.055 | 0.037 | Pos. value | |
| **Neg.control** | 0.000 | 0.000 | 0.005 | -0.006 | 0.012 | over: | 0.088 |

The results of the ELISA assay with the sera of 20 patients are summarized in Table 20. All values were calculated by subtracting the mean of the buffer blank from the mean of the duplicates., which led to negative values in some wells. The limit for positive values was calculated separately for each plate, using the results of the three NHS. The red-marked cells contain values that exceed the limit showing that there was IgE. The first column, coated with Bet v 1, was positive in all patients as expected, because they had clinically confirmed allergy to birch and were sensitized to Bet v 1. The result of patient 3 with Bet v 1 was so high that the signal was outside the range of the equipment and couldn't be quantified. TTH was positive

only with patient 1, which is unusual as TTH was used as a negative control. Patient 1 was probably overly sensitized, because their serum delivered positive values with all proteins. There were three patients positive for TB5 and two of them were also positive for TB8 and TB14. The highest positive values among the chimeras were still less than 10% of the signal with Bet v 1 which can be explained by the fact that the patches grafted onto the chimeras accounted only for about one eight of the total molecular surface of Bet v 1. Hence, only a minor fraction of the total Bet v 1-specific IgE is expected to bind to each of the chimeras.

### 4.2.2. Inhibition ELISA with sera of allergic patients

The aim of this experiment was to study whether Bet v 1-specific IgE was inhibited by binding the chimeric proteins in solution. The amount of inhibition corresponds to the percentage of Betv1-specific IgE that binds to a specific epitope. The fraction of IgE that recognizes the chimeric proteins will not be able to bind to the coated Bet v 1.

A 96-well-plate was coated with Bet v 1 and incubated with mixtures of diluted serum and chimeric protein. The results, calculated in Table 21 and plotted in Figure 46, show that Bet v 1 inhibited almost 100% of IgE binding to Bet v 1 in the two sera when highly concentrated. Interestingly, there was still around 50% inhibition by TTH-His-C/His-N, which was used to calculate the baseline, defining the lower limit for positive inhibition. The results with the chimeric proteins TB5 and TB14 were thus negative, as they were in the same range as the inhibition by the negative control (TTH-HisC/HisN). There was no concentration-dependent inhibition. This leads to the conclusion that Bet v 1-specific IgE has a lower affinity to the chimeric proteins TB5 and TB14 than to Bet v 1. The reason for that could be that structure of the patches differs from the corresponding region in the native Bet v 1 structure. Moreover, a part of the chimera-specific IgE might bind to the edge of the patch, which leads to reduced affinity as a part of the bound epitope is formed by residues of TTH.

*Table 21: Results of inhibition an ELISA with 2 sera of allergic patients using Bet v 1, TTH, TB5 and TB14, showing the calculated inhibition in %.*

| | ***Inhibitor*** | **1 ng/ml** | **10 ng/ml** | **100 ng/ml** | **1 µg/ml** | **10 µg/ml** |
|---|---|---|---|---|---|---|
| ***Patient 1*** | **Bet v 1** | 75 | 85 | 91 | 97 | 100 |
| | **TTH** | 52 | 53 | 53 | 52 | 51 |
| | **TB5** | 49 | 50 | 49 | 49 | 51 |
| | **TB14** | 52 | 52 | 55 | 51 | 51 |

| | ***Inhibitor:*** | **1 ng/ml** | **10 ng/ml** | **100 ng/ml** | **1 µg/ml** | **10 µg/ml** |
|---|---|---|---|---|---|---|
| ***Patient 2*** | **Bet v 1** | 68 | 77 | 87 | 97 | 99 |
| | **TTH** | 58 | 57 | 57 | 56 | 56 |
| | **TB5** | 54 | 54 | 54 | 55 | 55 |
| | **TB14** | 60 | 59 | 59 | 60 | 58 |

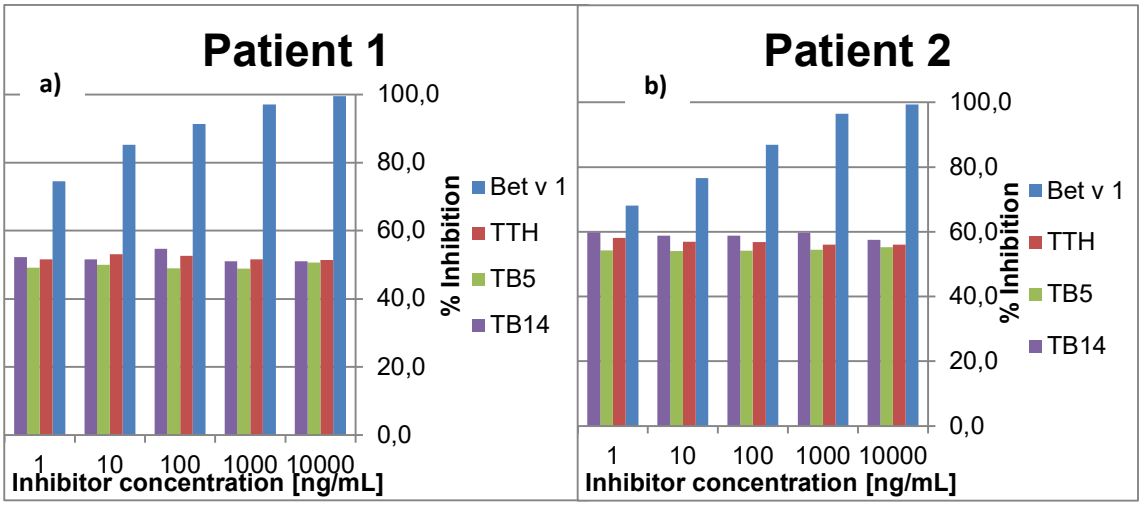



*Figure 46: Plotted results of inhibition ELISA: % inhibition, dependent on concentration and added protein: a) results of patient 1; b) results of patient 2.*

## 4.3. Generation of Bet v 1-specific recombinant antibodies

### 4.3.1. In vitro enrichment of Bet v 1-specific antibodies

Three rounds of biopanning with Bet v 1 of a single-domain antibody phage library were performed in order to select and amplify phages carrying a Betv1-specific antibody. A sample after each elution, before propagation, was taken in order to determine the amount of eluted phages after each round. The samples were used to infect TG1 *E. coli*, which were then quantified by titration (Table 22). The results indicate a successful enrichment of specific phages during the selection process.

*Table 22: Amount of total eluted phages after each round of biopanning.*

| Panning round | Eluted phages |
|---|---|
| Before panning | $2.1x10^{14}$ |
| After 1. round | $2.2x10^{4}$ |
| After 2. round | $5.9x10^{6}$ |
| After 3. round | $3.7x10^{8}$ |

A polyclonal phage ELISA was performed with samples from each round of biopanning, after propagation. It was done in order to verify that Bet v 1-specific phages were enriched in the selection process. A sample from each selection round, including the original library, was diluted in a tenfold dilution series. Two of the dilutions to each round were applied to a plate coated with Bet v 1. The sample from the third selection round was also applied to wells, coated with an anti-M13 coat protein g8P antibody. It was used as a positive control as it would bind all phages, regardless of their affinity. The helper phages were used as a negative control as they should not bind Bet v 1. The detection of the bound phages was achieved by using the anti-M13-HRP antibody.

The means of the duplicated of the measured signal, buffer blank subtracted, are summarized in Table 23 and pictured in Figure 47. There was a visible enrichment of Bet v 1-binding phages between the original library and the first round of selection, but it plateaued in the subsequent rounds. Only the positive control showed a concentration-dependent increase in the signal, which was expected as anti-M13 coat protein g8P antibody binds all phages. This means that the enrichment in the later rounds of selection was insufficient.

*Table 23: Results of the polyclonal phage ELISA after 30 minutes - binding of phages from different round of selection to the coated Bet v 1 or to anti M-13 coat protein g8P as a positive control.*

| | $OD_{450nm}$ | |
|---|---|---|
| Sample | Dilution 1 $10^{10}$ pfu | Dilution 2 $10^{11}$ pfu |
| Original library | 0.061 | 0.143 |
| 1. Round | 0.191 | 0.186 |
| 2. Round | 0.193 | 0.186 |
| 3. Round | 0.190 | 0.185 |
| Helper Phages (negative control) | 0.077 | 0.038 |
| | | |
| 3. Round with anti-M13 coat protein g8P | 0.149 | 0.257 |

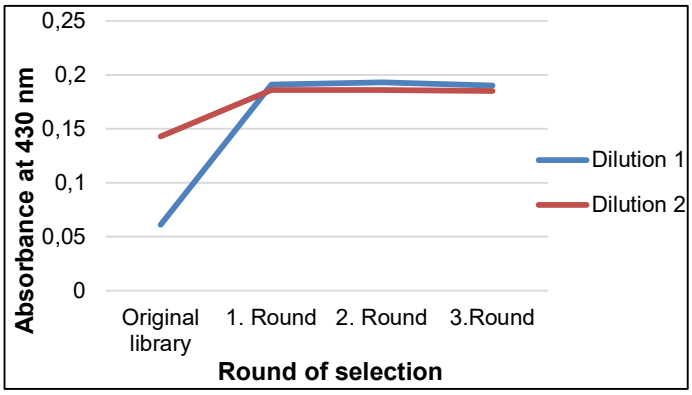



*Figure 47: Plotted results of polyclonal phage ELISA*

### 4.3.2. Monoclonal phage ELISA

This immunoassay was performed in order to find the phage clones that bind the highest amount of Bet v 1.

70 different colonies from the second and third round of selection were picked and propagated. A Bet v 1-coated plate was then incubated with the phages in order to find the clones which bind Bet v 1. The bound phages were then detected using an anti-M13-HRP antibody.

There were large differences in the measured signals between the clones. 15 clones with the highest signals were deemed of interest (28.5% of the clones produced in the second round and 18% from the third round). They are marked orange in Table 24. The clones of interest were named after the round of selection, the row and the column on the plate. They were propagated, purified and used for further experiments.

*Table 24: Results of $OD_{450nm}$ measurement of the monoclonal phage ELISA as measured on the plate.*

| <> | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | Second round | | | Third round | | | | | | |
| B | 0.1583 | 0.7718 | 0.2569 | 0.1089 | 0.249 | 0.1268 | 0.9252 | 0.1187 | 0.6163 | 0.1268 |
| C | 0.1342 | 0.6279 | 0.5927 | 0.1435 | 0.1046 | 0.6058 | 0.1098 | 0.5170 | 0.5548 | 0.1876 |
| D | 0.7623 | 0.0984 | 0.3308 | 0.4300 | 0.1116 | 0.1004 | 0.6477 | 0.1629 | 0.1213 | 0.3233 |
| E | 0.1034 | 0.1572 | 0.6655 | 0.2026 | 0.1105 | 0.7467 | 0.6938 | 0.3312 | 0.1177 | 0.3599 |
| F | 0.2589 | 0.5669 | 0.1299 | 0.1843 | 0.0996 | 0.1944 | 0.1764 | 0.1726 | 0.2530 | 0.1053 |
| G | 0.0988 | 0.2047 | 0.0905 | 0.7102 | 0.2541 | 0.2237 | 0.3133 | 0.1097 | 0.1674 | 0.0997 |
| H | 0.1045 | 0.0906 | 0.0923 | 0.099 | 0.1023 | 0.1027 | 0.0601 | 0.0612 | 0.0486 | 0.0582 |

### 4.3.3. Single clone ELISA with Bet v 1 homologue proteins

This assay was performed in order to check if the phage clones of interest bind to Bet v 1 homologue proteins. A 96-well-plate was coated with Bet v 1, the homologue proteins Cor a 1.04 mut (Cor a 1.04 with two surface exposed cysteines mutated to serine), Api g 1.01, Mal d 1 and Gly m 4. TTH was used as a negative control. Non-coated wells were also used as a negative control in order to determine if the phages bind to the plastic surface of the plate as well as to the blocked surface. The plate was incubated with different phage clones, selected in the monoclonal phage ELISA, and the bound phages were then detected using the anti-M13-HRP antibody.

The results, shown in Figure 48, are the mean values of the duplicates with subtracted buffer (background) value.

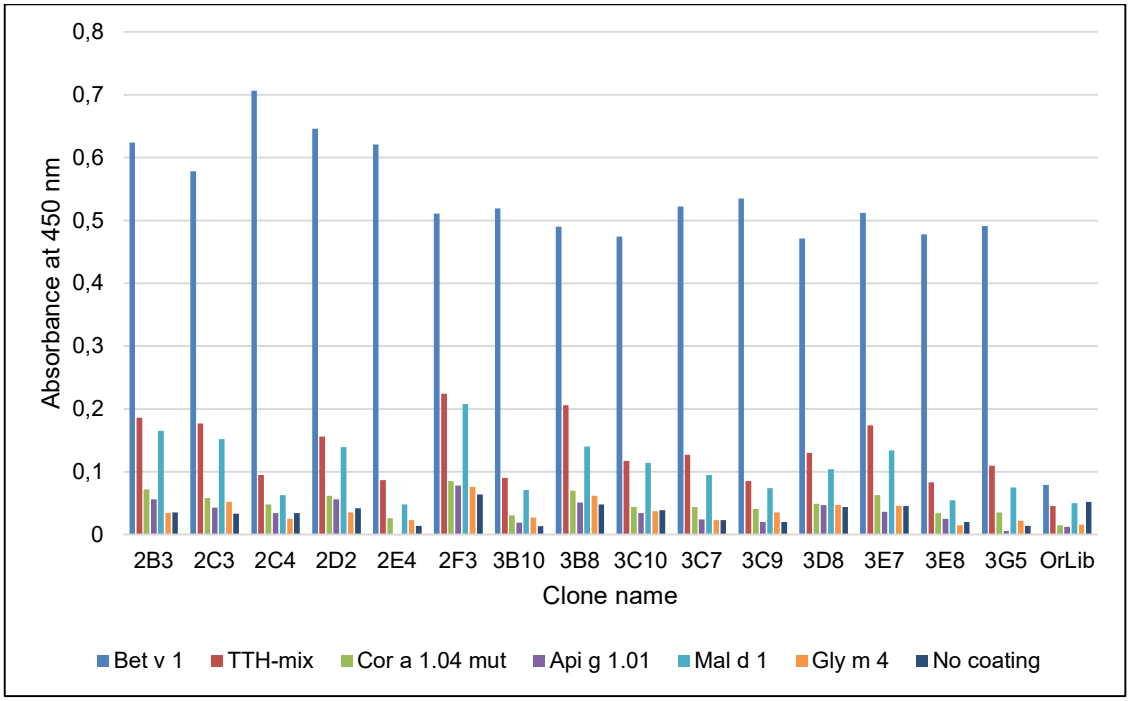



*Figure 48: Results of the single clone ELISA with Bet v 1 and its homologous proteins.*

It was unexpected that the wells coated with TTH had the second highest signal after Bet v 1, it was used as a negative control and its sequence is not similar to Bet v 1. Mal d 1 also had a moderately high signal with some clones. The rest of the Bet v 1-homologous proteins had low signal in the noise range. It is unclear if this is due to lower binding affinity as the biopanning was only done with Bet v 1 or if the proteins had been somehow damaged due to very long storage time.

3 clones were chosen for further experiments – 2C4, due to its monoreactivity to Bet v 1, and 2F3 and 3B8, due to their cross-reactivity.

### 4.3.4. Phage ELISA with chimeric proteins and homologue proteins

This assay was performed in order to check whether the phage clones of interest bind to the chimeric proteins. 96-well-plates were coated with the chimeric proteins, the homologous proteins as well as lysozyme, Bet v 2 (birch pollen profilin) and BSA as negative controls. It was then incubated with three different phage clones, as well as the phages from the original library as a negative control. The bound phages were then detected using an anti-M13-HRP antibody.

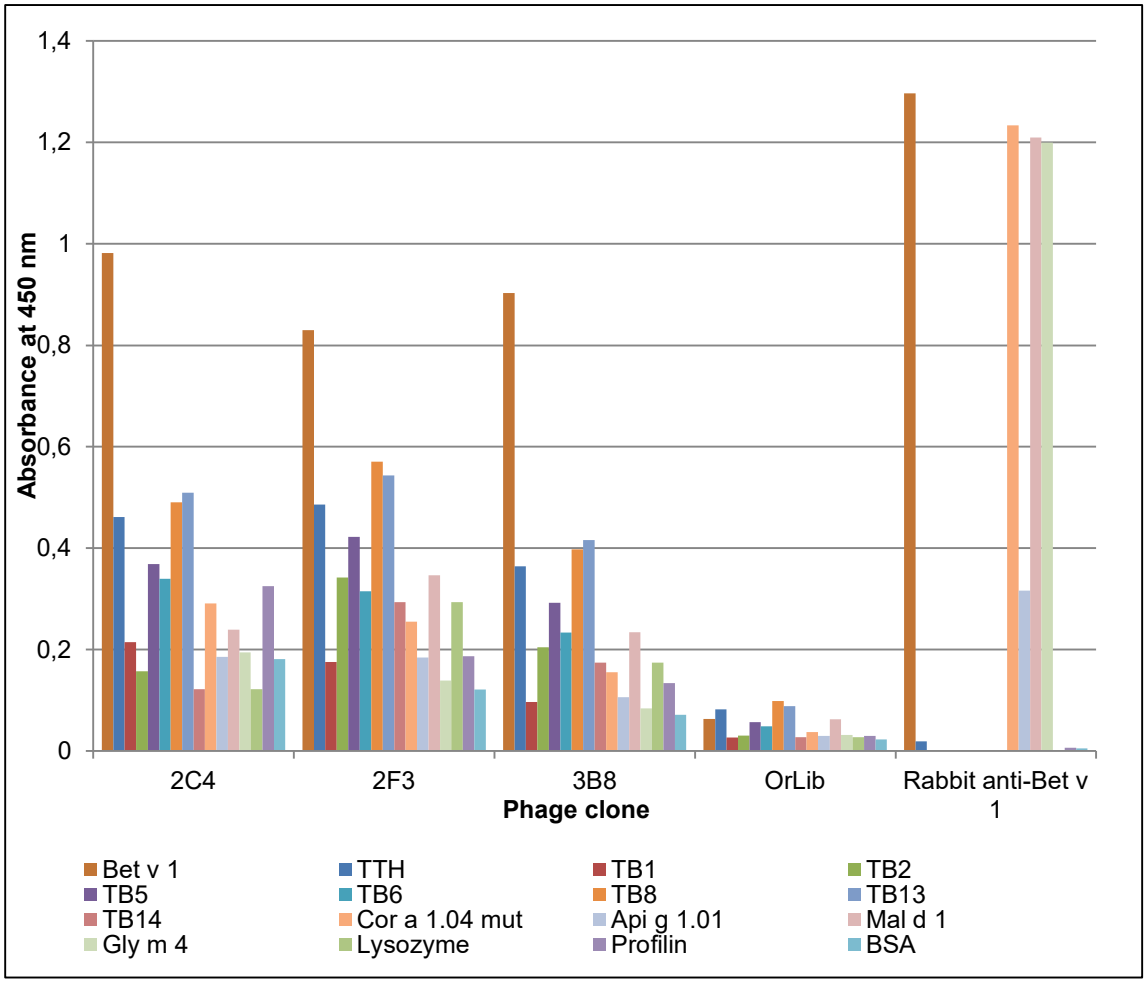



*Figure 49: Results of the phage ELISA with chimeric and homologous proteins.*

The results of the ELISA with chimeric and homologous proteins, presented in Figure 49, are the mean absorbance values of the duplicate wells with the buffer blank signals subtracted. As expected, Bet v 1 had the highest signal with all three phage clones. TTH, used as a negative control, had the second highest signals, which means that none of the chimeric proteins showed specific binding, the observed signal can be explained by binding of the phages to the TTH-derived part of the chimeras. There were also very high signals with lysozyme, Bet v 2 (profilin) and BSA, which were used as negative controls. This was another sign for very

unspecific interactions. The rabbit anti-Bet v 1, which was used as a positive control, bound only Bet v 1 and its homologues and showed no cross-reactivity with TTH, as expected.

### 4.3.5. ELISA with phage coating

This test was done as troubleshooting of the previous immunoassays which showed no binding of the phage clones to the chimeric proteins. In this assay, the 96-well-plate was coated with phages and then incubated with the proteins of interest. The detection of the bound proteins was done with either anti-His-HRP or rabbit anti-Bet v 1 detection antibody.

The results, presented in Table 25, are the mean values of the duplicate wells with subtracted buffer blank. As the measured signal was mostly very low, a lot of negative values were present after subtracting the blank value. The cells containing values over 0.1 were marked in red. Both phage clones show higher signal with TB8, but the binding was not specific because the signal to the original library (OrLib) was very similar. Specific bind would have yielded higher signal after the biopanning. The other proteins delivered a signal which was probably measurement noise.

*Table 25: Results of ELISA with phage coating: phage clones 2C4 and 3B8 and the original library. The cells with values over 0.1 are marked in red.*

| | 2C4 | 3B8 | OrLib |
|---|---|---|---|
| **Detection with anti-His antibody** | | | |
| **Bet v 1** | -0.001 | -0.003 | 0.007 |
| **TTH** | 0.001 | -0.001 | 0.008 |
| **TB1** | -0.006 | -0.007 | -0.009 |
| **TB2** | -0.008 | -0.017 | -0.009 |
| **TB5** | -0.010 | -0.015 | -0.006 |
| **TB6** | -0.013 | -0.020 | -0.008 |
| **TB8** | 0.193 | 0.196 | 0.201 |
| **TB13** | 0.031 | 0.028 | 0.034 |
| **TB14** | -0.007 | -0.009 | -0.003 |
| **Detection with rabbit anti-Bet v 1 antibody** | | | |
| **Cor a 1.04 mut** | 0.002 | 0.017 | 0.030 |
| **Api g 1.01** | -0.029 | 0.025 | 0.022 |
| **Mal d 1** | 0 | 0.048 | 0.049 |
| **Gly m 4** | -0.025 | 0.040 | 0.040 |
| **Lyc e 2** | -0.028 | 0.038 | 0.043 |
| **Phl p 5** | -0.015 | 0.050 | 0.031 |
| **Hev b 11** | -0.011 | -0.006 | 0.003 |

This experiment did not work, probably due to a conformational change in the phage structure during coating, because previous assays showed that there was high signal with Bet v 1, which was also used as a positive control. In this assay, the signal of Bet v 1 was also near 0. The design of the experiment needs to be changed and it needs to be repeated.

### 4.3.6. Sequencing

The sequences of 6 different phage clones were determined. It turned out that five out of the six clones shared the same sequence. The exact sequences are listed below.

- **2C4 protein sequence**

QVQLLESGGGLVQPGGSLRLSCAASGDKFNSQVMSWVRQAPGKGLEWVSSIVDRNGSTY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAA*KPGQEPLHYWGQGTLVTVSSA AA

- **2F3/3B8/3G5/2E4/2B3 protein sequence**

QVQLLESGGGLVQPGGSLRLSCAASGYRINNQFMSWVR*APGKGLEWVSTIFGTNGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGEATDRPKSVHFWGQGTLVTVSSA AA

### 4.3.7. Sequence alignments

In order to compare the sequences of both phages, a pairwise sequence alignment was done (Figure 50). The mismatches in the two sequences were exclusively in the three complementarity-determining regions.

```
2C4                1 QVQLLESGGGLVQPGGSLRLSCAASGDKFNSQVMSWVRQAPGKGLEWVSS     50
                     |||||||||||||||||||||||||.:.|:|.|||||.||||||||||:
2F3                1 QVQLLESGGGLVQPGGSLRLSCAASGYRINNQFMSWVR*APGKGLEWVST     50

2C4               51 IVDRNGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAA*K    100
                     |...|||||||||||||||||||||||||||||||||||||||||||||
2F3               51 IFGTNGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA--     97

2C4              101 PGQ-----EPLHYWGQGTLVTVSSAAA    122
                      |:     :.:|:|||||||||||||
2F3               98 -GEATDRPKSVHFWGQGTLVTVSSAAA    123
```

*Figure 50: Pairwise alignment of the sequences of 2C4 and 2F3. The 3 CDRs are marked in green, the suppressed stop codons, translated as Q, are marked in red.*

# 5. Discussion

The topic of this master thesis was to research the conformational epitopes on the surface of the major birch pollen allergen Bet v 1 as well as to look for a connection between them and the cross-reactivity of patients' IgE to homologue plant-related proteins.

The production of the chimeric proteins TB5, TB7, TB8 and TB14 carrying grafted patches from the surface of Bet v 1 was the first step in order to start the analytical experiments. The chimeric protein TB5-HisN was purified successfully in its native form, and it was mostly eluted at 100 mM imidazole, which produced an impure fraction. A second purification with an anion exchange chromatography was performed. The physico-chemical analysis of the protein showed that it was correctly folded and relatively stable in all tested buffers. The MALDI-TOF-MS results provided insight in the molecular structure of the protein and showed that the N-terminal methionine was cleaved during expression in a larger part of the proteins. The results for the molecules with methionine were unusual as there was a difference of 43.6 Da between the empirically determined molecular mass and the calculated one. One possible explanation would be a formyl group accompanied by oxidation of the N-terminal fMet to fMet sulfoxide. This is a question which could be pursued in future experiments in order to bring more knowledge to how *E. coli* processes the different chimeric proteins and whether this modification has an impact on the overall protein structure.

The purification of the TB7 chimeric protein led to complications. The first tests showed that the protein was expressed by the *E. coli* in large amounts but it was insoluble. One reason could be that due to the fast expression of the proteins, they could not fold correctly on time and thus aggregated in inclusion bodies. Different approaches were taken in order to counteract this – e.g. time and temperature tests to determine the optimal conditions. This method was also integrated as an optimization step for the expression of the other chimeric proteins and led to a successful expression and purification of TB8. The problem with the protein aggregation of TB7 remained unsolved.

The protein purification of TB7 was also tried under denaturing conditions to allow the proteins to refold correctly. This yielded a large amount of protein, which then precipitated in the resin during refolding. As both experiments did not lead to the desired results, it was decided that the protein needs to be redesigned in order to achieve a more stable protein structure. One suggested possibility would be to insert mutations in the protein scaffold which would not affect the grafted patch, but this experiment was not performed in the scope of this thesis.

The initial tests for the most suitable media, time and temperature showed that TB8 could also be insoluble. This was contradicted by the successful purification with a Ni-NTA column. Hence the results from the optimization tests could be explained by insufficient lysis of the cells. The

analysis showed that TB8 was not stable in a buffer without sodium chloride as it precipitated during dialysis. Thus, the test to determine the most suitable storage buffer was introduced for this and the other chimeric proteins. The physico-chemical analysis showed that the chimeric protein was folded correctly, and that the N-terminal methionine was not cleaved off.

TB14 was the last chimeric protein to be produced. The media test showed that it was expressed in sufficient amounts and was also soluble. The fractions from the IMAC purification still contained impurities and thus, a second purification using chitin resin was done, which did not yield the expected result as the impurities were apparently diluted when the fractions were pooled. The protein was very stable in all buffers and could be characterized. Circular dichroism showed that it is folded correctly. The mass spectrometry showed that a large part of the molecules had their N-terminal methionine cleaved off.

Some questions arose during the production of the chimeric proteins. As those are of synthetic origin, their performance during purification and the subsequent experiments could not be predicted. We hypothesized that Bet v 1-TTH chimeras that had a C-terminal hexa His-tag were more stable than the ones with a N-terminal one. This was just an observation made during the production of the first chimeric proteins for the project but there are also studies that research the influence of the His-tag on the protein structure. According to one of them, an N-terminal His-tag can negatively influence the thermal stability, the oligomeric states as well as the function of proteins compared to their native state (Booth et al., 2018). Another study covers the influence of a C-terminal His-tag. It was observed that there also were slight protein-dependent changes in the thermal stability, but they were positive in some cases and negative in others. The presence of C-terminal His-tag led to a modified processing of the N-terminus of the protein (a β-lactamase) which resulted in a different fold that brought the N- and C-termini in close proximity to each other. This apparently affected the active site of the protein (Briza et al., 1997). The influence of the His-tag on protein stability and function is a topic that needs to be further researched. One option would be to forgo the purification by IMAC and create chimeric proteins with the same patches but without a His-tag. The purification can be done using anion exchange chromatography or size exclusion chromatography instead, and it would be meaningful to compare not only the yields after expression and purification but also their recognition by IgE and selected antibodies in immunoassays.

There were also different circumstances and difficulties with each chimera that led to the introduction of new optimization tests and methods in order to prevent the loss of the produced protein. The needed tests were determined by trial and error but could prove useful for the purification of the rest of the 14 chimeras. The question arises if all methods were suitable for the chimeric proteins – e.g. the lysis of the cells with a Retsch ball mill was proven insufficient, probably due to the robustness of the cell membranes of *E.coli*. The ion exchange

chromatography also did not deliver the expected results to remove impurities and needs to be optimized by, for example, using a different resin or buffers with a different ionic strength or pH.

The purified proteins were then used to compare their IgE-binding to the one of Bet v 1, TTH and different Bet v 1-homologous proteins. The results of the immunoassays showed that IgE from only two patients produced positive signals with the chimeric proteins, but the IgE of all patients bound to Bet v 1, which was used as a positive control. The results of the inhibition ELISA also turned out negative, because IgE binding to Bet v 1 was only inhibited by Bet v 1 itself. This raises the question if the negative results of most patients are genuinely negative or there is another reason why most patients did not react to the chimeras. It is possible that all of the chimeric proteins, covered in this thesis, carry a non-antigenic surface patch that is usually not bound by IgE. Another reason could be that conformational changes happened during adsorption of the protein to the ELISA plate that disturbed the structure of the grafted patches. This was disproven by the inhibition ELISA in which the inhibitory proteins reacted with IgE in solution. It is also possible that small changes in the tertiary structure of the epitopes happened during protein expression that were not detected during the physico-chemical analyses. This needs to be further researched by using methods that deliver precise data about the protein structure, e.g. nuclear magnetic resonance spectroscopy. There are already studies that use NMR assessment as a quality control for purified allergens. One of them compares modelled and experimental structures of allergens from peanut, hazelnut, apple and peach and showed the presence of mobile parts and extended loops as differences (Alessandri et al., 2012). These differences could be induced during expression or purification and could disturb the patches and thus make them unrecognizable for Bet v 1-specific IgE.

The second part of this thesis consisted of the production of single domain anti-Bet v 1 antibodies with phage display. It was started by three rounds of selection for the affinity to Bet v 1. A single clone ELISA with Bet v 1 and homologous proteins was performed in order to choose the most suitable clones for further experiments. These were then used in different assays in order to compare their binding to Bet v 1 with their binding to other proteins (chimeric proteins, homologous proteins, unrelated proteins). The results of the assay were unexpected as some of the negative controls yielded high signal and the chimeric proteins did not bind the phages. The results of a sandwich ELISA with phage coating were even more unexpected, as Bet v 1 and TTH did not bind to the coated phages, but TB8 yielded a high signal. There are a few possible causes of this problem - it is possible that the conformation of the phages changed during coating so that the antibodies could not bind proteins anymore, but this is rather unlikely, because the sdAb is rather stable. It is also doubtful that the antibody gets covered up or concealed during adsorption because it is fused to the large phage particle. Some studies

mention that the selection of unspecific peptides is a major issue in phage display (Christiansen et al., 2015). Our results showed that the phages bound some unrelated proteins such as lysozyme, but they still did not bind the proteins in the blocking solution.

At the end of the phage display part of this thesis, six clones were sent for sequencing. The results showed that only one clone has a different sequence and the other five were identical. This might have been caused by the too stringent selection, in which only Bet v 1 was used. Although the amplification in multiple rounds is needed to produce well-binding clones, it also leads to a decrease of the diversity of the library and the number of binding clones (Derda et al., 2011). Something that might need to be considered in further experiments is that in order to produce more diverse and cross-reactive antibodies, the selection needs to be done with a mixture of different proteins. There is also the need to carry out additional experiments in order to optimize the assay conditions, prevent technical problems such as unspecific binding and impurities and deliver reliable results.

In summary, it was shown that the creation of chimeric proteins by epitope grafting is a practicable method to gain insight into the repertoire of conformational epitopes of Bet v 1, which appears to be at least partly patient-specific. Furthermore, phage display proved to be a reliable method for providing single-domain Bet v 1-binding antibodies. Both methods need optimization but could nevertheless be used to investigate the causes of the variable clinical picture of Bet v 1-sensitization as well as its accompanying cross-reactions.

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# 7. Appendix

## 7.1. List of used media and solutions

**Studier medium (ZYM-5052) for auto-induction**

| **ZYM-5052** | |
|---|---|
| ZY stock solution | 958 mL |
| 50x M | 20 mL |
| 50x 5052 | 20 mL |
| 500x $MgSO_4$ | 2 mL |
| 5000x trace elements | 0.2 mL |

**Stock solutions for Studier medium (ZYM-5052) for auto-induction**

| **ZY Stock Solution (autoclaved)** | |
|---|---|
| Tryptone | 10 g/L |
| Yeast extract | 5 g/L |

| **50x M (sterile filtered)** | |
|---|---|
| $Na_2HPO_4$-$7H_2O$ | 335 g/L |
| $KH_2PO_4$ | 170 g/L |
| $NH_4Cl$ | 134 g/L |
| $Na_2SO_4$ | 35.5 g/L |

| **50x 5052 (sterile filtered)** | |
|---|---|
| Glycerol | 250 g/L |
| Glucose monohydrate | 25 g/L |
| α–lactose | 100 g/L |

| **500x $MgSO_4$ (sterile filtered)** | |
|---|---|
| $MgSO_4$-7 $H_2O$ | 246.5 g/L |

| **5000 x Trace metals (sterile filtered)** | |
|---|---|
| $FeCl_3$-6 $H_2O$ | 14.41 g/L |
| $CaCl_2$-2 $H_2O$ | 2.94 g/L |
| $MnCl_2$-4 $H_2O$ | 1.98 g/L |
| $ZnSO_4$-7 $H_2O$ | 2.86 g/L |
| $CoCl_2$-6 $H_2O$ | 0.48 g/L |
| $CuCl_2$-2 $H_2O$ | 0.34 g/L |
| $NiCl_2$-6 $H_2O$ | 0.48 g/L |
| $Na_2MoO_4$ -2 $H_2O$ | 0.49 g/L |
| $Na_2SeO_3$ | 0.35 g/L |
| $H_3BO_3$ | 0.12 g/L |

**Other growth media:**

| **Luria-Bertani medium (LB) (autoclaved)** | |
|---|---|
| Peptone | 10 g/L |
| Yeast extract | 5 g/L |
| NaCl | 10 g/L |
| Adjust pH to 7.4 with NaOH | |

| **Terrific Broth (TB)** | |
|---|---|
| Tryptone | 12 g/L |
| Yeast extract | 24 g/L |
| Glycerol | 10 g/L |
| Sterile water | Fill up to 900 mL |
| *Autoclave* | |
| 10x TB salts | 100 mL |

| **10x TB salts (sterile filtered)** | |
|---|---|
| $KH_2PO_4$ | 23.1 g/L |
| $K_2HPO_4$ | 125.4 g/L |

| **Super Broth (SB) (autoclaved)** | |
|---|---|
| Tryptone | 30 g/L |
| Yeast extract | 20 g/L |
| MOPS | 10 g/L |
| Adjust pH to 7.0 with NaOH | |

| **Rich (sterile filtered)** | |
|---|---|
| Peptone | 10 g/L |
| Yeast extract | 5 g/L |
| NaCl | 5 g/L |
| Glucose monohydrate | 2 g/L |

| **SOC (sterile filtered)** | |
|---|---|
| Peptone | 20 g/L |
| Yeast extract | 5 g/L |
| NaCl | 0.58 g/L |
| KCl | 0.19 g/L |
| $MgCl_2$ x 6 $H_2O$ | 2.03 g/L |
| $MgSO_4$ x 7 $H_2O$ | 2.46 g/L |
| Glucose monohydrate | 3.97 g/L |
| Adjust pH to 7.0 with NaOH | |

**Additives for bacterial media**

| **1000x Kanamycin A (Kan) stock solution (sterile filtered)** | |
|---|---|
| Kanamycin A | 25 g/L |

| **1000x Ampicillin (Amp) stock solution (sterile filtered)** | |
|---|---|
| Ampicillin | 100 g/L |

| **1000x IPTG stock solution (sterile filtered)** | |
|---|---|
| Isopropyl β-D-1-thiogalactopyranosid | 1 M |

**Purification by IMAC under native conditions:**

| **Buffer A (Lysis buffer)** | |
|---|---|
| Tris HCl | 25 mM |
| NaCl | 300 mM |
| adjust pH to 8.0 with HCl | |

| **Buffer B (Elution buffer)** | |
|---|---|
| Tris HCl | 25 mM |
| NaCl | 300 mM |
| Imidazole | 250 mM |
| adjust pH to 8.0 with HCl | |

| **Regenerating Nickel Solution** | |
|---|---|
| NiCl2 | 100 mM |

| **Stripping buffer** | |
|---|---|
| EDTA | 100 mM |
| adjust pH to 7.5 with NaOH | |

**Purification by IMAC under denaturing conditions:**

| **Buffer B (Lysis buffer)** | |
|---|---|
| $NaH_2PO_4$ | 100 mM |
| Tris | 10 mM |
| Urea | 8 M |
| Adjust pH to 8.0 using NaOH | |

| **Buffer C (Washing buffer)** | |
|---|---|
| $NaH_2PO_4$ | 100 mM |
| Tris | 10 mM |
| Urea | 8 M |
| Adjust pH to 6.3 using HCl | |

| **Buffer D (Elution buffer 1)** | |
|---|---|
| $NaH_2PO_4$ | 100 mM |
| Tris | 10 mM |
| Urea | 8 M |
| Adjust pH to 5.9 using HCl | |

| **Buffer E (Elution buffer 2)** | |
|---|---|
| $NaH_2PO_4$ | 100 mM |
| Tris | 10 mM |
| Urea | 8 M |
| Adjust pH to 4.5 using HCl | |

**Note**: Due to the dissociation of urea, the pH of Buffers B, C, D, and E were adjusted immediately prior to use. All buffer solutions were sterile filtered.

| **R6 (Renaturing buffer 1)** | |
|---|---|
| Urea | 6 M |
| NaCl | 500 mM |
| Glycerol | 20% |
| Tris | 20 mM |
| Adjust pH to 7.4 using HCl | |

| R0 (Renaturing buffer 2) | |
|---|---|
| NaCl | 500 mM |
| Glycerol | 20% |
| Tris | 20 mM |
| Adjust pH to 7.4 using HCl | |

**Ion exchange chromatography**

| Buffer A | |
|---|---|
| Bis Tris | 25 mM |
| Adjust pH to 7.0 using HCl | |

| Buffer B | |
|---|---|
| Bis Tris | 25 mM |
| NaCl | 1 M |
| Adjust pH to 7.0 using HCl | |

**SDS-PAGE**

| 4x Lower buffer | |
|---|---|
| Tris | 1.5 M |
| Sodium dodecyl sulfate (SDS) | 0.4% |
| adjust pH to 8.8 with HCl | |

| 4x Upper buffer | |
|---|---|
| Tris | 500 mM |
| SDS | 0.4% |
| adjust pH to 6.8 with HCl | |

| 10% Ammonium peroxodisulfate (APS) | |
|---|---|
| APS | 100 g/L |
| Aliquote and store at -20°C | |

| 4x Sample buffer | |
|---|---|
| Tris | 200 mM |
| Dithiothreitol (DTT) | 300 mM |
| adjust pH 6.8 with HCl | |
| SDS | 4% |
| Glycerol | 40% |
| Bromophenol blue | 0.04% |

| 10x Electrophoresis buffer | |
|---|---|
| Tris | 250 mM |
| Glycine | 192 mM |
| SDS | 10 g/L |
| DO NOT adjust pH | |

| **Resolving gel 12%** | |
|---|---|
| Acrylamide/Bis Solution (30%) | 2.0 ml |
| 4x Lower buffer | 1.25 ml |
| $H_2O$ | 1.75 ml |
| Tetramethylethylenediamine (TEMED) | 2.5 µl |
| 10% APS | 25 µl |

| **Stacking gel 4.5 %** | |
|---|---|
| Acrylamide/Bis Solution (30%) | 300 µl |
| 4x Upper buffer | 500 µl |
| $H_2O$ | 1.2 ml |
| Tetramethylethylenediamine (TEMED) | 1 µl |
| 10% APS | 20 µl |

**Staining with Coomassie Brilliant Blue**

| **Coomassie Brilliant Blue (CBB) Staining Solution** | |
|---|---|
| CBB R-250 | 0.125% |
| Methanol | 50% |
| stir thoroughly, filter through folded filter | |
| 100% Acetic acid | 10% |
| aqua dest. | 40% |

| **Destaining solution** | |
|---|---|
| Methanol | 20% |
| 100% Acetic acid | 15% |
| aqua dest. | 65% |

| **Storing solution** | |
|---|---|
| Glycerol | 5% |
| 100% Acetic acid | 5% |
| aqua dest. | 90% |

**Western blot**

| **10x Transfer buffer** | |
|---|---|
| Tris | 250 mM |
| Glycine | 1.92 M |
| DO NOT adjust pH | |

| **1x Transfer buffer** | |
|---|---|
| 10x Transfer buffer | 10% |
| Methanol | 20% |
| dd $H_2O$ | 70% |

| **TBS** | |
|---|---|
| Tris | 50 mM |
| NaCl | 150 mM |
| Adjust pH to 7.6 with HCl | |

| TBST (Washing buffer) | |
|---|---|
| TBS | 1x |
| Tween 20 | 0.05% |

| MTBST 3% | |
|---|---|
| TBS | 1x |
| Tween 20 | 0.05% |
| Milk powder | 3% |

| MTBST 5% | |
|---|---|
| TBS | 1x |
| Tween 20 | 0.5% |
| Milk powder | 5% |

| AP buffer | |
|---|---|
| Tris | 100 mM |
| NaCl | 100 mM |
| Adjust pH to 9.5 with HCl | |
| $MgCl_2$ | 5 mM |

| Substrate solution | |
|---|---|
| Nitroblue tetrazolium chloride (0.487 mM) | 60 µL |
| 5-bromo-4-chloro-3-indolylphosphate (2.31 mM) | 60 µL |
| AP-buffer | 10 mL |

**ELISA**

| Coating buffer | |
|---|---|
| Sodium carbonate | 50 mM |
| Adjust pH to 9.6 with NaOH | |

| Blocking buffer | |
|---|---|
| TBS | 1x |
| Tween 20 | 0.05% |
| Milk powder | 3% |

| Dilution buffer | |
|---|---|
| TBS | 1x |
| Tween 20 | 0.05% |
| Bovine serum albumin (BSA) | 0.5% |

**Phage display**

| **TYE agar (autoclaved)** | |
|---|---|
| Agar | 15 g/L |
| NaCl | 8 g/L |
| Peptone | 10 g/L |
| Yeast extract | 5 g/L |

| **M9 minimal medium glucose plates** | |
|---|---|
| Agar | 15 g |
| dd$H_2O$ | 800 mL |
| *Autoclave* | |
| 5x M9 salts | 200 mL |
| 20% glucose | 10 mL |
| 1 M $MgSO_4$ | 1 mL |
| 1 M $CaCl_2$ | 0.1 mL |
| Thiamine | 1 mg |

| **5x M9 salts** | |
|---|---|
| $Na_2HPO_4$ | 64 g/L |
| $KH_2PO_4$ | 15 g/L |
| $NH_4Cl$ | 5 g/L |
| NaCl | 2.5 g/L |

| **2x TY medium** | |
|---|---|
| Peptone | 16 g/L |
| Yeast extract | 10 g/L |
| NaCl | 5 g/L |

| **PEG solution** | |
|---|---|
| Polyethylene glycol | 20 % |
| NaCl | 2.5 M |

| **H-top agar** | |
|---|---|
| Agar | 7 g/L |
| NaCl | 8 g/L |
| Tryptone | 10 g/L |

| **PBS** | |
|---|---|
| $Na_2HPO_4$ | 10 mM |
| $KH_2PO_4$ | 1.8 mM |
| KCl | 2.7 mM |
| NaCl | 137 mM |
| Adjust pH to 7.4 with HCl | |

| **Sodium phosphate buffer** | |
|---|---|
| $Na_2HPO_4$ | 118 mM |
| $NaH_2PO_4$ | 200 mM |
| Adjust pH to 7.5 with HCl | |

| **Blocking solution** | |
|---|---|
| Sodium phosphate buffer | 1x |
| Milk powder | 5 % |

| **Washing buffer** | |
|---|---|
| Sodium phosphate buffer | 1x |
| Tween 20 | 0.1 % |

| **TBSC buffer** | |
|---|---|
| Tris | 10 mM |
| NaCl | 137 mM |
| $CaCl_2$ | 1 mM |
| adjust pH to 7.4 with HCl | |

| **Trypsin solution** | |
|---|---|
| TBSC buffer | 1x |
| Trypsin | 100 mg/L |

| **Proteolysis reducing solution (20x)** | |
|---|---|
| BSA | 2 g/L |
| EDTA | 200 mM |

## 7.2. List of figures

## 7.3. List of tables

## 7.4. List of abbreviations

| | |
|---|---|
| Amp | Ampicillin |
| BSA | Bovine serum albumin |
| CBB | Coomassie Brilliant Blue |
| CD | Circular dichroism |
| CDR | Complementarity-determining regions |
| dAb | Domain antibody |
| $ddH_2O$ | Double distilled water |
| DLS | Dynamic light scattering |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| fMet | Formylmethionine |
| FPLC | Fast protein liquid chromatography |
| FT | Flow-through |
| Fv | Variable fragment (of an antibody) |
| HisC | Hexa-Histidine tag on C-terminus |
| HisN | Hexa-Histidine tag on N-terminus |
| HRP | Horseradish peroxidase |
| IgE | Immunoglobulin E |
| IMAC | Immobilized metal affinity chromatography |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| Kan | Kanamycin |
| mAb | Monoclonal antibody |
| MALDI-TOF | Matrix-assisted laser desorption/ionization time of flight |
| mAU | Milli absorbance units |
| MS | Mass spectrometry |
| Ni-NTA | Nickel nitrilotriacetic acid |
| OD | Optic density |
| OPD | o-Phenylenediamine dihydrochloride |
| PEG | Polyethylene glycol |
| pfu | Plaque forming unit |
| pI | Isoelectric point |
| PR-10 | Pathogenesis related class 10 proteins |
| rpm | Revolutions per minute |
| RT | Room temperature (22-24 °C) |
| sdAb | Single domain antibody |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TTH | TTHA0849 from *Thermus thermophilus* |