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GENEESKUNDE

master in de biomedische wetenschappen: bio-elektronica en nanotechnologie

2010
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Masterproef

Covalent and site-specific coupling of nanobodies onto solid substrates for biosensor applications

Promotor :
dr. Ties STEEN REDEKER
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting bio-elektronica en nanotechnologie

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Summary

Nowadays detection of (bio)chemical parameters at low concentrations is becoming increasingly important. Biosensors and protein arrays are interesting devices to detect and measure them. For these devices, oriented immobilization of the peptide-based receptors plays an important role. Proteins however, are often immobilized in a random orientation. This means that not all binding sites are available. In addition, immobilization can disturb the structure resulting in a loss of activity of the proteins. Therefore, the main goal of this study was to introduce bioorthogonal "click" functionalities into proteins. These "click" functionalities will allow for oriented and covalent attachment of proteins onto solid substrates.

The modification will be introduced by making use of an enzymatic system called the intein. This will catalyze the addition of a cysteine with a "click" functionality to the C-terminus of proteins.

First a proof of concept was done with maltose binding protein (MBP). MBP was used first since it is a protein which is easy to manipulate and is well described in literature. A MBP-intein-chitin binding domain (CBD) fusion protein was first expressed in *E.coli* cells and extracted. The yield for this expression was 50 mg/l culture. The solubility of the MBP fusion protein was assessed via SDS-PAGE. After checking the solubility, the MBP fusion protein was purified with chitin bead affinity chromatography and modified on column with a cysteine modified with an alkyne functionality. After modification, a click reaction with the modified MBP and a biotin modified with an azide functionality was performed in solution. This was transferred to a polyvinylidene fluoride (pvdf) membrane via Western blot. From this Western blot it was shown that the click reaction was successful and the degradation of MBP after modification was limited.

Next the same experiments were performed with a BCII10 nanobody. BCII10 is a nanobody which will recognise ß-lactamase. Because of stability reasons, the nanobodies are targeted to the periplasm after expression by a pelB tag. BCII10 nanobodies were successfully expressed in *E.coli* cells. After expression, a few problems were encountered such as a high degree of in vivo cleavage and formation of inclusion bodies. From literature, it was clear that the susceptibility to in vivo cleavage was dependant on the final C-terminal amino acid(s). For the BCII10 nanobody this is a serine which is very susceptible to in vivo cleavage. Therefore BCII10 nanobodies were engineered in which this serine is changed with (an)other amino acid(s). First, the constructs were cloned in a pTXB1 vector which allows for expression in *BL21(DE3)pLysS E.coli* cells. The ideal induction conditions for expression of soluble nanobodies were overnight induction at 28°C with 1 mM IPTG. The solubility and degree of in vivo cleavage were assessed after induction and were promising. However, there still was much formation of inclusion bodies Therefore, constructs were cloned in a pHEN6a vector which allows for expression in *WK6 E.coli* cells. These cells have a lower expression yield which should be beneficial to reduce formation of inclusion bodies. A protocol was optimized to prepare a periplasmatic extract via an osmotic shock. The nanobodies were purified out of the periplasmatic extracts via affinity chromatography with chitin beads. The yield of this purification was 50 µg/l culture. This is a low yield and can be explained because either there is a lot of formation of BCII10 nanobody inclusion bodies or because the periplasmatic trafficking was not possible.

Samenvatting

Tegenwoordig wordt de detectie van laag geconcentreerde (bio)chemische parameters steeds belangrijker. Biosensoren en eiwit arrays zijn interessante instrumenten om deze parameters te detecteren en meten. Bij deze instrumenten speelt de georiënteerde immobilisatie van peptide-gebaseerde receptoren een belangrijke rol. De receptoren worden echter vaak met een willekeurige oriëntatie geïmmobiliseerd. Dit houdt in dat niet alle bindingsplaatsen beschikbaar zijn. Daarbij komt ook dat willekeurige immobilisatie hun structuur kan verstoren wat tot een verlies van functie kan leiden. Daarom is het hoofddoel van dit onderzoek bioorthogonale "click" functionaliteiten te introduceren in eiwitten. Deze "click" functionaliteiten kunnen vervolgens gebruikt worden voor de georiënteerde, covalente koppeling van eiwitten op vaste dragers.

Om deze modificatie te introduceren wordt het inteïne systeem gebruikt. Dit zal de additie van een cysteïne gemodificeerd met een "click" functionaliteit aan de C-terminus van een eiwit katalyseren.

Eerst werd het concept van deze studie aangetoond met maltose binding protein (MBP). MBP werd gebruikt omdat het een goed handelbaar eiwit is dat uitgebreid beschreven is in de literatuur. Eerst werd een MBP-inteïne-chitine binding domein (CBD) fusie eiwit tot expressie gebracht in *E.coli* en geëxtraheerd. De opbrengst hiervan bedroeg 50 mg/l cultuur. De oplosbaarheid van het MBP fusie eiwit werd onderzocht door SDS-PAGE. Hierna werd het fusie eiwit opgezuiverd met behulp van chitine bead affiniteitschromatografie en gemodificeerd met een cysteïne gefunctionaliseerd met een alkyne functionaliteit. Vervolgens werd een "click" reactie in oplossing gedaan met het gemodificeerde MBP en een biotine gefunctionaliseerd met een azide. Dit werd overgebracht naar een polyvinylidene fluoride (pvdf) membraan via Western blotting. Hiermee werd aangetoond dat de "click" reactie succesvol was en dat de degradatie van MBP na de modificatie beperkt was.

Vervolgens werden dezelfde experimenten uitgevoerd met het BCII10 nanobody. Dit is een nanbody gericht tegen ß-lactamase. Om wille van stabiliteitsredenen, werd het nanobody naar het periplasma getarget door middel van een pelB tag. BCII10 nanobodies werden succesvol tot expressie gebracht in *E.coli*. Na de expressie werden er echter enkele problemen vastgesteld. Er was veel in vivo afbraak van het fusie eiwit en er werden inclusie lichamen gevormd. Uit de literatuur blijkt dat de in vivo afbraak afhankelijk was van het laaste C-terminaal aminozuur. Daarom werden BCII10 nanobodies gemaakt waarin het natieve, C-terminale serine vervangen werd door (een) ander(e) amino zu(u)r(en) die hier minder gevoelig voor zijn. Eerst werden de constructen gekloond in een pTXB1 vector. Deze vector laat expressie in *BL21(DE3)pLysS* cellen toe. De ideale inductie omstandigheden waren overnacht bij 28°C met 1 mM IPTG. Vervolgens werd de graad van in vivo afbraak bestudeerd. Deze was verbeterd maar er werden nog steeds veel inclusie lichamen gevormd. Daarom werden constructen in een pHEN6a vector gekloneerd voor expressie in *WK6* cellen. Deze cellen hebben lagere expressie niveaus wat de oplosbaarheid ten goede zou moeten komen. Een protocol werd geoptimaliseerd voor het bereiden van periplasmatische extracten via een osmotische shock. Na extractie werden de nanobodies opgezuiverd via chitine bead affiniteitschromatografie. De opbrengst na opzuivering bedroeg 50 µg/l cultuur. Dit is een lage opbrengst en is te verklaren door het feit dat een groot deel van de nanobodies inclusie lichamen vormt of doordat de transfer naar het periplasma niet mogelijk was.

Table of contents

1. List of abbreviations

IgG: Immunoglobulin G
(k)Da: (kilo)Dalton
Fab: Fragment, antigen binding domain
Fc: Fragment, crystallisable domain
V_L/V_H: Light chain/Heavy chain
HCAb: Heavy-Chain Antibody
sdAb: Single Domain Antibody
scFv: Single Chain Variable Fragment
CDR: Complementarity Determining Region
His_6: Hexahistidine
EDC/NHS: 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide/N-Hydroxy Succinimide
NTA: NitriloTriacetic Acid
E.coli: *Escherichia coli*
NCL: Native Chemical Ligation
ELISA: Enzyme Linked ImmunoSorbent Assay
SPR: Surface Plasmon Resonance
RU: Resonance Units
PCR: Polymerase Chain Reaction
LB medium: Luria Bertani medium
TB medium: Terrific Broth medium
SDS-PAGE: Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
MESNA: sodium 2-MercaptoEthaneSulfoNAte
DTT: DiThioThreitol
PIPES: PIPerazin-1,4-bis-(2-EthaneSulfonic acid)
IPTG: IsoPropyl β-D-1-ThioGalactopyranoside
APS: Ammonium PerSulfate
BSA: Bovine Serum Albumin
NBT/BCIP: NitroTetrazolium Blue chloride/5-Bromo-4-Chloro-3-Inodyl Phosphate disodium salt
EDTA: EthyleneDiamineTetraAcetic acid
HEPES: 4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid
TCEP: Tris(2-CarboxyEthyl)Phosphine
TEMED: TEtraMethylEthyleneDiamine
CBD: Chitin Binding Domain
MBP: Maltose Binding Protein
B-PER: Bacterial Protein Extraction Reagent
TBTA: Tris-(BenzylTriazolylmethyl)Amine
PVDF: PolyVinyliDene Fluoride
pLysS: BL21DE3pLysS
TBS: Tris Buffered Saline
VUB: Brussels University

2. Acknowledgements

Many years have passed since I started this education. Finally, all those years of hard studying have culminated into this: a master thesis. Eagerly, I started my last few months as a student. During the internship, there were moments of joy when experiments were successful and times of frustration, when something was not working at all or in the way we wanted them to work. Now that the internship is almost over, I can look back to an exciting time in the biomolecule design group of the institute for materials research.

Because a thesis is a lot more then just writing a 50 page long paper, there are a few people that I would like to thank. Without their help and advice, the results would not have been of the same quality as they are now.

To start of I would like to thank Dr. Erik Steen Redeker, Drs. Gunter Reekmans and Drs. David Cortens for their explanations and helping with occurring problems. Without them, the working atmosphere would not have been the same. The enthusiasm they had when discussing data or other topics was catchy. Finally I would also like to give special thanks to Erik and Gunter for critically reviewing this thesis and giving suggestions to further improve it.

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Last but not least I want to thank my parents, my brother and the rest of my family for their interest and unconditional support during my whole education. Special thanks to my parents for giving me this opportunity and for making my life as easy as it has been during this period.

Brecht Willems

3. Introduction

Nowadays detection of a whole plethora of (bio)chemical parameters at low concentrations is becoming increasingly important for applications such as protein arrays and biosensors. This is why biosensors are becoming interesting tools for their detection. They allow for fast measurements of these biochemical reactions, both qualitatively and quantitatively. In a quantitative setup, biosensors can measure allergen levels in food whereas in a qualitative setup they can be used for detection of microbial contamination.

Biosensors are composed of a biological recognition element for an analyte in a medium. The presence of the biosensor-target in the medium will cause a change in the output signal through a matching combination of the recognition element and a physical transducer system. This change is converted by the transducer to an electric signal. The output from the transducer is then amplified, processed and displayed as an electric signal. An overview is shown in figure 1.[1-2]

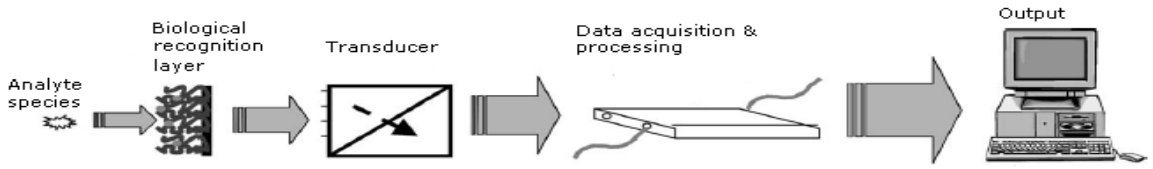

Figure 1: Schematic overview of a typical biosensor. [1]

Biosensors can measure biomarkers rapidly and at very low concentrations. Fast detection is obviously a big advantage in a diagnostic setting since in some cases, it can reduce or avoid hospitalization time, treatment costs and sometimes even permanent symptoms. In order for this fast detection to be possible however, the biological recognition layer has to be functionally active and sensitive. After all, this layer is the interface between the sensing part and the analyte. Typically, the biological recognition layer is built up out of a biological recognition element and a coupling layer. This coupling layer is optional however since coupling of the biological recognition element can also occur directly to the sensor surface. For example the coupling of DNA onto a diamond surface treated with hydrogen plasma.

Examples of biological target recognition elements are DNA (aptamers) or proteins (antibodies, enzymes).[3-6] These biological recognition elements are attached to the sensor surface. Attachment can be done in both a covalent or non-covalent way, oriented or random as described in table 1. The sensor surface is often a thin gold layer spincoated onto a glass substrate. Gold is used for its biocompatibility and surface chemistry possibilities such as chemisorption and self assemblage of monolayers.[7]

This study will focus on the use of proteins, more specifically nanobodies, in biosensor applications as biological recognition element. Biosensors with an antibody as receptor for their target are also called immunosensors.

Proteins are macromolecules that compromise about 60% of a typical mammalian cell.[8] They constitute most of a cell's dry mass and consist of polypeptides. Polypeptides are built up out of amino acids. These amino acids are linked together in a linear chain by peptide bonds as shown in figure 2. Proteins are the building blocks of the cell and perform virtually all of the cell's functions. They play an important role in both health and disease. In order for a person to be healthy, proteins need to function correct. This requires them to be folded into the appropriate conformation. Molecular chaperones can help proteins achieve this conformation.[9]

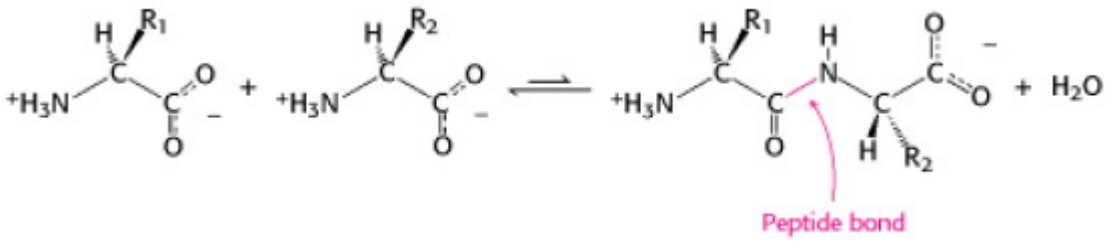

Figure 2: Representation of a peptide bond between 2 amino acids.[10]

When the human body encounters foreign antibodies or substances, a special type of proteins play an important role.[11] These proteins are antibodies. Antibodies are produced by the human body in response to the presence of foreign molecules, known as antigens. They circulate in the blood and lymph where they recognize and bind their antigen target to evoke an immune response by the body. The antigen-antibody complex is a very strong, specific interaction which makes these molecules such an interesting tool for biosensors.

Even though there is a big variety of antibodies, they share some general characteristics. Antibodies are all able to bind both antigens and specialized cells from the immune system. Structurally, conventional antibodies are composed of 2 heavy chains (±55 kDa) and 2 light chains (±25 kDa). They are Y-shaped as is shown in figure 3.[12] The antibodies that belong to the immunoglobulin G (IgG) class, which is the most abundant class of antibodies in humans, are often used to describe their structure. An IgG antibody is composed of different protein domains. From these domains, there are 2 that are identical to each other. These are called the Fab domains and form the 2 arms of the Y-shaped IgG antibody as shown in figure 3. The Fab domain contains the antigen binding site. The other domain is called the Fc domain and forms the base of the Y-shape. This part of the antibody plays an important role in directing the immune response against antigens that get encountered by the antibody.[12]

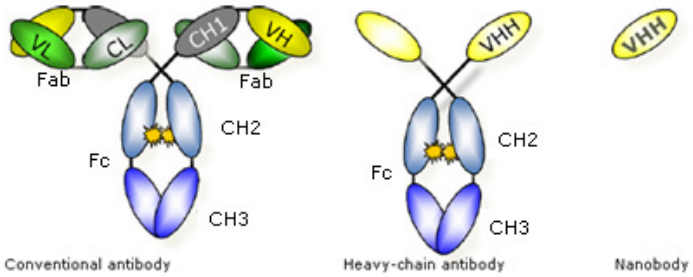

Figure 3: Comparison of nanobodies with conventional and heavy-chain antibodies.[13]

The antibodies described so far appear in humans among others. In some mammals like camelids however, a second type of antibodies is found in the blood and lymph. Apart from the conventional antibodies, some of the antibodies present in camelids lack a light chain shown as VL on figure 3. Because of the lack of their light chain these antibodies are called heavy-chain antibodies (HCAbs). HCAbs are composed of only 3 globular domains as opposed to conventional antibodies which have 4 globular domains.[13] The fourth domain is spliced out while processing the HCAb mRNA.[14] They contain a single variable domain (VHH) and two constant domains (CH2 and CH3). Because of their natural function, to protect the organism from disease, antibodies are very interesting proteins to use as therapeutical agents for a wide spectrum of diseases. In the past, mainly monoclonal antibodies have been used since they are very antigen-specific and show a high affinity for their target. These full-scale antibodies have some disadvantages however. They are big, have a complex nature and because of their immunogenicity they encounter a lot of restraints when being used for therapy. They are prone to destabilization as well. Because of these reasons, a lot of effort has been put in reducing their size.[2] This has resulted in the development of antigen-binding fragments (Fabs) and variable fragments (Fv). The simplest variant of them all however is the single-domain antibody (sdAb), also known as nanobody or VHH, which is retrieved from heavy-chain antibodies.[2]

Nanobodies are particularly interesting as they offer a lot of advantages over conventional antibodies. They have a high expression yield and are easy to produce since they are so well expressed in microorganisms.[15] Nanobodies are only 11-15 kDa in size which is roughly ten times less then conventional antibodies while retaining the full antigen-binding capacity of the original heavy chain antibodies in their naturally occurring counterparts.[16] This allows for attachment of more nanobodies onto the same surface area as compared to when conventional antibodies would be used. They are more stable and have an increased shelf life. Their solubility is better as well. Nanobodies do not have the tendency to aggregate unlike single chain variable fragments (scFv) and can survive in harsh conditions such as the acidic environment of the stomach.[2,17] It is easier to make immunoconjugates of nanobodies by joining the genes of one nanobody with those of another nanobody, toxin or enzyme. On top of that nanobodies target epitopes that are uncommon, also known as hidden epitopes, or epitopes which are not accessible for conventional antibodies due to steric hindrance. Nanobodies can penetrate into tissues where conventional antibodies cannot go.[18] Also, they are more homogeneous which means that they are less prone to spontaneous dimerisation.[16]

Apart from biosensors, nanobodies can be used for a wide range of other applications. A first application is to use them for research in different therapeutic or diagnostic settings.[13,16,19] Nanobodies allow for fast drug discovery and development.[20] Many diseases have been treated successfully using nanobodies. Some examples are sleeping sickness and cancers. [21-23] Apart from cancer therapy, nanobodies can also be used for diagnostic cancer tests. Saerens et al. described a nanobody that can discriminate between different forms of prostate-specific antigen which helps in determining the stage of prostate cancer in a patient.[18] Nanobodies can be used in a biotechnological setting. For example, a fluorescent tag can be attached to nanobodies to allow for targeting of any desired antigen in a living cell.[24]

During this study a nanobody with affinity for β-lactamase will be used. This nanobody is known as the BCII10 nanobody and was chosen for its very high degree of stability (47 kJ/mol), good

expression yield (5 mg/ l culture in *E.coli)* and the property that it remains functional in absence of a disulfide bridge. The disulfide bridge helps forming the loop structure of the nanobody. In most nanobodies, the CDR1 and 3 domains contain a cysteine. These cysteines form a disulfide bridge. The BCII10 nanobodies however form an exception: As can be seen here, the BCII10 nanobodies also have a disulfide bridge however it is not connecting CDR1 and 3, but it connects 2 cysteins upstream of CDR1 and 3. The disulfide bridge of the BCII10 nanobody is shown as the orange line on figure 4. On the bottom part of figure 4, the 3D structure of the nanobody is shown. It can be deducted from this structure that modifications at the C-terminus will have little effect on the functioning of the nanobody whereas modifications at the N-terminus will drastically affect the binding capacity of the nanobody via sterical hindrance. By modifications of the N-terminus, the binding pocket of the nanobody can become inaccessible because of steric hindrance. The spatial structure is, as shown in figure 4, very important for the functioning and the activity of the nanobody.[13,25,26]

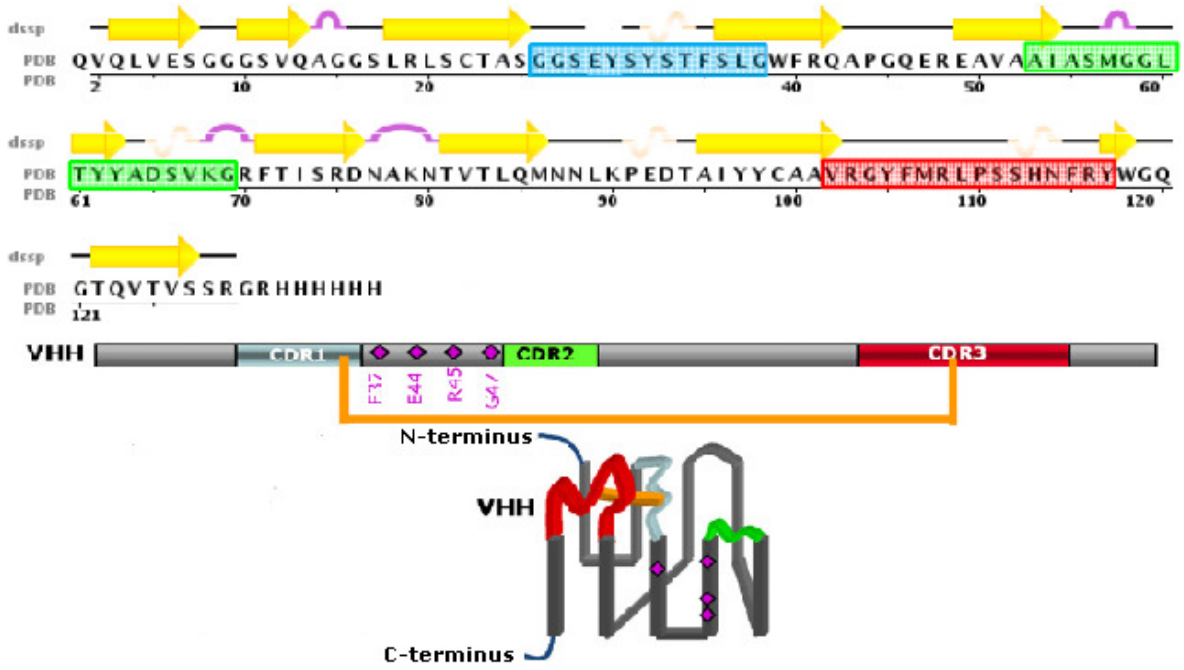

Figure 4: Amino acid sequence of the BCII10 nanobody with a C-terminal His_6 tag. Depicted in different colours are the CDR domains. At the bottom of the picture, a schematic overview of the 3D structure is shown.[13]

Nowadays, immobilization of proteins, for example nanobodies, onto substrates has gained much interest because of its importance in protein activity. Different immobilization strategies are shown in table 1. Applications such as biosensors and affinity chromatography require the protein to be attached to a surface in order to be successful. The proteins can be attached using several methods. Traditionally, proteins are immobilized non-specifically to surfaces. This results in proteins oriented randomly on the surface. The drawback of this random orientation is that proteins may no longer be functional. On top of that, the proteins are only attached to the surface via weak interactions. This results in dissociation of the proteins from the surface.[27] Another method, in which proteins can be attached randomly to surfaces, is via naturally occurring thiol or amine groups. This attachment is in most cases still not oriented because a protein can have several of these groups. Immobilization on the surface however occurs via covalent bonds which is already a

stronger interaction. The use of thiol or amine groups allows for more control over the coupling reaction.[28] The advantage of random attachment is that these methods are often straightforward and easy to perform.[27-29] The disadvantage of random protein immobilisation is that it gives rise to proteins with a reduced activity and affinity because the protein folding can be affected.

The most feasible way to immobilize proteins is to attach them in an oriented way via a stable, covalent bond. An overview of different methods is shown in table 1.

Table 1: Overview of different chemical methods for attachment of biomolecules to surfaces. Depicted in red is the strategy that will be employed during this project.

	Random Coupling	**Oriented Coupling**
Non-covalent Attachment	Pysical absorption	Hexahistidine tail
Covalent Attachment	EDC/NHS coupling	Bioorthogonal chemisty ("Click" chemistry)

As mentioned in table 1, a non-covalent way of attaching a protein in an oriented way to a surface is by use of a hexahistidine or His_6 tagged proteins using a nitrilotriacetic acid (NTA) treated surface. There are some drawbacks to this method however. The NTA/His_6 interaction is a slow immobilization method in which continuous dissociation of proteins from the surface occurs.[30] This will be detrimental when one wants to store a biosensor for a longer period of time until it is being used.

The advantages of oriented and covalent coupling are that the binding sites of the proteins (and the nanobody) will be available. This is beneficial for the activity of the protein and consequently also the sensitivity of the biosensor and the surface. Another advantage is that via oriented coupling, spacer and linker molecules can be used.[28] Once proteins have been attached to a surface, this functionalized surface will interact with their surroundings. Compared to DNA however, there still is a lot of work to be done for site-specific protein immobilization. An example of oriented DNA coupling is the ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxy succinimide (NHS) coupling. For proteins however, this task is much more complex. If the EDC/NHS method is used for proteins, random immobilization will occur. This is because proteins have a high amount of reoccurring chemical groups as compared to DNA. Because of their reoccurrence, these groups do not allow for oriented attachment to substrates. Apart from this random orientation problem a lot of other problems often occur when using EDC/NHS coupling methods for proteins. Some of these are related to the surface type, the pH of the buffer or due to the structure and molecular properties of the protein. The stability of the active tertiary structure of a protein is often very low. [28, 31]

A solution to random protein immobilization can be found by using bioorthogonal chemistry. Using bioorthogonal chemistry a unique chemical functionality, also called a bioorthogonal group, can be introduced in the protein. A bioorthogonal group does not occur naturally in proteins and is often just a single functional group such as an azide group. Azide functionalities are absent from natural proteins, nucleic acids and carbohydrates.[32] By introducing this bioorthogonal group site-specifically, for example at the C-terminus of a protein, it can be targeted for oriented immobilization of the protein on surfaces. Bioorthogonal chemistry thus allows for attachment of chemical tags to proteins which do not display cross-reactivity with other biomolecules. Other advantages of bioorthogonal chemistry are that it is a versatile technique which can be applied to

different kinds of molecules. It can also be used to label targets in living cells and on top of that, the chemical tags involved are sufficiently small allowing tolerance by biosynthetic enzymes.[33] By using this bioorthogonal group for surface attachment, the attached protein will be oriented since the bioorthogonal group occurs only once in the protein.[8] Other applications of bioorthogonal chemistry are metabolic labelling or activity-based labelling of enzymes. Bioorthogonal chemistry can also be used for modification of other biomolecules such as glycans, lipids and nucleic acids which will not be discussed here.[8]

The goal of this study is to introduce a site-specific, bioorthogonal "click" functionality at the C-terminus of the nanobody using an in vitro post-translational modification technique. A BCII10 nanobody, directed against ß-lactamase, will be modified site-specifically with a bioorthogonal "click" functionality to allow for covalent and oriented immobilization of the nanobody onto solid substrates.[17] The C-terminus is chosen because manipulations here have no effect on the functionality and activity of the nanobody. The in vivo domain is being researched in the biomolecule design group as well. Here an in vivo system in *Saccharomyces Cerevisiae* that introduces bioorthogonal "click" functionalities in nanobodies will be developed.

"Click" chemistry is a set of powerful, highly reliable and selective reactions that allow for fast synthesis of new compounds through heteroatom links.[34,35] "Click" reactions usually have a high yield, are wide in scope, stereospecific and produce byproducts that can be easily removed. "Click" chemistry is an easy-to-perform biocompatible method for the formation of stable covalent bonds. Amongst "click" chemistry there are a lot of different reactions. A well known "click" reaction, that also will be used in this study, is the Huisgen 1,3-dipolar cycloaddition as shown in figure 5. Through this reaction, an azide moiety is coupled to an alkyne in presence of a catalyst (e.g. Cu^{I}). These azide moieties do not readily react with water and are resistant to oxidation. In addition, they are electrophiles that do not react with amines from peptide bonds, which are abundant in a biological system. It has also been shown that organic azides have no intrinsic toxicity.[8] "Click" reactions have been successfully used for immobilization of modified proteins containing an azide or alkyne functionality onto alkyne or azide-coated surfaces.[36]

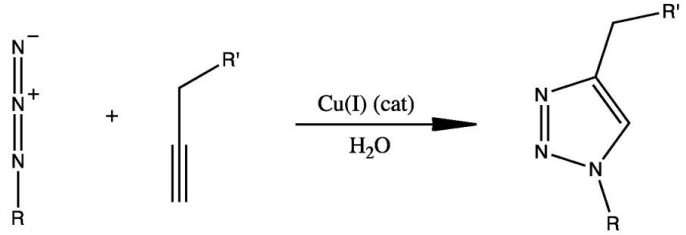

Figure 5: The copper (I) catalyzed [3+2] Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes.[37]

The in vitro post-translational modification method used in this present study makes use of the intein system. Inteins are enzymes with a self-splicing activity. This activity is autocatalytic and leaves no intein fragments in the nanobody. The intein system is a simple, fast, high-throughput method that allows for site-specific modification of proteins at the C- or N-terminus. Some examples of these modifications are biotinylation, introduction of a "click" functionality or attachment of a peptide. In vitro modification relies on native chemical ligation (NCL) between a reactive thioester group at the C-terminus of a protein and the N-terminus of a cysteine. This cysteine can be modified with a bioorthogonal group.

Modification of proteins using the intein system is started by transformation of the plasmid vector containing the desired protein cDNA, the intein DNA and the chitin binding domain DNA. Expression of the protein is then activated in the transformed cell type (Step 1). An example of cells that can be used for these experiments are *E.coli* cells. For this study, it is important that the intein is situated C-terminally with respect to the protein. The intein domain can also be used for N-terminal modification of proteins but this will not be discussed here. After expression of the proteins, the chitin binding domain (CBD) allows for purification of proteins by use of affinity chromatography with chitin beads (figure 6A). The protein sticking on the column can then be eluted and modified by addition of a thiolated substrate (e.g. MESNA) and a modified cystein to the column. The inteins catalytic activity will shift the chemical equilibrium present from a peptide bond towards a stable C-terminal thioester. In other circumstances the N-S shift strongly favours peptide bond formation. After cleavage of the intein by addition of a thiolated substrate and a modified cysteine, the thiolated substrate will be hydrolized from the C-terminus of the nanobody whereas the cysteine remains covalently attached to the nanobody (figure 6B).[38, 39] The activity of the intein will result in ligation of the protein and modified cysteine. This ligation reaction will yield a native peptide bond under aqueous conditions. After the transthioesterification with the cysteine, an S-N acyl transfer will regenerate the thiol and form a stable amide bond. However, this thiol can cause problems. It can be an agent in undesirable side reactions since it is one of the most reactive residues towards disulfide bonds.[32] An example of such a side reaction is a redox reaction with O_2. Research by Kalia and Raines has shown that this problem can be avoided by using nitrogen nucleophiles instead of sulphur nucleophiles. An example of a modified cysteine is a cysteine modified with a "click" functionality. The "click" functionality acts as a bioorthogonal group and will allow for "click" chemistry to be performed with the modified nanobody.

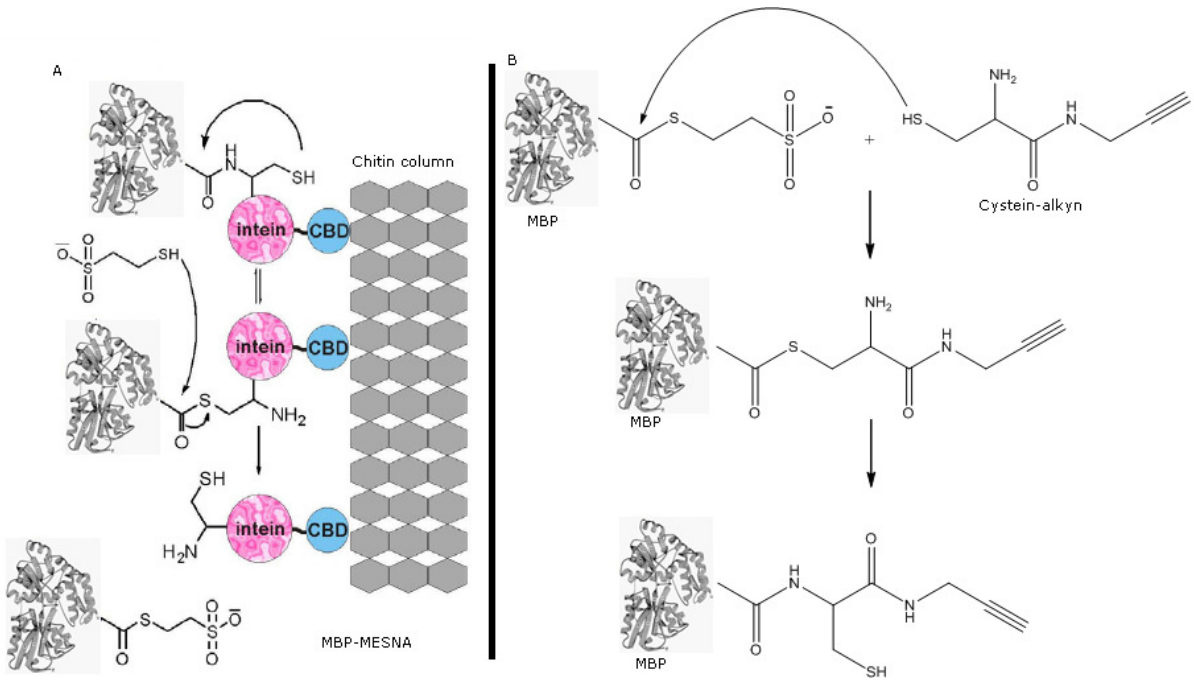

Figure 6: Overview of the functioning of the whole in vitro system which makes use of the self-cleavage activity of an intein. Part A shows the cleavage of the bond between MBP and the intein. Part B shows the C-terminal modification of MBP via a cysteine modified with an alkyne.[39]

After modification of the nanobodies with a bioorthogonal group, it is important to verify if they are still active. This can be done by making use of techniques such as Quartz Crystal Microbalance (QCM), ELISA, ellipsometry or SPR. The main emphasis of the present study was to introduce site-specific, bioorthogonal "click" functionalities in the BCII10 nanobody that allow for oriented and covalent attachment to solid substrates e.g. a biosensor surface.

First, a proof of concept will be given with maltose binding protein (MBP). MBP is chosen because it is well described in literature and is an easy-to-manipulate protein. For expression, a pMXB10 vector containing MBP will be used. Protein expression will be done in *BL21(DE3)pLysS E.coli* cells followed by purification out of B-PER extracts with chitin bead affinity chromatography. B-PER extracts are a combination of cytoplasmatic and periplasmatic extracts. At the same time of purification, a C-terminal modification will be introduced by making use of the intein system as shown in figure 6. The modification will be done with a cysteine modified with an alkyne functionality. Next a "click" reaction will be performed in solution with the modified MBP and biotin modified with an azide. Finally the "click" reaction will be checked by Western blot analysis.

The same strategy will be employed to modify BCII10 nanobodies with a bioorthogonal "click" functionality. Because the stability of nanobodies often depends on formation of disulfide bridges between cysteine residues, it is important that they are targeted to the periplasm. This is done by a pelB tag which is present at the N-terminus of the nanobody and gets cleaved off after periplasmic transfer. First, BCII10 nanobodies will be expressed by *BL21(DE3)pLysS E.coli* cells. Here it is possible that some problems occur. The first being in vivo cleavage of the nanobody fusion protein and the second being formation of nanobody inclusion bodies. For solving possible in vivo cleavage problems, it was found after literature search, that the susceptibility of the fusion proteins to in vivo cleavage is dependant on the last C-terminal amino acid(s).[41-43] To reduce in vivo cleavage, different BCII10 nanobody constructs will be developed in which the C-terminal serine is changed to (an)other amino acid(s). These constructs are shown in appendix 7. Not all the constructs will be used in the present study due to time constraints. The SA, SSA and LEY constructs however will be cloned in a pTXB1 vector (SA and SSA) or a pMXB10 vector (LEY). These vectors allow for expression in *BL21(DE3)pLysS* cells. In order to assess possible solubility problems (inclusion bodies), a balance will have to be found between the induction temperature, duration and concentration of IPTG. The in vivo cleavage will be assessed after induction with SDS-PAGE and Western blot. B-PER and periplasmatic extracts will be made of the modified nanobodies and purified with chitin bead affinity chromatography. For this periplasmatic extraction, two different protocols will be used and optimized. Another way to solve possible inclusion body formation will be by cloning the SA construct into a pHEN6a vector. This vector allows for expression in *WK6 E.coli* cells. *WK6* cells have a reduced expression as compared to *pLysS* cells which should be beneficial for the solubility of the nanobodies. Finally the goal of the present study is to obtain high yields of pure, active, C-terminally modified BCII10 nanobodies.

4. Materials

4.1 Chemicals & devices

For the experiments described, agarose, gel electrophoresis equipment, SDS-PAGE and Western blot equipment were obtained from Bio-Rad (Nazareth Eke, Belgium). Bacto Yeast, Bacto Tryptone and agar were purchased from Becton Dickinson and company (MD, USA). NaCl, D-glucose, antibiotics, $CaCl_2$, glycerol, KAc, $MnCl_2$, piperazin-1,4-bis-(2-ethanesulfonic acid) (PIPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), ammonium persulfate (APS), trizma base, sodium dodecyl sulphate (SDS), polyoxyethylenesorbitan monolaurate (Tween-20), bovine serum albumin (BSA), nitrotetrazolium blue chloride (NBT), 5-bromo-4-chloro-3-inodyl phosphate disodium salt (BCIP), ethylenediaminetetraacetic acid (EDTA), sucrose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dithiothreitol (DTT), sodium 2-mercaptoethanesulfonate (MESNA), tris(2-carboxyethyl)phosphine (TCEP), $CuSO_4$, glycine, NaH_2PO_4, KH_2PO_4, tetramethylethylenediamine (TEMED), bromo-chloro-indolyl-galactopyranoside (xGal) and brilliant blue G and R were obtained from Sigma-Aldrich (Bornem, Belgium). $MgCl_2$, K_2HPO_4 and KCl were purchased from Fluka (Bornem, Belgium). Taq polymerase, restriction enzymes and their corresponding buffers were provided by Fermentas (St. Leon Roth, Germany). The TA cloning kit including pCR2.1 and T4 DNA ligase was obtained from Invitrogen (Merelbeke, Belgium). Finally NaOH and Na_2HPO_4 were provided by Acros Organics (Geel, Belgium).
Other providers of the materials are stated elsewhere.

4.2 Cell culture media and agar plates

Different types of *E.coli* cells were cultured in two different types of media. The first medium was Luria Bertani (LB) medium. This was supplemented with antibiotics where necessary. The other medium was Terrific Broth (TB) medium. This medium was supplemented with 20% D-Glucose, antibiotics where necessary and $MgCl_2$. After the media were made they were autoclaved and stored at 4°C. LB medium was used for *E.coli* growth and propagation, competent cell preparation, transformation and plasmid DNA preparation and production. TB medium was used for protein expression. The exact composition of the media is mentioned in appendix 1.
For the agar plates only LB medium was used. To make the LB medium become solid, 15 grams of agar was added per liter of LB medium prior to autoclaving. After autoclaving the medium was allowed to cool down and antibiotics were added where necessary. Plates were poured in a sterile environment to avoid contamination. The final concentration of the different antibiotics used in the experiments were ampicillin in water (100 µg/ml), chloramphenicol in ethanol (34 µg/ml) or tetracycline in 50% ethanol (12,5 µg/ml). After the plates were poured they were stored at 4°C. For the *TOP10F'* cells the media were supplemented with tetracycline. The media of the *BL21(DE3)pLysS* cells were supplemented with chloramphenicol. The *WK6* cells had no innate resistance to antibiotics. The pTXB1, pCR2.1, pMXB10 and pHEN6a vectors all had an ampicillin resistance gene.

5.Methods

5.1 Molecular biology methods

5.1.1 Preparation of chemically competent cells

For preparation of competent *E.coli,* two different protocols were used. *E.coli* cells had to be made competent in order to transform plasmid DNA into them.

The first protocol used $CaCl_2$. This method was used for all types of cells mentioned throughout the report. A single colony was picked from a plate containing *E.coli* cells. This colony was placed in a sterile flask containing 30 ml of LB medium and antibiotics depending on the type of *E.coli* cells. The culture is allowed to grow overnight at 37°C while shaking at 220 rpm in a KS 400 ic control incubator shaker (IKA). After overnight growth, 1 ml of the overnight culture was inoculated in a flask containing 100 ml fresh LB medium and antibiotics where required. This culture is grown at 37°C while shaking at 220 rpm until the optical density at 600 nm (OD_{600}) was approximately 0.4. The density was measured using a visible Novaspec Plus spectrophotometer (Amersham Biosciences). It usually took about 2 to 3 hours until this value was reached. Next the cells were transferred to 50 ml tubes (Sarsted) and incubated on ice for 20 minutes. After incubation the cells were harvested by centrifugation at 4°C (10 minutes at 3000g) using a MR 23i centrifuge (Jouan). The supernatant was discarded and each pellet was resuspended in 6 ml of cold 0.1M $CaCl_2$. This mixture was allowed to chill on ice for 30 minutes after which it was centrifuged at 4°C (10 minutes at 3000g). The supernatant was poured off and each cell pellet was resuspended in 1.6 ml of cold 0.1M CaCl2 supplemented with 15% glycerol. Finally, cells were stored at -80°C.

In the second protocol, cells were made competent using RbCl (Sigma). The RbCl method was used to prepare super competent *TOP10F'* and *WK6* cells. Again a single colony was picked and added to a sterile flask containing 30 ml of LB medium and antibiotics. This was allowed to grow overnight at 37°C while shaking. After overnight growth the bacteria were inoculated in a 1/100 dilution with 100 ml of LB supplemented with antibiotics where necessary. This was grown until OD_{550} was about 0.5. The cells were chilled on ice for 10 minutes and then centrifuged at 4°C (5 minutes at 3600g). The supernatant was discarded and cells were resuspended in 40 ml of ice-cold TfbI buffer. The pH of TfbI buffer was adjusted to 5.8 using 0.2M acetic acid (VWR Probalo). Before use, the buffer was filtered using 0.22 µm syringe filters (Pall Corporation). This was allowed to chill on ice for 5 minutes followed by centrifugation at 4°C (5 minutes at 3600g). Each pellet was resuspended in 4 ml of ice-cold TfbII buffer. The pH of TfbII buffer was adjusted to 6.5 using 1M KOH (Merck) followed by filtration using 0.22 µm syringe filters. The mixture was incubated for 10 minutes on ice and finally distributed over precooled aliquots. The aliquots were stored at -80°C until further use. The composition of the TfbI and TfbII buffers are shown in appendix 2.

5.1.2 Isolation of plasmid DNA

In order to isolate the plasmid DNA containing the nanobody DNA single *TOP10F'* colonies containing the desired plasmid were grown overnight at 37°C while shaking in a KS 400 ic control incubator shaker (IKA). After growing, the plasmid DNA is extracted out of the *TOP10F'* cells and

isolated using the QIAprep Spin Miniprep kit (Qiagen, #27106), the extraction is performed according to manufacturer's instructions.

5.1.3 Verifying the plasmid DNA extraction

To check if the isolated plasmid DNA was indeed correct, two different methods were used. The first method to check the DNA extraction was by use of restriction enzyme digestion. Here for, 10 µl of miniprep DNA was digested with different restriction enzymes for 1 hour at 37°C. After digestion, the DNA was then analysed using agarose gel electrophoresis. The fragment length retrieved from the gel analysis was compared to the theoretically expected length. Restriction enzymes that were frequently used are EcoRI, SapI, NdeI, PstI, XhoI, NcoI and BamHI. More info about the precise reaction mixtures can be found in appendix 5.
The second method to check the plasmid DNA was by sequencing. Here for, a dilution of 8 µl miniprep DNA and 12 µl sterile water was made. This was then sent to Macrogen (The Netherlands) for sequencing. After sequencing, the sequences were aligned and compared to the wild type BCII10 nanobody cDNA sequence. Results are shown in appendix 6.

5.1.4 Transformation of plasmid DNA into competent E.coli cells

Plasmid vectors containing the MBP DNA or nanobody (construct) DNA were transformed into chemically competent *E.coli* cells. This was done by adding 1 µl of miniprep DNA to 100 µl of competent *E.coli* cells. Next the mixture was incubated on ice for 45 minutes after which a heat shock was given to the cells by submerging them in water at 42°C. Depending on the cell type the heat shock was either 1 minute for *TOP10F' and BL21(DE3)pLysS* or 30 seconds when *WK6 E.coli* cells were used. After the heat shock the cells are chilled shortly on ice followed by addition of 900 µl of preheated (37°C) LB medium without supplements. This is then incubated for one hour at 37°C while being shaken at 220 rpm. After centrifugation at room temperature (5 minutes at 3000g) the pellet was resuspended in 80 µl of the remaining supernatant. Finally, the cell suspension was plated on agar plates and incubated overnight at 37°C.

5.1.5 Engineering of BCII10 nanobodies less prone to in vivo cleavage by introduction of C-terminal modifications using PCR

Because high amounts of in vivo cleavage were observed in the BCII10 nanobody, the DNA of the nanobody was modified to have different C-terminal amino acids using polymerase chain reaction (PCR). Because of this mutation the enzymatic activity of the intein will be altered which results in a fusion protein less susceptible to in vivo cleavage. Therefore different constructs are made in which the C-terminal serine is replaced e.g. to an alanine, pelB-BCII1O-SA-C-intein-CBD (Bio6). In another construct (Bio5), an extra alanine was added C-terminally behind the serine, pelB-SSA-C-intein-CBD. For these 2 a forward primer called 1.1 was designed and used. Apart from these constructs, 5 other constructs were made with another forward primer called Bio48. The reverse primers were different for every construct. Primers and the constructs are shown in appendix 3.

The constructs that were designed with the Bio48 forward primer were pelB-BCII10-SQ-C-intein-CBD (Bio30), pelB-BCII10-ST-intein-C-CBD (Bio32), pelB-BCII10-SG-intein-C-CBD (Bio44), pelB-BCII10-SD-C-intein-CBD (Bio45) and pelB-BCII10-SS-LEY-C-intein-CBD (Bio54). In Bio30 serine is replaced by glutamine, in Bio32 serine is replaced by threonine, in Bio44 serine is replaced by glycine, in Bio45 serine is replaced by asparctic acid and in Bio54 a leucine, glutamine acid and tyrosine are added to the wild type BCII10 DNA. Bio30, 32, 33, 44 and 45 will be cloned in a pTXB1 vector. Bio54 will be cloned in a pMXB10 vector. The tyrosine in the Bio54 clone was not introduced via primers. It was still present in the pMXB10 vector after the original construct was excised with restriction enzymes.
Three of these constructs were intensively used during the project. The first construct was Bio6 (SA), the second construct was Bio5 (SSA) and the final construct was Bio54(LEY).
Since each amino acid can be encoded by different codons, the codons of the new amino acid(s) in the primers were selected that occur most for each specific amino acid in *E.coli*. In addition to the preferred mutation, the reverse primer also introduces a restriction site for SapI restriction digestion. The forward primer introduces a restriction site for NdeI restriction digestion. These restriction sites will be used later on for cloning the construct into other vectors. All primers were purchased from Eurogentec.
By using PCR, the pelB-BCII10-SS-RGR-intein-CBD nanobody cDNA was amplified. This DNA was kindly provided by prof. Muyldermans (VUB). The PCR protocol is described in appendix 3. After the PCR, 10 µl of each PCR sample was mixed with 2 µl 6x DNA loading buffer (appendix 2). This was loaded onto agarose gels containing 1/10000 Gel Red Nucleic Acid Stain (Biotium) and ran for 1 hour at 120V. After running, the PCR fragments were checked using the Gel Doc XR system for PC (Bio-Rad).
Once the fragments were found to be correct via agarose gel electrophoresis, they were ligated into vector pCR2.1 overnight at 14°C. Composition of these ligation mixtures is mentioned in appendix 4. These vectors were transformed into *TOP10F'* cells as described in section 5.1.4. The *TOP10F'* cells were plated on agar plates containing ampicillin and tetracycline. The plates were treated with 40 µl 100 mM IPTG in water and 40 µl xGal in dimethylformamide (40 mg/ml). Finally they were grown overnight at 37°C. The next day blue/white screening could be performed. The white colonies are the ones containing the vector with the PCR product ligated into them. The blue colonies were transformed with an empty vector. Multiple white colonies were picked and transferred to 4 ml of LB medium supplemented with ampicillin and tetracycline. These were grown overnight at 37°C while shaking. After overnight growth the plasmid DNA was extracted and verified as described in section 5.1.2.

5.1.6 Purification of DNA fragments after separation using agarose gel electrophoresis.

During cloning procedures, the DNA fragments were checked using restriction analysis with appropriate restriction enzymes depending on the construct. After quickly checking the fragment length on agarose gel with UV, the desired fragments were cut out of the agarose gel using a sterile scalpel. The DNA was purified from the agarose gel using the QIAquick Gel Extraction kit (Qiagen #28704) according to the manufacturer's instructions. The purified DNA was then used for further cloning.

5.2 Protein expression, purification and characterization methods

5.2.1 Induction of nanobody and maltose-binding protein expression using IPTG

To test the induction of maltose-binding protein (MBP) and the different nanobody constructs, single colonies were picked from agar plates using a sterile toothpick and incubated in flasks containing 30 ml of LB medium supplemented with antibiotics where necessary. The cultures were grown overnight at 37°C. Next they were inoculated (1/100) in larger cultures of either TB or LB medium and grown until the OD_{600} was about 0.5-0.6. Next IPTG was added and the cells were incubated overnight at 37°C while shaking at 220 rpm. IPTG will activate the promoter region of the plasmid vector, resulting in high amounts of protein being produced. The expression of nanobodies and MBP was done using different concentrations of IPTG and different temperatures for 3 hours or overnight. Details about the exact conditions in which the nanobodies were expressed are mentioned in the results & discussion section per experiment.
Prior to induction of big cultures, small test inductions were performed. This was done by growing single colonies until the OD_{600} was about 0.5-0.6 after which 1 mM IPTG was added. After overnight induction at 28°C, cells were harvested by centrifugation of 1 ml culture at room temperature (1 minute at 16000g) in a 5415R centrifuge (Eppendorf). The pellets were dissolved in 2x SDS-PAGE sample buffer followed by analysis using SDS-PAGE. For the negative control samples, no IPTG was added.

5.2.2 Extraction of BCII10 and MBP out of E.coli cells after induction of protein expression

Prior to purification of the proteins, they were extracted out of the *E.coli* cells. Here 3 different methods were used. One method was using bacterial protein extraction reagent (B-PER, Thermo scientific). Using B-PER, a cytoplasmatic extract is produced. To extract proteins using B-PER, *E.coli* cells were first harvested by centrifugation at room temperature (10 minutes at 5000g). Next the supernatant was discarded and 6 ml of B-PER with 1/1000 DNAseI (Thermo Scientific) was added. The pellet was resuspended and the suspension was homogenized by repeated pipetting up and down. This was incubated for 15 minutes at room temperature after which it was centrifuged at 4°C (30 minutes at 15000g). The goal of centrifugation was to separate soluble and insoluble proteins. After centrifugation the supernatant was taken off and analyzed on a 12% SDS-PAGE gel. This method was used for both MBP and BCII10 extraction.
To obtain periplasmatic extracts, two different methods were used. Periplasmatic extraction was done for the BCII10 nanobody because the pelB sequence targets them to the periplasm. To produce this extract, an osmotic shock is given to the *E.coli* cells. Because of this shock the weaker outer membrane will rupture while the inner membrane remains intact. These 2 methods are different from the method using B-PER. In the first of these methods, which was a protocol provided by Brussels University (VUB), the *E.coli* cells from 1 liter of culture were harvested by centrifugation at 14°C (30 minutes at 8000g). Next the supernatant was discarded and the pellets were resuspended in a total volume of 28 ml TES1 buffer. (Appendix 2) This suspension was incubated for 2 hours while shaking on ice after which double the amount (56 ml) of a 1:4 dilution of TES1 buffer in water was added to the suspension. This was incubated overnight on ice while

shaking. The next day, the suspension was centrifuged at 4°C (30 minutes at 8000g). The supernatant, containing the periplasmatic extract, was taken off and used for purification. This method was based on the protocol provided by Brussels University (VUB). The second protocol was based on the method used by Reulen et al.[40] Here cells from 500 ml of culture are first harvested by centrifugation at 4°C (10 minutes at 8000g). The supernatant is then discarded and the cells are resuspended in 85 ml of TES2 buffer. (Appendix 2) This suspension is incubated at room temperature for 10 minutes while being shaken. The suspension was again centrifuged at 4°C (10 minutes at 8000g) after which the pellet was resuspended in 85 ml of a 5 mM $MgCl_2$ solution. This was incubated on ice for 10 minutes while shaking periodically. Finally this mixture was centrifuged at 4°C (10 minutes at 8000g) after which the supernatant was discarded and stored at 4°C for further purification and modification.

5.2.3 Purification of MBP and BCII10 using affinity chromatography

Purification was performed using a 50% slurry solution of chitin beads (New England Biolabs) in 20% ethanol. First a frit (Alltech) was put in a 15 ml column (Bio-Rad) and prewetted using distilled water. Next 8 ml of the slurry bead solution was loaded on the column. This resulted in a total of 4 ml bead volume in the column. The beads were then washed with 10 column volumes of column buffer. This buffer's composition is described in appendix 2. After washing, the cellular extracts, prepared as discussed in section 5.2.2, were incubated with the beads for 1 hour while constantly being inverted at 4°C on a spinning wheel. After incubation, the beads were distributed over smaller 5 ml columns (Bio-Rad) and flushed with a column volume of column buffer. A second frit was carefully placed on top of the beads. Next the columns were eluted using 3 different types of cleavage buffer and a negative control. The cleavage buffers were 50 mM DTT in column buffer, 30 mM MESNA and one mixture composed of 30 mM MESNA, 1 mM TCEP and 1 mM cysteine-alkyne. In the column flushed with the buffer containing cysteine, C-terminal modification of the MBP will occur.

First, the columns were flushed with one column volume of the different cleavage buffers. The flow through was captured and stored on ice. Next a second column volume of the cleavage buffers was loaded on the column and incubated overnight at 4°C. After overnight incubation the columns were eluted twice with column buffer. The elution was captured in falcon tubes.

For MBP, the proteins were separated from the additives (MESNA, DTT, cysteine alkyne). This was done by gel filtration over a 50% solution of sephadex G25 beads (GE Healthcare) in water. 3 columns were prepared and washed with 10 column volumes of phosphate buffered saline (PBS). The composition of PBS is described in appendix 2. Hereafter 250 µl of each sample was loaded on the columns. This was washed with 250 µl PBS and finally eluted with 700 µl PBS.

Finally, a "click" reaction was performed by adding 21,8 µl of a mixture consisting of 12 µl 20 mM Biotin-azide, 24 µl 50 mM TCEP, 70,8 µl 1.7 mM tris-(benzyltriazolylmethyl)amine (TBTA) and 24 µl 50 mM $CuSO_4$ to 200 µl of the protein solution. This was incubated for 2 hours while constantly inverting on a spinning wheel.

For purification of BCII10 fusion proteins, 10 ml of a 50% slurry chitin bead solution in 20% ethanol was pipetted in a 15 ml column. This was incubated for 1 hour at 4°C with a periplasmatic or B-PER extract while being inverted on a spinning wheel. After incubation, the beads were added

to a 5ml column. The supernatant was allowed to flow through the column after which it was captured and stored. The column was washed with column buffer until OD_{280} was approximately 0.01. Next the column was washed with 2 column volumes of 30 mM MESNA after which a small level of MESNA was left above the beads. This was incubated overnight at 4°C to allow cleavage. The next day the MESNA was eluted and captured. Finally the column was eluted 5 more times with 1 ml of column buffer. For the BCII10 fusion proteins no "click" reaction was performed.

5.2.4 Characterization of MBP and BCII10 using SDS-PAGE analysis.

In this study, mainly 12% SDS-PAGE gels were used for analysis. To prepare total extract samples for SDS-PAGE analysis, 1 ml of each culture was taken and centrifuged at room temperature (1 minute at 13000g) using a 5415R centrifuge (Eppendorf). The pellets were resuspended in 200 µl 2x sample buffer with SDS (Appendix 2). For other extracts, the samples were diluted with 5x sample buffer with SDS until a final concentration of 1x sample buffer with SDS. Finally the pellets were boiled in water for 5 minutes after which 5µl of a SDS7 protein marker and 10 µl of each sample were loaded on the gel. The gels were run for 10 minutes at 100V followed by 1 hour at 120V. After running the gel was stained using a Coomassie Brilliant Blue staining solution by boiling it for 5 minutes and then cooling it down while shaking. (Appendix 2) Finally the gel was destained 3 times by boiling it for 5 minutes in destaining solution and then cooling it down while shaking. (Appendix 2)

5.2.5 Characterization of MBP and BCII10 using Western blot.

To start a Western blot analysis, SDS-PAGE gels were incubated in ice-cold towbin blot buffer for 20 minutes. (Appendix 2) Next polyvinylidene fluoride (pvdf) membranes (GE Healthcare) were prepared for the blotting. First the membranes were soaked in methanol for 20 seconds followed by 20 seconds of rinsing the membrane in distilled water. Finally the membrane was soaked in towbin blot buffer for 5 minutes. The blotting papers (Whatman) were soaked in towbin blot buffer while the membranes were being prepared.
After the membranes were ready, the blotting setup was built up. First the electro cassette was prepared. The cassette was loaded starting at the cathodic side with a sponge soaked in towbin blot buffer, followed by a blotting paper. Next the SDS-PAGE gel was placed on the blot paper with the membrane positioned on top of the gel. Excess air between the membrane and gel was removed after which another blotting paper and sponge were put on top of the membrane. The cassette was closed and positioned with the correct orientation in the electro blotting tank. Finally the tank was filled with ice-cold blotting buffer and a cooling element. The blot was run overnight at 20V while being stirred with a magnetic stirrer.
After overnight blotting the membranes were removed from the cassette. Different types of markers were used. The first type of marker was a prestained protein marker, PageRuler™ Prestained Protein Ladder (Fermentas). The other marker was the SDS7 marker (Sigma). The SDS7 markers were stained using a Coomassie and Ponceau staining. The marker in Coomassie staining was boiled and destained just like the SDS-PAGE gel staining. The Ponceau staining was done by adding 10 ml of Ponceau staining solution to the pvdf membranes and incubating them for

5 minutes while shaking. After the reaction, the Ponceau staining solution was recovered and the membranes were washed with distilled water. The markers were dried and stored between 2 dry blotting papers.

Next the pvdf membranes were incubated for 1.5 hour in a 5% solution of skim milk powder (Merck) in Tris Buffered Saline (TBS) -Tween. (Appendix 2) After 1.5 hour the 5% solution was poured off and the membranes were incubated in a 2% solution of skim milk powder containing 1/5000 rabbit anti-CBD (New England Biolabs) polyclonal antibody. Negative control membranes were incubated in a 2% solution of skim milk powder containing no antibody. The membranes were incubated for 2 hours with the solution after which they were poured off. On the negative control membranes no bands should develop after adding the substrate to this membrane since there is no antibody added that can bind to the proteins. The membranes were washed 3 times for at least 5 minutes with TBS-Tween. Next they were incubated with the secondary antibody. This was done using a 2% solution of skim milk powder in TBS-Tween containing 1/15000 goat anti-rabbit alkaline phosphatase (Sigma) for 2 hours. The secondary antibody was poured off and the membranes were washed at least 5 times for 5 minutes using TBS-Tween. For the experiments with biotin a solution of BSA was used instead of skim milk powder. After washing, the substrate for the alkaline phosphatase was added to colour the membrane. This substrate was a solution of NBT (0.3 mg/ml) and BCIP (0.15 mg/ml) in a buffer, which is here called NBT/BCIP buffer. (Appendix 2) 10 ml of this substrate was added to each membrane and allowed to react for 2 minutes. The incubation period of the substrate is varied depending on how fast the colour develops and might be extended or shortened depending on this time. Pictures of the SDS-PAGE gels and the Western blots were taken using a canoscan 5600F (Canon).

6. Results and discussion

There are two parts in this section. The first section is regarding MBP which was used for a proof of concept. MBP was chosen since it is a protein which is well described in literature. It is stable, easily soluble and easy to manipulate. More details about MBP are displayed in appendix 8 and 9. The second section contains the results obtained for the BCII10 nanobody. When using the nanobody some problems were encountered that were not present when using MBP. In addition to *pLysS* cells, *WK6* cells will be used for the expression.

6.1 Proof of concept using MBP

6.1.1 Expression of MBP-intein-CBD in BL21(DE3)pLysS E.coli cells

In order to induce expression of MBP in a pMXB10 vector, *BL21(DE3)pLysS* cells were transformed with isolated MBP DNA. Expression was induced by addition of 0.4mM IPTG to a culture supplemented with ampicillin and chloramphenicol. This was incubated for 4 hours while shaking at 30°C. The result of MBP expression is shown in figure 8.

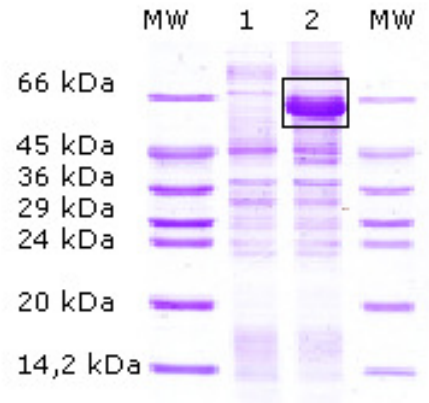

Figure 8: 12% SDS-PAGE analysis of MBP expression. Lanes with MW are the SDS7 protein marker. Lane 1 contained a total extract in which no IPTG was added (negative control). Lane 2 was a total extract of the induced culture.

On figure 8, the outer lanes contain the SDS7 protein marker. By comparison of lane 1 and 2, it can be seen that the fragment that is marked, which corresponds to the weight of MBP, is not present in lane 1. Lane 1 contains the control sample. This is an extract of the MBP culture, taken prior to addition of IPTG, obtained via B-PER reagent. These B-PER extracts contain both the proteins present in a cytoplasmatic and periplasmatic extract. For the induced culture in lane 2, it would be expected that a band shows up at around 66 kDa. This because the effective mass of the MBP fusion protein is very close to 66 kDa hence why they will appear to be at the same height on figure 8. The exact mass of the MBP-intein-CBD is 70274 Da. The MBP itself is only 42436 Da but the rest of the mass is accounted for by the intein and CBD (27856 Da). From figure 8 it can be concluded that expression of the MBP fusion protein in *BL21(DE3)pLysS* cells with IPTG was successful. Next it will be checked if the recombinant MBP is still soluble after extraction and purification. This is described in section 6.1.2.

6.1.2 Purification and modification of MBP-intein-CBD

After expression, MBP-intein-CBD was purified using a chitin bead column and eluted using 3 different elution buffers. The elution buffers were column buffer (lane 6 in figure 9), 50 mM DTT in column buffer (lane 7 in figure 9), 30 mM MESNA in column buffer (lane 8 in figure 9) and a mixture for the actual modification containing 30 mM MESNA, 1mM TCEP and 1 mM cysteine-alkyne in column buffer (lane 9 in figure 9). TCEP is a reducing agent which breaks disulfide bonds and is added to the elution mixture to prevent dimerisation of the modified cysteines. Elution was performed after overnight incubation at 4°C in the elution buffer. The results of purification and elution are shown in figure 9.

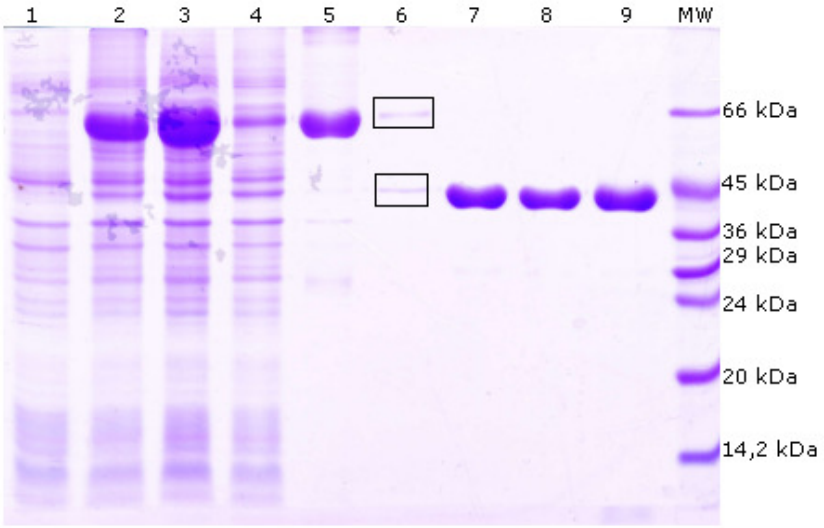

Figure 9: 12% SDS-PAGE gel analysis of MBP-intein-CBD purification and modification. MW is the SDS7 marker. Lane 1 and 2 are MBP-intein-CBD before and after induction (total extracts). Lane 3 is the extract obtained using B-PER. Lane 4 contains flow through of the chitin binding column. Lane 5 is a sample of the chitin beads prior to elution. Lane 6 till 9 contains the elutions obtained using the negative control (column buffer) and 3 different elution buffers.

In the first 2 lanes of figure 9, the same result as in figure 8 is shown. This is done to make comparison of the other results in this 12% SDS-PAGE gel easier. Lane 3 shows the extract obtained using B-PER extraction reagent. As can be seen the band here is thicker then in lane 2. This is because the sample gets diluted less as compared to the total extract. In lane 4 the column flow through is shown. If the MBP-intein-CBD had successfully bound to the chitin beads, the band should disappear in this lane. And indeed, as can be seen in lane 4, the band has almost completely disappeared which indicates that binding to the chitin beads was successful. The fragment which is still present in lane 4 at the height of 66 kDa is most likely due to endogenous proteins. To confirm this, Western blot analysis should be performed. In lane 5, a small fraction of the protein bound to the beads is shown. The result here confirms what was already concluded from the result in lane 4. MBP-intein-CBD has successfully bound to the chitin beads. In addition the binding of the recombinant MBP was very specific. No other fragments could be observed in lane 5 indicating that no other proteins were present on the beads. Next, in lane 6, the negative control for the elution of MBP is shown. The elution buffer used was column buffer which should not cleave the MBP of the chitin beads as can be seen in lane 6. When taking a closer look however, 2

thin bands can be distinguished (highlighted by black boxes): One band at the height of 66 kDa, which corresponds to MBP-intein-CBD that came loose again since an equilibrium sets and another band at the height of 45 kDa. This band corresponds to the mass of the MBP without intein or CBD. Lanes 7, 8 and 9 contain the results after overnight incubation with the different elution buffers and then eluting them. In lane 7 the results of elution with 50 mM DTT are shown. As can be seen a thick band shows up at approximately 45 kDa indicating that cleavage of the intein-CBD from the MBP was successful. The same happened in lane 8 and 9 where respectively 30 mM MESNA and a mixture of 30 mM MESNA with 1 mM TCEP and 1 mM cysteine-alkyn were used. The elution buffer used in lane 9 is the one that will be actually used in the future for "click" reactions to attach modified nanobodies to surfaces. From figure 9 it can be concluded that the intein-CBD system can indeed be used for purification of the MBP fusion protein out of a complex mixture using affinity chromatography. Another important conclusion is that the MBP-intein-CBD still is soluble after expression and that high yields of MBP-intein-CBD expression can be achieved. The yield was calculated to be approximately 50 mg/liter of culture. The next step was to check if the MBP from lane 9 effectively contained an alkyne and to check if this alkyne is available to perform a click reaction. (Section 6.1.3)

After proving that the intein system could be used for purification of MBP, it was evaluated if one elution step was sufficient or if multiple elution steps were required to elute the modified MBP. Here for, the amount of elution steps required after overnight incubation in the different elution buffers was optimized. This was done with one more elution after the first elution simply by adding 1,5 column volume of column buffer. The results are shown in figure 10.

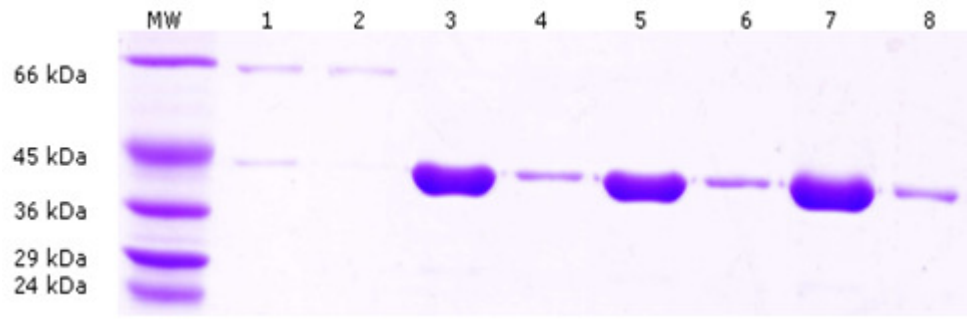

Figure 10: 12% SDS-PAGE gel for optimization of MBP elution. Lane 1 and 2 are the negative controls. Lane 3 and 4 contain elutions done using 50 mM DTT. Lane 5 and 6 contain elutions with 30 mM MESNA and lane 7 and 8 are the elutions done with cysteine-alkyn.

The goal of this experiment was to optimize the elution of C-terminally modified MBP. Once again lane 1 and 2 respectively were the first and second elution of the control buffer. Lanes 3, 5 and 7 were the first elutions of the elution buffers after overnight incubation at 4°C. Lanes 4, 6 and 8 were the second elutions of the elution buffers. On the SDS-PAGE gel shown on figure 10, it is clear that for each of the 3 different elution buffers, it is sufficient to elute just once with 1,5 column volume of column buffer. By looking at the bands of 45 kDa it is clear that the major fraction is eluted. There is only a thin band visible in the lanes containing the second elution (lane 4, 6, 8) as compared to the thickness of the bands from the first elution (lane 3, 5, 7). In this case, future elutions with 1,5 column volume will be sufficient.

6.1.3 Western blot analysis of the "click" reaction of C-terminally modified MBP with biotin-azide

Finally, it was checked if the alkyne functionality was incorporated into MBP. Here for a Western blot was performed. To do a proof of concept with this Western blot, an azide modified biotin (Appendix 7) was "clicked" with the C-terminally, alkyne modified MBP. The "click" reaction was performed in solution as described in section 5.2.3.
After running the gel, containing the MBP-biotin complex, was used for Western blotting as described in section 5.2.5. The differences here with the conventional Western blot protocol are that blocking was done with BSA instead of skim milk powder because this contains biotin as well. The blot was incubated with a 1/1000 dilution of streptavidine-alkaline phosphatase (Sigma) in TBS-Tween for 1 hour. Bands were developed by addition of NBT/BCIP reagents as described in section 5.2.5. The result is shown in figure 11. After the blot was obtained the marker was cut of and stained using a Coomassie brilliant blue staining.

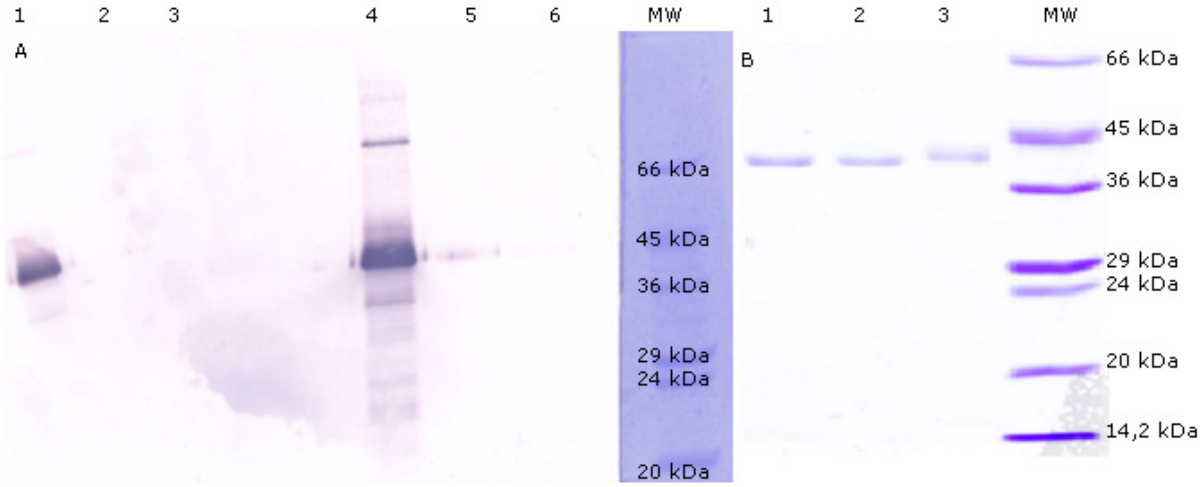

Figure 11: **A.**Western blot demonstrating that a click reaction between the alkyne modified MBP and the azide modified biotin can be performed. In lane 1 and 4 the MBP modified with cysteine-alkyne is shown. Lane 2, 3, 5 and 6 were loaded with the elutions where no C-terminal modification was performed. **B.** 12% SDS-PAGE gel run for analysis of the same samples as in part A. Here the samples were not diluted.

In lane 1 and lane 4 on figure 11 the 1/100 and 1/10 dilutions respectively of the C-terminally modified MBP were loaded. Since it was modified with cysteine-alkyne, development of bands on the pvdf membrane is to be expected. The biotin-azide will be capable of binding to the alkyne via a 1,3 Huisgen dipolar cycloaddition as shown on figure 5. After the alkyne and azide have "clicked" MBP with the biotin, streptavidine will bind the biotin. Since this streptavidine was modified with alkaline phosphatase, a band will develop after addition of the substrate for alkaline phosphatase if modification was successful. On lane 2, 3, 5 and 6 the 1/100 and 1/10 dilutions of the MBP eluted with DTT and MESNA were loaded. In these lanes the MBP present was not C-terminally modified with a "click" functionality hence why no bands are expected. It was only cleaved from the intein by adding small nucleophiles such as DTT and MESNA. As can be seen on figure 11, bands did indeed develop in lane 1 and 4 containing the 1/10 and 1/100 dilutions of modified proteins. It was opted to use dilutions instead of the pure mixture because a first a blot was done using the undiluted samples. The development of bands there was too strong. This is because streptavidine

and biotin have a high affinity for each other. The streptavidine-biotine interaction is an exceptionally strong interaction ($K_d=10^{-15}M$).[44]
Another reason is because the yield of MBP fusion proteins produced by the *pLysS* cells is very high (50 mg/l culture) and thus a high amount of MBP is present. Since there was development of bands in both the 1/10 and 1/100 dilution, it was concluded that the C-terminal, post-translational, in vitro modification of MBP with a bioorthogonal group for site specific attachment was possible using the intein system. The band which is situated the highest in lane 4 is possibly due to dimerisation of modified MBP. The few other bands that are visible in lane 1 and 4 of part A on figure 11 are possibly due to breakdown of MBP due to the modification. By comparing part A with part B however, it can be concluded that the concentration of these breakdown products is very low since they barely show up on the SDS-PAGE gel in lane 3 (1/100 dilution). Since Western blot is such a sensitive technique these breakdown products will show up there even though their concentration is very low. In the 1/100 dilution, the bands' intensity is very weak since their concentration is below the detection range.
Generally, what can be concluded from these results is that MBP can be produced with *pLysS* cells in concentrations of approximately 50 mg/liter culture. After purification, soluble MBP is obtained in which an alkyne functionality can be introduced by the intein system. On top of that, this click functionality is still reactive after incorporation into MBP. The "click" functionality does not cause significant breakdown of MBP. To assess the effectiveness of this "click" modification however, further experiments should be conducted such as mass spectroscopy or streptavidine affinity purification of the modified MBP.
Based on this proof of concept, the same techniques will be used to modify a BCII10 nanobody C-terminally with an alkyne functionality. This is described in the paragraphs below.

6.2 C-terminal, post-translational modification of BCII10 nanobodies in a pTXB1/pMXB10 vector using the intein system

6.2.1 Expression of wild type BCI-10-intein-CBD in BL21(DE3)pLysS E.coli cells

In order to express pelB-BCII10-intein-CBD using *BL21(DE3)pLysS* cells, miniprep DNA was transformed into them. The *pLysS* cells were once again used for their ability to survive while toxic proteins are being produced and because there is very little to no leakage of the promoters in these cells as described earlier. The construct used was pelB-BCII10-SS-RGR-C-intein-CBD. It is not the BCII10 wild type DNA since the final amino acids of the wild type DNA are the two serines (S) and was kindly provided by VUB. It will be referred to as native DNA however. The RGR sequence is an arginine (R), glycine (G) and another arginine (R). Nevertheless, first induction experiments were performed with this DNA. Colonies of *pLysS* cells were induced overnight at 19.5°C with 0.5 mM IPTG. This low induction temperature was chosen because in the past, a lot of problems were encountered with the formation of inclusion bodies by the nanobodies. By reducing the induction temperature, it was attempted to reduce the formation of inclusion bodies. Total extracts of the cells were analyzed using SDS-PAGE.

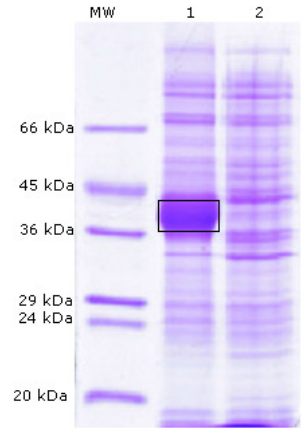

Figure 12: 12% SDS-PAGE gel of wild type pelB-BCII-10-intein-CBD expressed in *pLysS E.coli* cells. Lane 1 is a total cell extract of a culture induced with IPTG. Lane 2 contains the total cell extract from a culture where no IPTG was added.

On figure 12, lane 2 contains a control sample which has gone through the same process as the sample that was loaded in lane 1 with the exception that no IPTG was added to activate the promoter of the pTXB1 vector. As can be seen, a thicker band shows up at the height of approximately 45 kDa indicating that the BCII10 nanobody was successfully expressed by the *pLysS* cells. Because no IPTG was added, we would not expect a band to show up in lane 2 at a height of approximately 45 kDa, which corresponds to the combined weight of the pelB-BCII10-SS-RGR-C-intein-CBD fusion protein. After proving that expression of the recombinant nanobody was possible by addition of IPTG, the next step was to check if the modified BCII10 nanobody was still soluble once it was expressed and to check if indeed the correct fusion protein was produced. Therefore a Western blot was performed. This is necessary since if the BCII10 nanobodies still form inclusion bodies or if the intein is not present, C-terminal modification is not possible.

6.2.2 Verification of BCII10-intein-CBD expression by Western blot

To further verify the production of the original BCII10-intein-CBD construct, the proteins from an SDS-PAGE gel were transferred overnight to a pvdf membrane. Here fore, B-PER extracts were produced. The blocking was done using skim milk powder, the primary antibody was a polyclonal rabbit anti-CBD antibody and the secondary antibody was a polyclonal goat anti-rabbit antibody with alkaline phosphatase linked to it. After addition of the NBT/BCIP substrate, the blot was developed.

The Western blot analysis was performed, since on a SDS-PAGE gel, any protein with a mass equal to the mass of the desired protein can be sitting on that proteins position in the gel. With Western blotting, this is no longer the case since antibodies, directed against the CBD of the nanobody, will only bind to proteins containing a CBD. If bands show up on the blot, it is assured that the protein present is indeed the correct protein. It can be further assured by using monoclonal antibodies. It is important to compare the bands present in the control sample with those present in the other lanes. The bands that can be observed in the control sample are due to aspecific bonding of the primary antibody. This was tested but these results are not included here.

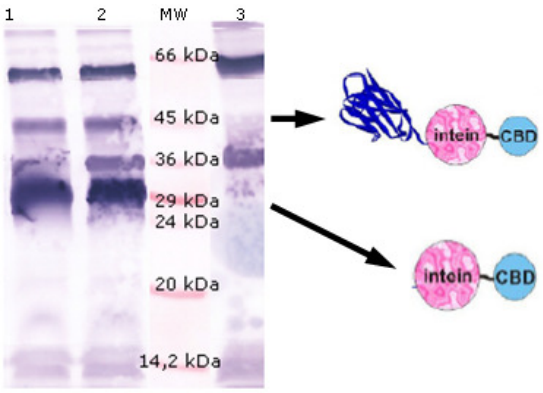

Figure 13: Western blot of BCII10-intein-CBD expressed in *pLys E.coli* cells. Lane 1 and 2 contain the extracts from induced *pLysS* cultures. Lane 3 contains a non-induced *pLysS* culture. The marker is the SDS-7 marker stained with Ponceau staining.

As can be seen on figure 13, bands appeared at the expected height of 45 kDa. This once more confirms that the BCII10 nanobody can be expressed in *pLysS* cells. The intensity of the 45 kDa bands however is relatively low. On figure 13, another very thick band, at the height of 29 kDa, can be observed in lane 1 and 2. This is equal to the mass of the intein-CBD part of our construct. The presence of this band indicates that there is a lot of in vivo cleavage in the *pLysS* cells. This in vivo cleavage means that the BCII10 nanobody and the intein-CBD get separated from each other by protease activity.[41-43] Separation of the BCII10 nanobody and intein-CBD fragment at this stage is problematic since if they get separated, affinity purification against the CBD domain and subsequent post-translational modification using the intein activity are no longer possible. The susceptibility of the original BCII10-intein-CBD construct has been described. In order to solve this problem, the nanobody was engineered to change the C-terminal amino acid(s) to amino acids that are less prone to in vivo cleavage such as alanine or glycine.[41-43] This means that the RGR and the last serine of the pelB-BCII10-SS-RGR-C-intein-CBD will be removed by PCR with specially designed primers and replaced by other amino acids.

6.2.3 Modification of native-BCII10 cDNA to reduce in vivo cleavage using PCR

As mentioned earlier the native BCII10 nanobody ends with two serines. The RGR sequence which is present in the original construct that was used so far is prone to in vivo cleavage. There fore different constructs were made in which the C-terminal serine was replaced by an alanine by PCR. The exact constructs that were made are mentioned in section 5.1.5. An overview of all the constructs is also shown in appendix 3. Due to time constraints, not all constructs were used however. In this internship the main constructs that were used are the Bio5, Bio6 and Bio54 construct. The Bio44 construct was also used during the experiments with Bio54 for comparison. The combination of C-terminal amino acids that were chosen for Bio54 (LEY) are identical to the last 3 amino acids of MBP. These were chosen since good results were obtained with MBP. (Appendix 8) The first step was to amplify the native cDNA with PCR. After the PCR, the obtained fragments should be 477 base pairs (bp) for the serine to other amino acid constructs and 480 bp for the construct in which an alanine was added behind the 2 final serines. For the LEY construct, a fragment of 471 bp is expected. The result is shown on figure 14. With exception of figure 14, no more results will be shown for the Bio30, Bio32 and Bio45 constructs. As can be seen, the bands

are a little bit below the 500 bp band from the ladder. This indicates that amplification of the BCII10 cDNA was successful.

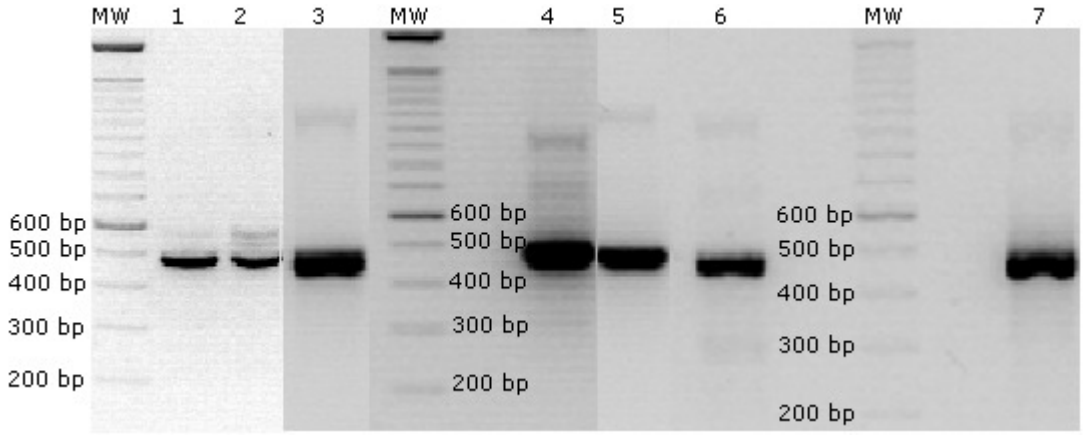

Figure 14: 1,5% agarose gel to check PCR fragments after PCR with primers to replace the C-terminal serine by another amino acid or to add an extra alanine C-terminally. Lane 1 contains the SA construct (Bio6). Lane 2 contains the SSA construct (Bio5). Lane 3 contains the SQ construct (Bio30). Lane 4 and 5 contains the ST (Bio32) and SG construct (Bio44). In lane 6 the SD construct (Bio45) is shown. Finally in lane 7 the LEY construct (Bio54) is shown.

One remark for figure 14 however, is the smear of DNA that can be observed in lane 1 and 2. This is most likely due to a too low annealing temperature (45°C) during the PCR reaction. In order to solve this, another PCR reaction was performed in which the annealing temperature was increased to 55°C. This result is shown in figure 15. As can be seen, the smear of DNA has been reduced by increasing the temperature. Though the smear is still present to a certain degree, further optimization was not performed. A possible way to reduce this later on is by performing a touch down PCR with fewer cycles or by developing a new primer. This was done for the samples in lanes 3 till 7. As can be seen here, the smear is almost completely gone. Because of time constraints and literature recommendations it was opted to start experiments first with the SA and SSA construct. In case the solubility and in vivo cleavage is not solved by making use of these constructs, the LEY construct will be assessed.

In the lane containing the negative control in figure 15, a small band at the 500 bp height is visible. This can be either due to contamination or because of carry over from one slot into the other while loading the gel. Nevertheless, it was decided to continue with this DNA to further develop the constructs. PCR reaction mixtures and conditions are displayed in appendix 3.

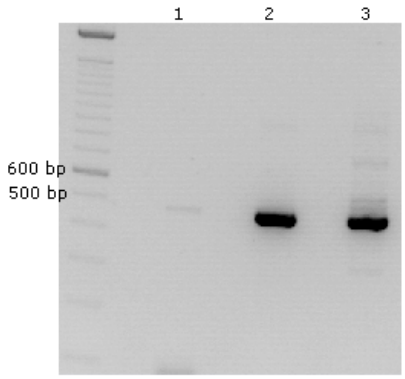

Figure 15:1,5% agarose gel containing the same PCR fragments as figure 14. Lane 1 contains a control in which water instead of DNA was used. Lane 2 contains the serine to alanine (SA) construct. Lane 3 contains the extra alanine (SSA) construct. Annealing was performed at 55°C.

After amplification, the PCR fragments were ligated into vector pCR2.1. The reaction mixture is shown in appendix 4. The T4 DNA ligase will ligate the A overhangs of the PCR fragment, which are put there by the DNA polymerase, and the T overhangs in the pCR2.1 vector. Ligation was performed overnight at 14°C. For these reasons, this procedure is also called a TA-cloning. After ligation into pCR2.1 vectors, the plasmid DNA is transformed into chemically competent *TOP10F'* cells. The transformed *TOP10F'* cells are grown overnight at 37°C on agar plates treated with IPTG/xGal. The morning after growth, single, white colonies of both the SA and SSA construct were picked and grown overnight at 37°C followed by plasmid DNA isolation as described in 5.1.2. The blue colonies were not used since they contain empty pCR2.1 vectors.
The isolated plasmid DNA was first checked by restriction enzyme digestion with EcoRI and agarose gel electrophoresis. The result of this analysis is shown on figure 16 and 17.

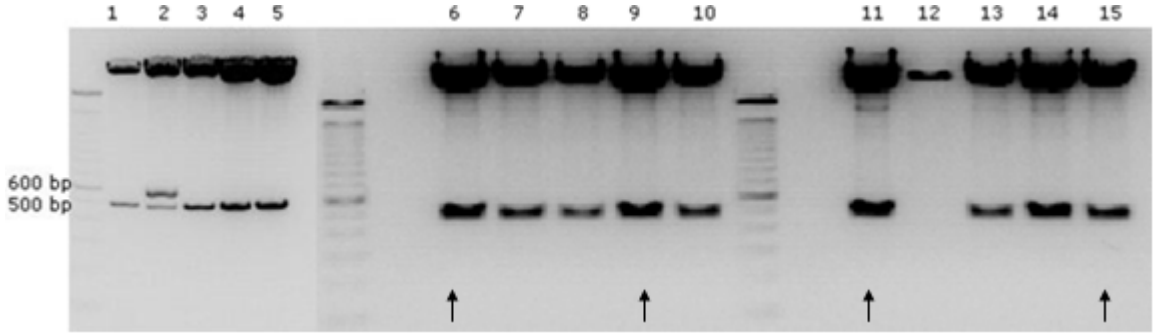

Figure 16: 1,5% agarose gel with the pCR2.1 vector containing the BCII10-SA-C-intein-CBD construct. The different lanes are all single colonies of *TOP10F'* cells transformed with the SA construct. After plasmid DNA extraction, digestion was performed with EcoRI. Arrows indicate the samples that were sequenced.

Theoretically, the length of the fragments obtained after digestion with EcoRI should be 493 bp and 3913 bp. On figure 16, it can be seen that for all SA constructs except for the one in lane 2 and 12, the fragment present matches the expectations. The fragment in lane 2 does not correspond to the expectations. As can be seen, 2 different bands are observed in the lane: One band of roughly 600 bp long and one of roughly 500 bp long. The one at the height of 500 bp is most likely the fragment of 493 bp, as expected from the calculation. The precise length of the 600 bp length fragment was not known nor determined. A possible explanation as to why this fragment is present is that a bigger PCR fragment had been cloned into the pCR2.1 vector. In lane 12 there is only one fragment visible. This fragment is situated at the same height as the other vectors. This indicates that there was no digestion of the vector. Four samples, indicated by the arrows, were chosen for sequencing. Details about the exact restriction mixes are mentioned in appendix 5. Sequencing primers and results are shown in appendix 3 and 6.

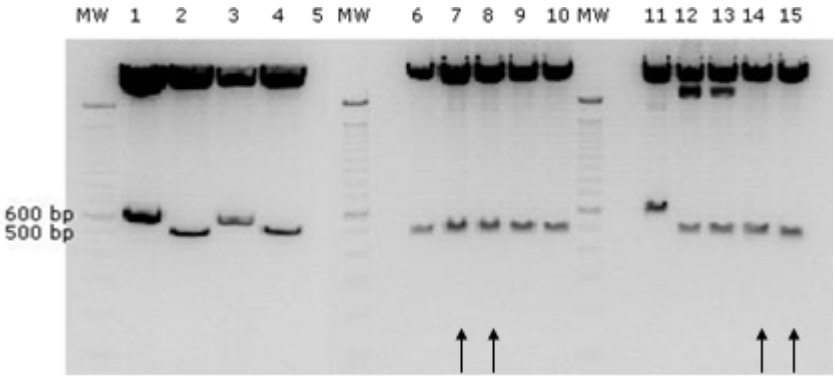

Figure 17: 1,5% agarose gel with the pCR2.1 vector containing the BCII10-SSA-C-inteind-CBD construct. The different lanes are all single colonies of *TOP10F'* cells transformed with the SSA construct. After plasmid DNA extraction, digestion was performed with EcoRI. Arrows indicate the samples that were sequenced.

On figure 17 the results for the restriction digestion of the SSA construct are shown. Here, a fragment with a length of 496 bp is expected. The samples loaded in lane 1, 3 and 11 were not at the same height as expected. Their length is at the same height as the 600 bp band from the marker. Once again this can be due to the ligation of a bigger PCR fragment as already was observed in figure 14 (lane 1 and 2). Another remark can be made about the samples loaded in lane 13 and 14. Here a second band can be observed near the top of the gel. A possible explanation as to why this fragment is there might be because of incomplete digestion by EcoRI. A final remark can be given about the sample in lane 5. Here nothing is visible. This is because there was probably no DNA loaded into the slot. After observation of both figure 16 and 17, the good clones from each gel were selected and prepared for sequencing by Macrogen. The results of this sequencing are shown in appendix 6 and serve 3 goals. The first was to check if no mutations had been introduced in the DNA during amplification with PCR. Secondly, they were used to check if the amino acid(s) modification was successfully introduced and finally it was assessed if the restriction digestion sites for SapI and NdeI were incorporated. It appeared that the isolated plasmid DNA from the minipreps in lane 7 and 15 for the SSA construct and the minipreps in lane 11 and 15 for the SA construct was suitable for further use. All other plasmid DNA was discarded and no longer used.

6.2.4 Engineering of a pelB- BCII10-intein-CBD construct by cloning modified BCII10 cDNA into a pTXB1 vector.

The next step in the construction of the recombinant proteins was to clone these modified constructs being pelB-BCII10-SA-C-intein-CBD and pelB-BCII10-SSA-C-intein-CBD into a pTXB1 vector. This vector allows for expression in cell types that are more suited for protein expression such as the *pLysS E.coli* cells. For the pTXB1 vector, the plasmid DNA from the minipreps in lane 11 for the SA construct and in lane 7 for the SSA construct were cut out of vector pCR2.1 by digestion with SapI and NdeI, their restriction sites were introduced with the primers. Apart from the constructs, the pTXB1 vector itself is cut open with NdeI and SapI. This will result in compatible overhangs in the pTXB1 vector and the insert. The results of this digestion are shown in figure 18.

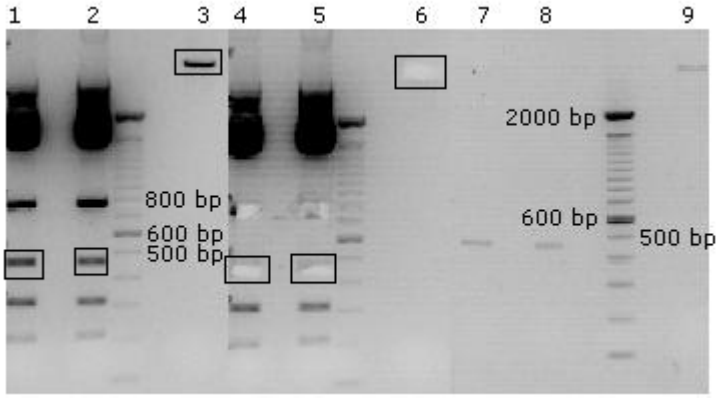

Figure 18: 1,5 % agarose gel providing an overview of all steps taken for the cloning of SA (lane 1,4,7) and SSA (lane 2,5,8)constructs into the pTXB1 (lane 3,6,9) vector. Lane 1,2 and 3 contain the fragments after digestion with NdeI and SapI. Lane 4,5 and 6 show the bands that were cut out prior to purification. Lane 7, 8 and 9 contain the selected samples after gel extraction.

Figure 18 provides an overview of the first 3 steps of the cloning procedure to clone the SA and SSA constructs in a pTXB1 vector for improved protein expression. As can be seen in lane 1 and 2, there are multiple fragments appearing after digestion. This is because there are multiple restriction sites present for both SapI and NdeI. The restriction enzymes will digest the pCR2.1 vector containing the construct into multiple fragments. The length of these fragments is respectively 210 bp, 316 bp, 452 bp, 1506 bp and 1922 bp. The fragment that is needed for cloning is the 452 bp fragment for the SA construct. For the SSA construct, this fragment is 455 bp long. The pTXB1 vector will be digested into a fragment of 6654 bp and 52 bp. The large fragment is the fragment which is required for the cloning. The 52 bp fragment is not visible on the gel. From lanes 1 and 2 of figure 18 it can be observed that another, unexpected fragment with a length of approximately 800 bp is present on the gel. This is most likely there due to incomplete digestion by SapI. This is since the concentration of SapI was low (2 units/µl).

After separating the digested fragments, they are excised out of the gel using a sterile scalpel and purified. The holes that are left after cutting out the gel are shown in lane 4, 5 and 6. The purified DNA was once more loaded on the gel to check if purification was successful. As can be seen in lane 7, 8 and 9, only the desired fragments are present. What one might notice however is that their intensity has decreased as compared to the same fragments in lane 1, 2 and 3. This is because a smaller amount of DNA was loaded onto the gel. From the results shown in figure 18, it was clear that the DNA was suited for continuing the cloning. The DNA fragments were ligated into a pTXB1 vector overnight at 16°C. The ligation mixes are shown in appendix 4. Next, the constructs were transformed into *TOP10F'* cells made competent using the RbCl method as described in section 5.1.1. It was opted to use cells made competent by RbCl over Ca_2Cl because in cells prepared with the RbCl method the transformation efficiency was much higher then in cells prepared with the Ca_2Cl method. After overnight growth, the plasmid DNA was extracted out of the *TOP10F'* cells and checked using restriction digestion. Digestion was performed with PstI. The result is shown in figure 19.

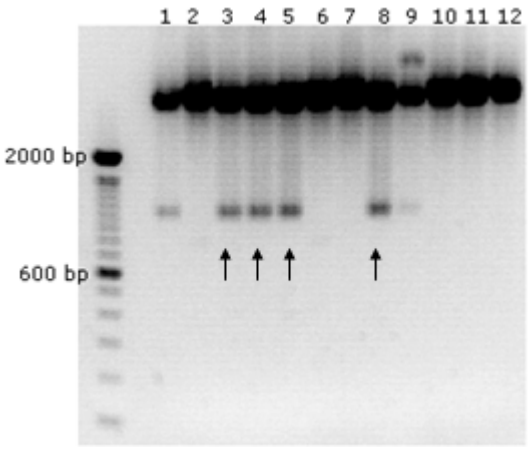

Figure 19: 1,5% agarose gel containing 6 single colonies of both the SSA and SA construct. Lane 1 till 6 contains the digestions from the SSA construct. Lane 7 till 12 contains the digestions from the SA construct. Digestion was performed with PstI.

Theoretically, 2 fragments are expected after digestion with PstI. These fragments should have a length of 918 bp and 6187 bp for the SA construct and 921 bp and 6187 bp for the SSA construct. On figure 19, it can be seen that the cloning was successful for the colonies in lane 1, 3, 4 and 5. In lanes 2 and 6 there is no second fragment visible. This is most likely because this is an empty vector. For the SA construct, analyzed in lanes 7 till 12, there is only 1 good construct present. This is the construct in lane 8. It is clearly visible here that there are 2 fragments visible here. In lane 9, one might say that there are 2 fragments visible as well. Though, by looking carefully, a third fragment can be observed at the far end of the gel. This was not further investigated. A possible explanation however is that only a part of the DNA was digested. The highest and biggest fragment is undigested, complete plasmid DNA. The 2 lower fragments are from the plasmid DNA that was digested by PstI. As can be noticed on figure 19, the fragments are situated a bit higher then theoretically expected after digestion. This is most likely due to non ideal running of the agarose gel because the agarose content was to high for proper separation of fragments with a length around 900 bp. To make sure however, that the cloning was indeed successful, it was decided to do a second restriction digestion analysis of the clone in lane 3 (SSA) and lane 8 (SA) by making use of NdeI and BamHI. Results of the digestion are shown in figure 20. Details about the restriction digestion mixes can be found in appendix 5.

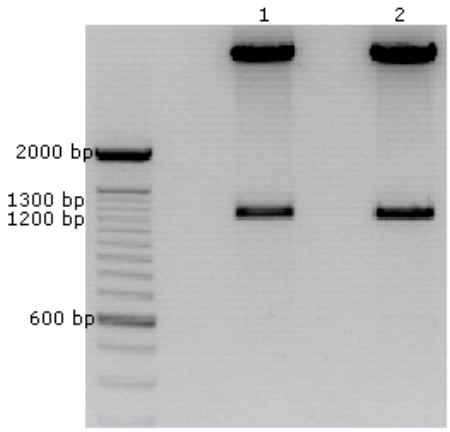

Figure 20:1% agarose gel containing the SSA construct from lane 3 in figure 19. In lane 2 the SA construct from lane 8 in figure 19 is shown. Digestion was performed with NdeI and BamHI.

As can be seen on the picture above, this time it is proven that cloning was successful. After digestion, 2 fragments with a length of 5859 bp and 1246 bp for the SA construct and 5859 bp and 1249 bp for the SSA construct are expected. After digestion, these fragment can be observed between the 1200 and 1300 fragments of the DNA ladder. Digestion mixtures can be found in appendix 5. These clones were transformed into different of *E.coli* cells for protein expression. The sequencing results and primers are shown in appendix 6 and 3.

6.2.5 Characterisation of the expression of BCII10-SA-intein-CBD in pTXB1 in different E.coli cell lines by SDS-PAGE and Western blot

In order to express the engineered nanobody, the plasmid DNA, constructed as described in section 6.2.4, was transformed into *pLysS and WK6 E.coli* cells. Transformation was performed as described in section 5.1.5. The *WK6* cells were grown on agar plates supplemented with ampicillin. The *pLysS* cells were grown on agar plates supplemented with ampicillin and chloramphenicol. Before starting large cultures, test inductions (4 ml) were performed by picking single colonies and growing them at 37°C until OD_{600} was equal 0.6. Next protein expression was induced by addition of 1 mM IPTG. To 1 colony, no IPTG was added. This colony served as a negative control. First, the results of induction are shown for the *pLysS* cells. This can be seen on figure 21.

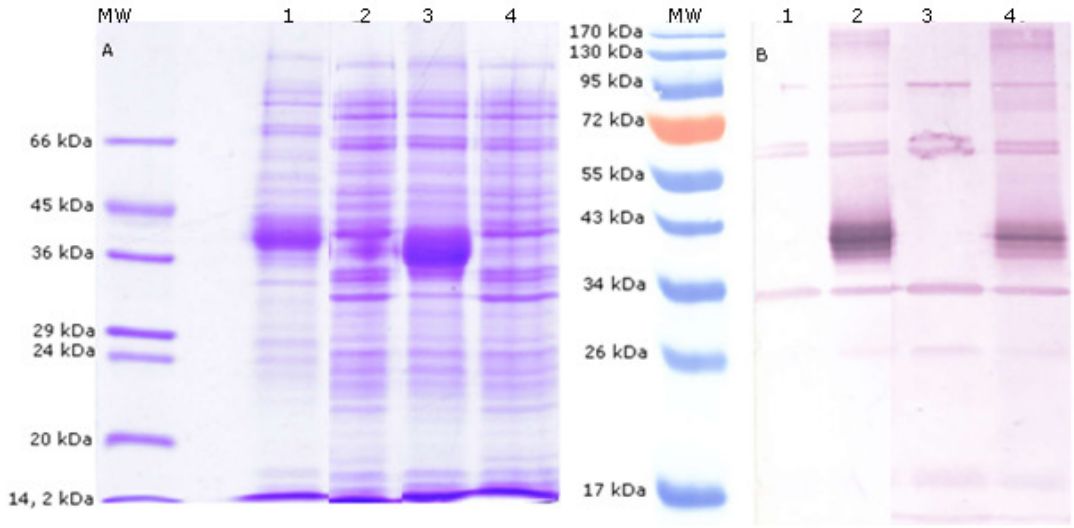

Figure 21: **A.** 12% SDS-PAGE gel containing total cell extracts from *pLysS* cells transformed with the SSA construct and the SA construct. Lane 1 and 2 contain the SSA construct. Lane 3 and 4,contain the SA construct. The negative controls are shown in lane 2 for the SSA construct and in lane 4 for the SA construct. **B.** Western blot analysis of the SDS-PAGE gel from part A. Lane 1 and 2 contain the SA construct. Lane 3 and 4 contain the SSA construct. Lane 5 contains the negative control for the SA construct and lane 7 contains the negative control for the SSA construct.

A first result from part A of figure 21 is that protein expression occurred successfully in the *pLysS E.coli* cells. This can be deducted from the thick bands that show up at the height of the 45 kDa fragment from the marker. These bands represent the pelB-BCII10-SSA-intein-CBD complex (lane 1) and the pelB-BCII10-SA-intein-CBD complex (lane 3). From the samples loaded in lane 2 and 4, it is also proven that the expression of the constructs can be activated by addition of IPTG to the cultures. To the samples in these lanes no IPTG was added hence why there is no band visible

around 45 kDa. The other fragments present in these lanes originate from other proteins, which are endogenously produced by the bacteria whether or not IPTG is present.

It was proven that the constructs, cloned into the pTXB1 vector, allow for expression of nanobodies with a different C-terminal amino acid in *pLysS E.coli* cells. To further strengthen this conclusion, a Western blot with polyclonal anti-CBD antibodies was performed. This is more specific due to the use of the antibodies for the detection. The Western blot is shown in part B of figure 21.

As can be seen, the difference between the negative controls (lane 1 and 3) and the induced samples is much easier to see as compared to the SDS-PAGE in figure 21A. Around the 43 kDa height of the prestained marker, there are no bands visible for the negative control samples (lane 1 and 3 in figure 21B) whereas a sharp band can be distinguished for the induced samples. Both the SA (lane 1 and 2) and SSA (lane 3 and 4) construct were expressed efficiently by the *pLysS* cells.

The band, which corresponds to the recombinant nanobody, is the sharp, darker line. The other light purple smear around this sharp, thin line is most likely due to different forms of the fusion protein. For example fusion proteins in which the pelB was completely removed or fusion proteins in which the pelB sequence was partially removed. One might think it is aspecific binding of the polyclonal antibodies but if this was the case however the purple smear should be present in the control samples too, where it was not present. In the Western blots later on, some will be stained using monoclonal antibodies. As can be seen in all lanes, there are 4 more fragments visible. What can be noticed is that these fragments are also visible in the lanes containing the negative controls. This indicates that these bands are just endogenous proteins that get recognized aspecifically by the antibody. This is further confirmed by Western blot experiments where no primary antibody was added yet these bands did show up there as well. By using monoclonal antibodies, this can be improved as will be shown later. A final, important conclusion, which can be drawn from part B of figure 21 is that there is no leakage of the promoter from the pTXB1 vector. This is because in the control lanes there is no band visible at the height of 43 kDa whereas in the induced cultures, the fragment is present.

Next the constructs were transformed in *WK6* cells. These cells were used to check if a better soluble nanobody could be obtained then with the *pLysS* cells. It was expected however that there would be no expression in *WK6* cells because the pTXB1 vector is not compatible with the *WK6* cells. Results of the protein expression via *WK6* cells are shown in figure 22.

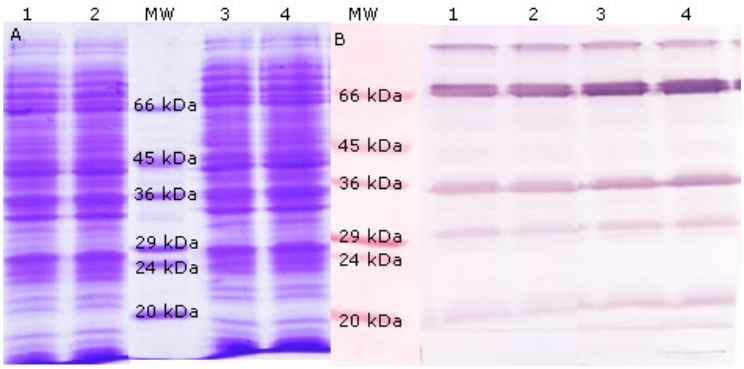

Figure 22: **A.** 12% SDS-PAGE gel of the *WK6* cell extracts. Lane 1 (negative control) and 2 contain the SSA construct. Lane 3 (negative control) and 4 contain the SA construct. **B.** Western blot of the SDS-PAGE gel from part A. Lane 1 and 2 contain the SSA constructs, lane 3 and 4 contain the SA constructs. Negative controls were lane 1 and lane 3.

The results in figure 22 match the expectations. A first observation, from part A, shows that there is very poor to no expression of the BCII10 nanobody in *WK6* cells. This is, as mentioned earlier, because the pTXB1 vector used is not appropriate for use in *WK6* cells. A very thin fragment can be observed at the 45 kDa height. A second conclusion from the picture is that there is no clear difference between total extracts from the colonies that were induced with IPTG and the extracts from the colonies that were not induced. As compared to the cell extracts from the *pLysS* cells in figure 21 however, there is a lot more expression of other proteins in the *WK6* cells because there are a lot more different bands visible all across every lane. In figure 22B, a Western blot of the SDS-PAGE gel in figure 22A is shown. This further confirms that expression of the BCII10-SSA and BCII10-SA constructs was not successful in *WK6* cells. As can be seen on figure 22B, the band that one would expect after induction with IPTG, at the height of 45 kDA, is not present. The other bands present on the blot are due to endogenous proteins that are recognised by the polyclonal antibodies.

After observing the results in figure 21 and 22 it was decided that the *pLysS* cells would be used for further protein expression of the SA and SSA constructs in the pTXB1 vector. The *WK6* cells were discarded for use with the pTXB1.

Due to time constraints, the SSA construct was not used for the rest of the project. The first reason as to why this usage of the SA construct over the SSA construct was because Chattopadhaya et al. described in literature that the SA construct is more likely to yield good results.[41-43] It was also opted to start with the SA construct because the amount of extra amino acids that are being added to the BCII10 C-terminus has to be as low as possible.

6.2.6 Testing the solubility of the BCII10-SA-intein-CBD fusion protein

In order to test the solubility and check the degree of in vivo cleavage of the pelB-BCII10-SA-intein-CBD fusion protein, a medium sized culture (300 ml) was prepared with transformed *pLysS* cells in LB medium. Protein expression was induced with 0.4 mM IPTG overnight at 16°C. Based on previous results, it was opted to use a low induction temperature to reduce the formation of inclusion bodies. After expression, periplasmatic and cytoplasmatic extracts were prepared. The obtained extracts were first analysed via SDS-PAGE analysis. These results are displayed in figure 23. In the future, periplasmatic extracts are preferred because the oxidizing environment of the periplasmic space is beneficial for the formation of disulfide bridges. These disulfide bridges are required for the stability of most nanobodies. The second reason why periplasmatic extracts will be used is because the pelB tag gets removed of the protein after transfer to the periplasm. If cytoplasmatic extracts were to be used, the tag would still be there which might impair functioning of the nanobody.

The samples in lane 1 and 2 of figure 23 are the total extracts of the *pLysS* cells before and after induction by IPTG. As can be seen, the protein bands present in these lanes are very thin as expected. This is because the temperature was only 16°C which will slow down the *E.coli* metabolism or even partially prevent it from functioning. The optimal temperature for *E.coli* cells is 37°C after all. Another observation on figure 23 is that there is no clear difference present between the culture that was induced with IPTG and the culture that was not induced with IPTG. This is

possibly due to the low induction temperature. In lane 3 of figure 23, the B-PER extract (cytoplasmatic and periplasmatic extract), is shown. This is not the same as a total extract however. A very thick smear of proteins is visible in this lane. The B-PER extract contains much more proteins than the other extracts. This is an expected result as the cytoplasmatic extract contains the proteins of both the periplasm and the cytoplasm. In lane 4 and 5, the periplasmatic extracts are loaded. For the periplasmatic extract the least bands are visible. This indicates that there is a very low amount of proteins present. It is because the periplasmatic space is only a small volume of the cell as compared to the whole cell or cytoplasmatic space. On top of that it has a completely different function. The difference between the periplasmatic extracts and the B-PER extract (lane 3) is so explicit because the B-PER extract contains proteins from both the periplasm and cytoplasm.

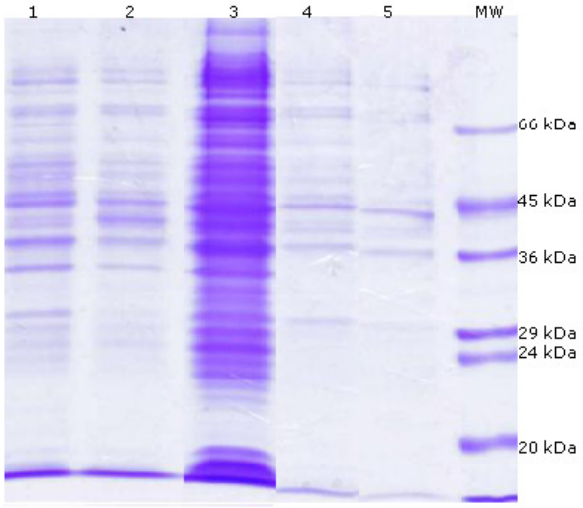

Figure 23: 12% SDS-PAGE analysis of cytoplasmatic and periplasmatic extracts of the BCII10-SA transformed *pLysS* cells. Lane 1 and 2 contains a total extract before and after induction of protein expression with IPTG. Lane 3 contains the cytoplasmatic extract obtained using B-PER reagent. Lane 4 contains the periplasmatic extract achieved using the VUB protocol. Lane 5 contains the extract obtained via the Reulen protocol.[40]

From figure 23 it is hard to conclude if induction of the nanobody was successful. This is because the concentration is very low and because there are endogenous proteins present at the same height as the fusion protein. Because of these reasons, a Western blot analysis was performed to check for the fusion protein. This result is shown in figure 24.

From figure 24 it is clear that by comparing the total extracts without and with IPTG in lane 1 and 2 respectively, the fusion protein was produced by the *pLysS* cells. In lane 1, the fragment at the height of 43 kDa, which corresponds to the weight of the pelB-BCII10-SA-intein-CBD fusion protein, is not visible. This is expected since no IPTG was added. In lane 2, this band is visible as expected. Other bands visible are due to aspecific binding of the polyclonal antibody to endogenous proteins. In lane 3 the B-PER extract can be seen. Here the band of the fusion proteins is visible as well. Apart from that a big smear of endogenously recognized proteins can be observed. One would expect the total extracts to be this concentrated as well yet as can be seen this is not the case. This can be explained by the dilution factor of the samples. The total extracts are diluted as if they come from a 50 µl culture. The B-PER extract, shown in lane 3 is diluted as if it came from a 350 µl culture and the periplasmatic extract is diluted as if t came from a 250 µl culture. This is why the difference is there. In order to really be able to compare them, the protein concentration would

have to be standardized but this was not performed within this project. It can be concluded from lane 2 and 3 that the BCII10 fusion protein is expressed well by the *pLysS E.coli* cells. Because of the dilution factors, a thicker band of fusion protein was expected for the B-PER extract. However, this is not the case indicating that there still are many inclusion bodies. There is however a good amount of fusion proteins that are in the cytoplasm in solution. In lane 4 and lane 5, the periplasmatic extracts are represented. The fusion protein is the very thin line at the height of 43 kDa. This line is surrounded by a purple haze as in figure 21. This is once again due to different forms of the fusion protein being present. The result from the extracts in lane 4 and 5 were not satisfactory however. If the thin fragment is compared to the fragments present in 3, it can be concluded that a lot of the recombinant BCII10 nanobody is not transferred to the periplasmic space. A possible explanation for this might be that the induction of 16°C is too low for the pathways that transfer proteins from the cytoplasm to the periplasm to be functional. Another explanation might be that the intein is preventing transfer to the periplasmic space. To confirm this however further experiments would have to be performed. In order to prevent or reduce formation of inclusion bodies, a balance has to be found between the concentration of IPTG used to induce protein expression on the one hand and the induction temperature on the other hand.
A positive result from figure 24 however, is that apparently the amount of in vivo cleavage has been reduced. This confirms that the strategy employed to solve the problem of in vivo cleavage was successful. Another possible explanation as to why there is no observation of in vivo cleavage is because the concentration of the fusion protein is very low, especially in the periplasmatic extracts.

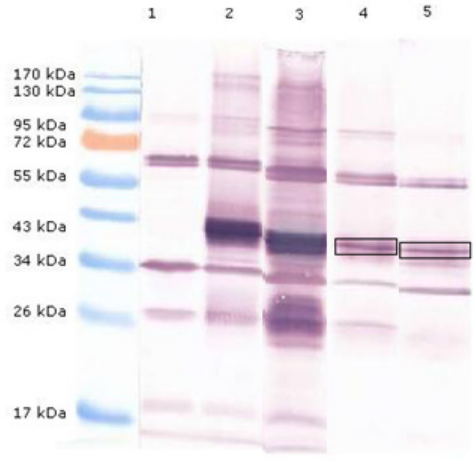

Figure 24: Western blot analysis of the SDS-PAGE gel shown in figure 23. Lane 1 contains the total extract before induction. Lane 2 contains the total extract after induction. The sample in lane 3 was the cytoplasmatic extract. In lane 4 and 5 the periplasmatic extracts are shown obtained using the protocol from VUB and Reulen respectively.[40]

In order to further verify these observations, induction was performed at higher temperatures to examine the effect on the protein transfer to the periplasm. This experiment will be discussed in section 6.2.7. Another experiment which is discussed there, is the search and optimization of a protocol which allows gaining a high amount of soluble proteins from the periplasm by an osmotic shock.

6.2.7 B-PER and periplasmatic extraction and characterization of BCII10-SA-intein-CBD

For the B-PER and periplasmatic extractions several cultures of *pLysS* cells transformed with the BCII10-SA construct were induced under different circumstances. Prior to induction the OD_{600} was approximately 0.7 for both the TB and LB cultures after growing for 3 hours at 37°C. Two cultures with 100 ml TB medium were induced and 2 cultures of 100 ml LB medium were induced. One LB and one TB culture were induced for 3 hours at 30°C with 0.2 mM IPTG. The other LB and TB culture were induced overnight at 28°C with 0.2 mM IPTG. These concentrations and induction temperatures were chosen because in two other protocols, the one from VUB and the one from Reulen et al., good results were obtained with them.[40] The concentration of IPTG was reduced as compared to the testinductions in section 6.2.6 to avoid formation of inclusion bodies. This was done because the induction temperature was increased almost two-fold. The periplasmatic extraction was performed using 2 different protocols and the cytoplasmatic extraction was done with B-PER reagents. These were described earlier. Results are shown in figure 25.
In lane 1 and 2 of figure 25, a band at around 43 kDa can be observed which corresponds to the BCII10-SA-intein-CBD fusion protein present in the total cell extracts. The band here is more intense then the bands observed in figure 23 for the same extracts. This is because induction was performed at higher temperatures.

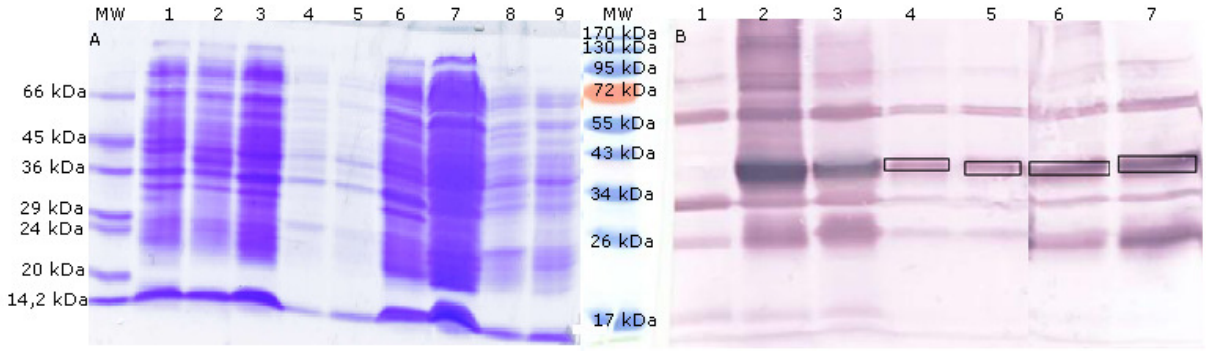

Figure 25: **A.** 12% SDS-PAGE gel containing the extracts of the *pLysS* cells in which BCII10 expression was induced under varying circumstances. Lane 1 and 2: Total extract before (1) and after (2) induction with IPTG. Lane 3: B-PER extract of LB cultures after 3 hours growth. Lane 4 and 5: VUB periplasmatic extract of LB (4) and TB (5) cultures after 3 hours growth. Lane 6 and 7: B-PER extracts from LB (6) and TB (7) cultures after overnight growth. Lane 8 and 9:VUB periplasmatic extracts from LB (8) and TB (9) cultures after overnight growth. **B.** Western blot of the gel in part A. Lane 1 and 2: Total extracts before and after induction with IPTG. Lane 3: B-PER extract form colonies in LB contains the after 3 hours growth. Lane 4 and 5: VUB periplasmatic extracts from LB (4) and TB (5) cultures after 3 hours growth. Lane 6 and 7: VUB periplasmatic extracts from LB (6) and TB (7) cultures after overnight growth.

For the B-PER extracts, present in lane 3, 6 and 7 of figure 25A, the same thick, smear of proteins can be observed once more. The periplasmatic extracts are shown in lane 4 and 5 for the culture that was induced for 3 hours. The ones in lane 8 and 9 are from the cultures that were induced overnight. As can be seen, the intensity of the correct fragments at the height of approximately 45 kDa in the overnight cultures has not increased much as compared to the fragment from the cultures that were only induced for 3 hours. It can be observed however that a lot more endogenous proteins have been expressed in the overnight-induced cultures. In order to further analyse these results, a Western blot analysis of the SDS-PAGE gel was done as shown in figure

25B. The difference between the B-PER extracts and the total extracts in amount of protein can once again be explained by the dilution factor while preparing the samples for gel analysis.
The periplasmatic extracts for the 3 hour cultures are shown in lane 4 and 5, the ones for the overnight cultures are shown in lane 8 and 9. By comparing the 3 hour cultures to the overnight cultures, it is already visible that the thin fragment at the 43 kDa line is more intense for the overnight culture. By comparing the periplasmatic extract obtained from the culture grown in LB and the culture grown in TB, it is visible that the fragment for the TB cultures is more intense. The difference is particularly clear for the overnight cultures and also matches expectations since the TB medium is further enriched with other substances as mentioned earlier. As compared to the results from the testinductions at 16°C, shown in figure 21, the fragments in the periplasmatic extract are more intense indicating that transfer of the fusion protein from the cytoplasm to the periplasm occurs more efficiently at elevated temperatures. Even though the transfer is more effective, there is still a very low concentration of the fusion protein in the periplasmatic extract as compared to the cytoplasmatic extract. This is most likely due to the type of *E.coli* cells that were used because better results were obtained in a parallel project running in the group in which *WK6 E.coli* cells were used for production of BCII10-His_6 nanobodies in a pHEN6a vector. It is important to note however that in this project no fusion proteins are being used. It is possible that the periplasmatic transfer is inefficient because of the intein. Nevertheless, it was decided to purify the BCII10-SA-intein-CBD from the periplasmatic extract. Another conclusion from this blot is that it is beneficial to use TB for the expression of BCII10 fusion proteins since some of the supplements in the TB medium, such as sucrose reduce the toxicity of recombinant proteins for the *E.coli* cells. The periplasmatic extractions based on the protocol provided by VUB were most successful and will therefore be used further. However, it is expected that there still is a high degree of inclusion body formation. This is concluded by comparison of the B-PER extracts to the periplasmic extracts. To further investigate this, the protocol was tested with a bigger culture. (Section 6.2.8) In case experiments with the SA (Bio6) and LEY (Bio54) constructs still are not satisfactory. A construct will be made in which the BCII10-SA-C-intein-CBD construct is cloned into a pHEN6a vector allowing for expression in *WK6* cells. This should reduce formation of inclusion bodies because the promoter of the pHEN6a vector has a lower expression level as compared to the promoter of pTXB1. This will be beneficial to reduce the amount of inclusion bodies. In addition the *WK6* cells are better suited for periplasmic expression and extraction as compared to the *pLysS* cells. This will be discussed in section 6.3.

6.2.8 Purification of the modified BCII10 nanobody from the periplasmatic extract by affinity chromatography

For purification of the BCII10-SA nanobody, a fresh culture (1 liter) of *pLysS* cells transformed with the pTXB1 vector containing the pelB-BCII10-SA-C-intein-CBD was started. Protein expression was induced by 0,2 mM IPTG overnight at 28°C. The periplasmatic extract was obtained via the VUB protocol. Results of the purification were analyzed by SDS-PAGE and are shown in figure 26 and 27.
Figure 26 shows an overview of all the steps in the purification of the BCII10-SA out of the periplasmatic extract. Lane 1 and 2 contain the total extracts once more for comparison. Lane 3

and 4 contain the periplasmatic extracts before and after induction with IPTG. As can be seen in lane 3, a fragment is visible at the height of 29 kDa. This protein is only visible in the periplasmatic extract prior to induction. It is most likely an endogenous protein but it might also be the intein-CBD complex. For further confirmation a Western blot analysis will be performed.

By comparing lane 6, 7 and 8, it can be concluded that cleavage of the BCII10-SA fusion protein from the intein-CBD can be performed successfully by MESNA. In lane 6, thick fragments can be observed. The expected fragment at the height of 45 kDa can be observed as well. After cleavage, it can be observed that the fragment at the height of 45 kDa is gone. The BCII10-SA has been cleaved off by MESNA. The fragment remaining at the height of 29 kDa in lane 7 corresponds to the intein-CBD complex which remains on the beads after elution. Lane 8 contains the elution of the column obtained by MESNA. The BCII10-SA nanobody is highlighted on figure 26A.

To further verify the result from figure 26 a Western blot analysis was performed. Finally, the purity of the extracts was checked. This result is shown in figure 27.

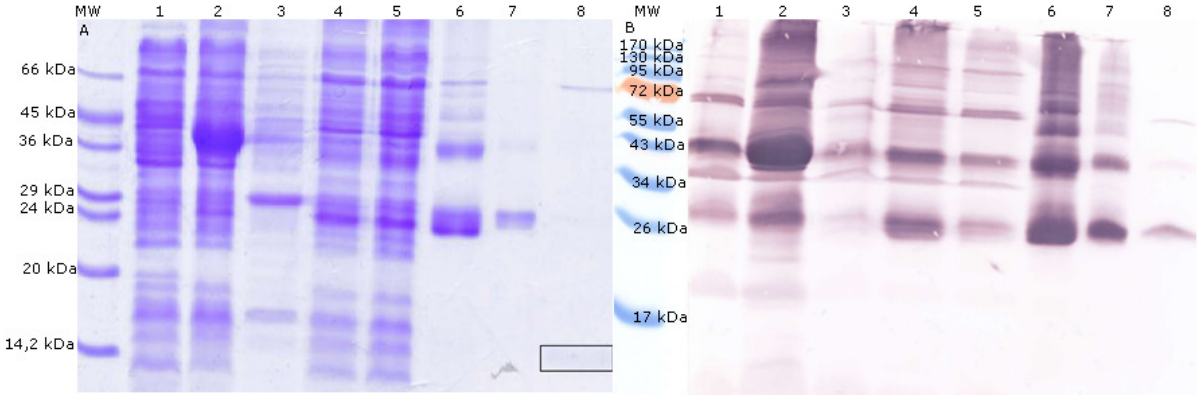

Figure 26: **A.** 12% SDS-PAGE analysis to check for the purification of BCII10-SA. Lane 1 and 2 contain total cell extracts before and after induction. Lane 3 and 4 contain the periplasmatic extract before and after induction. Lane 5 contains the flow through from the column. Lane 6 and 7 contain the extract of the chitin beads before and after cleavage with MESNA. Lane 8 contains the elution. **B.** Western blot analysis of the SDS-PAGE gel shown in figure 26A. Lane 1 and 2 contain the total extracts before and after induction. Lane 3 and 4 contain the periplasmatic extract before and after induction. Lane 5 contains the flow through. Lane 6 and 7 contain the beads before and after cleavage. In lane 8, the elution is shown. For recognition of the fusion proteins a polyclonal anti-CBD antibody was used.

As predicted, a clear difference can be observed between the total extracts before and after induction with IPTG. A very big, intense fragment can be observed in lane 2 (fig 26B). It was mentioned earlier that in lane 3 from figure 26A, a band could be observed at the height of the 29 kDa fragment of the marker. On figure 26B in lane 3, this band can no longer be observed confirming that it was an endogenous protein. If it contained the CBD, a band would have shown up. In lane 5 (figure 26B), the flow through of the beads was loaded. By comparison to lane 4 (figure 26B), it is clear that a certain amount of the BCII10-SA-intein-CBD fusion protein has bound to the chitin beads though there still is a lot of the protein that hasn't bound to the beads. This can be due to the setting of an equilibrium between the binding spots on the chitin beads and the periplasmic extract after which no more protein is bound. By comparison of lane 6 and 7 (figure 26B), it is further assured that the chitin beads bind the fusion protein. However, there still is some in vivo cleavage present since the band which matches the intein-CBD is present at the height of approximately 26 kDa before addition of MESNA to induce cleavage. In lane 6, thick fragments can

be observed for a sample of chitin beads that was taken prior to cleavage. It can be seen that the BCII10 fusion protein is present since there is a band with a high intensity at the height of the 43 kDa fragment of the marker. Lane 7 contains a sample of the beads after cleavage and elution. As can be seen there is a thick fragment present at the height of approximately 29 kDa. This corresponds to the intein-CBD complex that remains on the beads after cleavage. A small fraction of the intact BCII10-SA-intein-CBD can be observed as well. This indicates that the cleavage efficiency by MESNA is not 100%. (Appendix 7) Nevertheless, purification of the BCII10-SA protein is possible. Lane 8 contains the elution of the samples.

As can be seen on figure 27, the BCII10-SA modified protein was eluted successfully. The band corresponding to the protein is situated in between the 14,2 and 20 kDa fragments of the marker. Apart from the expected band however, other bands could be observed as well. The band at the height of the 29 kDa fragment of the marker is a small fraction of the intein-CBD complex that has come loose of the beads. Another fragment that can be observed, are the fragments at the height of 66 kDa. This can be either endogenous proteins or poly-mers that arise by spontaneous dimerisation of the nanobodies of the BCII10-SA nanobody. Further investigation to identify these bands was not performed. From figure 27, it is also clear that after eluting the column 5 times, almost all of the BCII10-SA is eluted. Further eluting of the column does not increase the yield drastically and is therefore not recommendable. This is concluded from the intensity of the fragments at the height of the 15 kDa fragment.

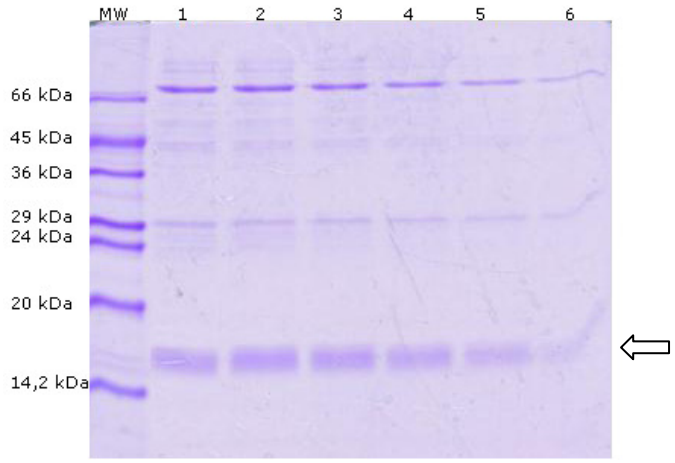

Figure 27: 12% SDS-PAGE analysis checking for the purity after affinity chromatography with chitin beads. Lane 1 contains the eluted MESNA after overnight incubation. Lane 2 till 6 contains the fractions that were eluted by adding 1 ml of column buffer.

The total yield after purification was approximately 50 µg/l culture. Because of this low yield in *pLysS*, *BL21DE3* cells were tested to check if the nanobody yield could be increased in this way. With the *BL21DE3* cells the expression and purification was performed as well. These cells were chosen because Reulen et al. obtained good results using these cells.[40] No improvement was observed here however. After the experiments performed with the construct in a pTXB1 vector for expression in *pLysS* cells and the unsatisfactory results, another strategy was employed.

This included cloning the pelB-BCII10-SA-intein-CBD construct into another type of vector. This vector was the pHEN6a vector which allows for expression of the fusion protein in *WK6 E.coli* cells. (Chapter 6.4) Further experiments with the pelB-BCII10-SS-LEY-C-intein-CBD construct were performed to check if better results could be obtained. (Chapter 6.3)

6.3 C-terminal, post-translational modification of BCII10 nanobodies in a pMXB10 vector

6.3.1 Amplification and modification of pelB-BCII10-SS-LE by PCR of native BCII10 DNA.

To try to further improve the solubility of the nanobodies, a construct was made in which the last 3 C-terminal amino acids match the last C-terminal amino acids of MBP. These amino acids are leucine (L), glutamic acid (E) and tyrosine (Y). This was done because, as is shown from the results in section 6.1, all the problems that arise when working with the BCII10 nanobody are non-existent when doing C-terminal coupling of MBP. Because of this a pMXB10 vector containing the MBP was digested with NdeI and XhoI with the exception that the last C terminal amino acid of MBP, tyrosine (Y) remained in the open vector. This is because of the location of the XhoI restriction site. Into this open vector, the BCII10 nanobody wild type DNA was ligated to which a leucine (L) and glutamic acid (E) were added by PCR. This results in construction of a pMXB10 vector containing pelB-BCII10-SS-LEY-C-intein-CBD.

In a third construct, Bio44, the C-terminal serine is changed to a glycine (G) via PCR. This construct, pelB-BCII10-SG-C-intein-CBD (Bio44), was ligated in the pTXB1 vector and is used here for means of comparison since glycine is known from literature to be very resistant to in vivo cleavage. [41-43]

To start of the plasmid DNA, here called Bio54 and Bio44, was amplified by PCR with primers designed to introduce the specific mutations. After amplification the DNA was ligated into a pCR2.1 vector and transformed into *TOP10F'* cells. Bacteria containing a vector with Bio54 were then selected via blue/white screening. The plasmid DNA was extracted for further use. Reaction circumstances for both the ligation and amplification are shown appendix 3 and 4.

Figure 28 shows the DNA samples after amplification. For Bio54 a fragment of 471 bp was expected. In the lane containing Bio44 a fragment of 477 bp is to be expected.

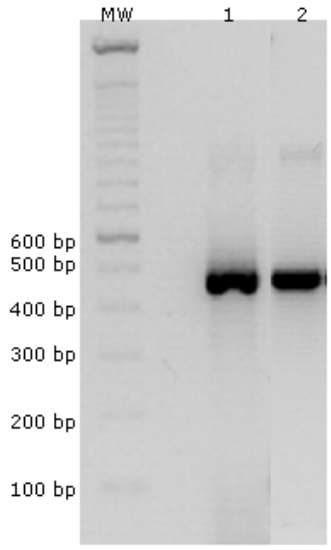

Figure 28: 1,5% agarose gel to check the amplification of BCII10 DNA. Lane 1 contains the Bio54 modified DNA. Lane 2 contains the Bio44 modified DNA.

The amplification of BCII10 DNA with the modified primers was successful. This can be concluded since the fragments observed in figure 28 are both situated just below the 500 bp fragment of the molecular weight marker. Next the DNA was transformed into *TOP10F'* cells. Via blue/white screening white colonies were picked and grown again overnight in LB medium. After overnight growth, the DNA was isolated and digested with EcoRI. (Appendix 5) For Bio54, the digestion should result in a fragment of 487 bp and a fragment of 3913 bp. For Bio44, a fragment of 493 bp and a fragment of 3913 bp should be observed. Results of the digestion are shown below in figure 29.

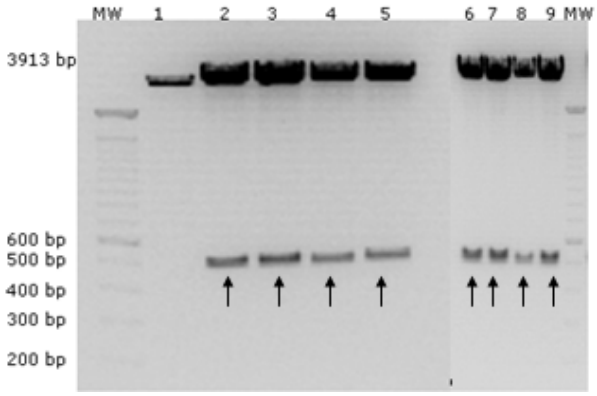

Figure 29: 1,5% agarose gel containing Bio44 and Bio54 in pCR2.1 after digestion with EcoRI. Digestion was performed to make a first selection of potentially successful clones. Lane 1 till 5 contains the Bio54 clones. Lane 6 till 9 contains the Bio44 clones.

For Bio54, the clones in lane 2, 3, 4 and 5 were successfully cloned. The clone in lane 1 did not contain an insert hence why only 1 fragment can be observed. The DNA from the clone in lane 1 was discarded from further experiments. After the digestion, the good clones were sequenced. (Appendix 3 and 6)

The results for Bio44 are represented in lanes 6, 7, 8 and 9. It appears that all of these samples were successfully cloned. The 493 bp fragment is there just below the 500 bp fragment of the molecular weight marker. All of these 4 clones will be included for now. Sequencing results can be found in appendix 6. The miniprep DNA that will be used for ligation into a pMXB10 (Bio54) or pTXB1 (Bio44) vector was the sample that was loaded in lane 2 (Bio54) and lane 7 (Bio44).

6.3.2 Cloning of pelB-BCII10-SS-LE into a pMXB10 vector

In order to clone Bio54 into a pMXB10 vector, the Bio54 construct had to be excised out of the pCR2.1 vector and the pMXB10 vector had to be cut open. This was done by using NdeI and XhoI. (Appendix 5) Because both the construct and the vector can be digested with the same enzymes, this will result in compatible sticky ends to perform the cloning reaction. After digestion of the pCR2.1 vector containing the Bio54 construct, 3 fragments of different lengths are expected. A 3897 bp fragment, a 454 bp fragment and a 49 bp fragment. The required fragment for the cloning reaction is the 454 bp fragment. After digestion of the pMXB10 vector containing MBP, a fragment of 6662 bp and a fragment of 1151 bp is expected. The 6662 bp fragment is used for further cloning procedures. Results of the digestion are shown in figure 30

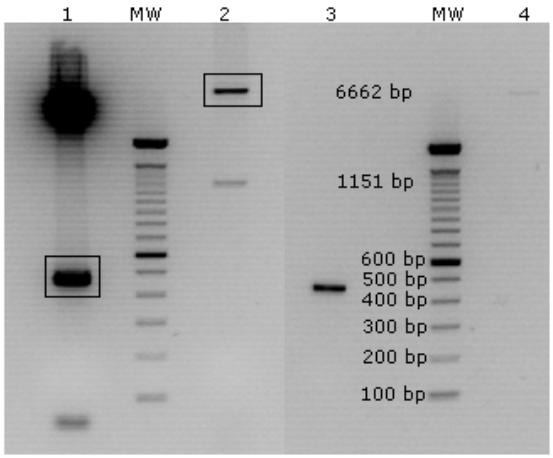

Figure 30: 1,5% agarose gel to check the digestion by NdeI/XhoI and to cut out the desired fragments for purification. Lane 1 contains the pCR2.1 vector containing Bio54. Lane 2 contains the pMXB10 vector containing MBP. Lane 3 contains the fragment from lane 1 after purification. Lane 4 contains the fragment from lane 2 after purification.

Digestion to extract the Bio54 construct was, as shown in lane 1, successful. The highest fragment is the 3897 bp fragment. This is what remains of the pCR2.1 vector after digestion. The desired fragment is the 454 bp fragment which is more specifically pelB-BCII10-SS-LE. This fragment will be excised and ligated into the pMXB10 vector. The 49 bp fragment at the bottom of the gel is a part of the multiple cloning site that gets cut out as well. In lane 2, the pMXB10 vector after digestion was loaded. Here, the experiment was successful as well since the 2 expected fragments can be observed. The desired fragment is highlighted by the black rectangle and is the pMXB10 vector without its original insert, MBP. The other fragment is MBP without the last C-terminal amino acid being tyrosine. Tyrosine will remain in the vector and will become the C-terminal amino acid of the BCII10 nanobody after its DNA is ligated into the pMXB10 vector. After ligation the full fusion protein, pelB-BCII10-SS-LEY-C-intein-CBD, is obtained. In lane 3 and 4, the DNA of Bio54 and pMXB10 is shown after purification. The intensity of the fragments has reduced as compared to lane 1 and 2. This is because less material was loaded onto the gel. The purified samples were used for ligation of the Bio54 construct into pMXB10. (Appendix 4) Next, the pMXB10 vector containing the Bio54 construct was transformed into *TOP10F'* cells. After overnight growth at 37°C, colonies were picked and grown in 3 ml of LB after which the DNA was isolated and digested by PstI. If a clone was successfully incorporated, 2 fragments should be visible after digestion. one fragment of approximately 6187 bp and one of 927 bp. Results of the digestion by PstI are shown in figure 31.

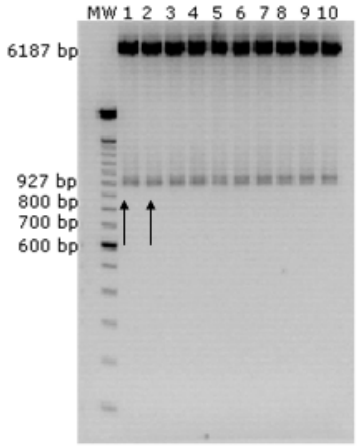

Figure 31: Restriction digestion of the pMXB10 vector containing the Bio54 construct. Digestion was performed with PstI for 1 hour at 37°C. Lane 1 till 10 contains all the different clones. Arrows indicate clones selected for sequencing.

As can be seen from the picture above, the cloning of BCII10-SS-LEY-C-intein-CBD into the pMXB10 was successful. After restriction digestion, every single one of the 10 colonies seemed to have a correct vector in them. As expected, 2 fragments can be observed on the gel: The first one being the pMXB10 vector and the second one being the 927 bp fragment that was excised out of the vector by PstI. From these successful clones, the DNA that was loaded in lane 1 and 2 was sequenced. Sequencing primers and results are shown in appendix 3 and 6. After sequencing it was further confirmed that both DNA samples were suited for further use. The miniprep DNA from the sample in lane 1 was used for protein expression discussed in section 6.3.3.

6.3.3 Characterisation of pelB-BCII10-SS-LEY-C-intein-CBD in pMXB10 testinductions in pLysS E.coli cells by SDS-PAGE and Western blot

In order to test the induction of BCII10, the Bio54 construct was transformed to *pLysS E.coli* cells. During the transformation a heat shock of only 45 seconds was given. This increased the transformation efficiency. The *pLysS* cells were grown overnight on agar plates supplemented with ampicillin and chloramphenicol. Next, single colonies of both the Bio54 and Bio44 were picked and grown for 5 hours at 37°C in TB medium. Finally, nanobody expression was induced overnight at 28°C by addition of 1 mM IPTG. The results are shown in figure 32.

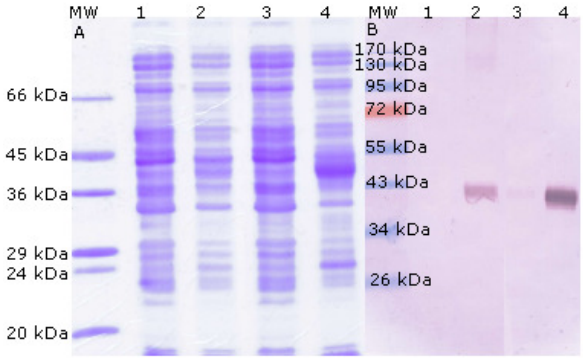

Figure 32: **A.** 12% SDS-PAGE analysis of the total extracts from the testinductions for Bio54 and Bio44. Lane 1 and 2 contain the Bio54 testinductions. Lane 1 was not induced with IPTG. Lane and 4 contain the Bio44 testinductions. Lane 3 was not induced with IPTG. **B.** Western blot of the SDS-PAGE from part A. Lane 1 and 2 contain the Bio54 testinductions. Lane 3 and 4 contain the Bio44 testinductions. Lane1 and 3 were not induced with IPTG and functioned as negative controls.

For the Bio54 cultures, which are loaded from lanes 1 and 2 on figure 32A, the results shown on the gel are remarkable. A first conclusion is that there appears to be no difference between the negative control in lane 1 and the other cultures that were induced. There is no clear band visible. Secondly, the fragment which is expected at a height of 45 kDa can not be distinguished clearly. For further conclusions, a Western blot analysis will be performed using monoclonal antibodies.
For the Bio44 construct, the difference between the induced and non-induced cultures is better visible. In the negative control (lane 3) there is no thick, more intense fragment present at a height of 45 kDa whereas in the induced cultures (lane 4), the fragment is present. A clear, thick, intense fragment can be distinguished in this lane at the height of 45 kDa. Further analysis of the testinductions will be discussed by the Western blot shown in figure 32B. For the Western blot a monoclonal antibody was used.
A first remark that can be made from the Western blot is that here, the difference between the induced and non-induced cultures of Bio54 is clearly visible. In lane 1, there is no band present, as would be expected. In lane 2 a clear band can be observed at the height of the 43 kDa fragment from the molecular weight marker. This band is the BCII10-SS-LEY-C-intein-CBD fusion protein. For the Bio44 cultures, the same observation can be done. For the Bio54 total extract in lane 2 the bands that can be seen have a low intensity. The fact that the intensity is low is beneficial for production of the nanobody in larger cultures. The intensity of the bands is low because the fusion protein concentration is low. Because of this the Bio54 fusion protein is less prone to formation of inclusion bodies and the solubility might increase. For further testing of this hypothesis however, a larger culture will have to be performed out of which periplasmatic extracts are made and then purified via chitin beads affinity chromatography. This was not performed during this study due to time constraints.
The fragments from the Bio44 extracts are a lot more intense then those of the Bio54 extracts. This indicates that the expression of this fusion protein is higher then the Bio54 fusion protein. The drawback of this however is that there is a higher chance for the Bio44 fusion protein to be insoluble or to form inclusion bodies. This would be detrimental for the modification of the nanobodies. To check for this, a large culture would have to be performed as well followed by purification of the periplasmatic extracts. This will be performed in the future where a balance will have to be found between good expression levels, solubility, in vivo cleavage and yields after purification.

6.4 C-terminal modification of BCII10 nanobodies in a pHEN6 vector

6.4.1 Engineering of a-BCII1-SA-intein-CBD by cloning modified BCII10 cDNA into a pHEN6a vector.

For this cloning, the BCII10-SA-intein-CBD construct was cut out of the pTXB1 vector constructs that were described in section 6.2 in 2 times. In order to do this, the pTXB1 vector containing the pelB-BCII10-SA-C-intein-CBD clone was cut open with BamHI.(Appendix 5). BamHI will digest the DNA just after the stop codon of pelB-BCII10-SA-C-intein-CBD. Since no empty pHEN6a vectors were available, a pHEN6a vector containing the BCII10 nanobody with a hexahistidine (His_6) tag was used. This vector was cut open with EcoRI. (Appendix 5) EcoRI will cut just after the stop

codon of pelB-BCII10-SS-RGR-His_6. Because of the different restriction enzymes that had to be used to cut in the pTXB1 and the pHEN6a vector, the sticky ends that are obtained after digestion are not compatible. The consequence of this is that to be able to clone the fragment into the pHEN6a vector, the sticky ends need to be blunted. (Appendix 5) This means that a T4 DNA polymerase will be used to fill up the sticky ends made by EcoRI and BamHI with nucleotides. Another consequence of this is that the digestion had to be performed in multiple steps. After digestion with BamHI (pTXB1) and EcoRI (pHEN6a), the sticky ends are blunted. Next the vectors are digested with NcoI to cut out the inserts. NcoI digests the DNA at the end of the pelB sequence. This digestion will result in compatible sticky ends in the BCII10-SA-intein-CBD construct and pHEN6a. Finally, ligation can be performed with T4 DNA ligase by adding the BCII10-SA-intein-CBD and open pHEN6a vector together.
First, the restriction digestion with BamHI and EcoRI will be discussed. The result of the digestion and blunting of pTXB1 and pHEN6a are shown in figure 33.

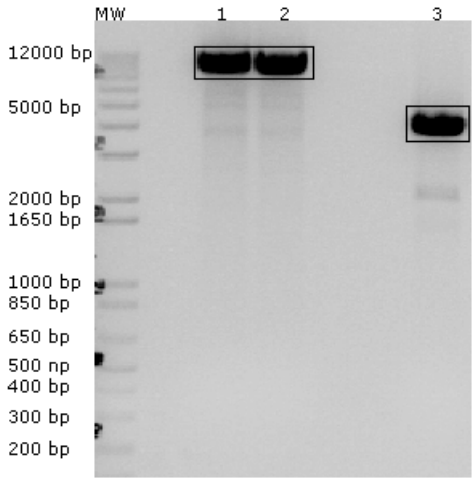

Figure 33: 0.8% agarose gel containing the pTXB1 vector and pHEN6a vector after digestion for 1 hour at 37°C. Lane 1 and 2 contain the pTXB1 vector with pelB-BCII10-SA-C-Intein-CBD. With pelB-VHH-SS-RGR-His_6 which was cut open with BamHI. Lane 3 contains the pHEN6a vector that was cut open with EcoRI.

After digestion of the pTXB1 and pHEN6a vector, a single fragment should be found on the agarose gel. As predicted, a thick, single fragment can be observed in each of the 3 lanes. The pTXB1 vector in lane 1 and 2 can then be cut out for purification. One can observe however that there are some other fragments visible on the gel. These fragments are most likely vector which was cut at more then 1 spot by BamHI star activity. Star activity is the aspecific digestion by restriction enzymes. For the pHEN6a, the same results can be observed. The fragments that were cut out of the gel are depicted by the black rectangles.
The next step in cloning the pelB-BCII10-SA-C-intein-CBD to a pHEN6a vector was digestion with NcoI. In this step, the pelB-BCII10-SA-C-intein-CBD will be excised out of the pTXB1 vector and the BCII10-His_6 will be excised of the pHEN6a vector. Because of this, it is expected that there will be different fragments visible on the gel. The pelB-BCII10-SA-intein-CBD fragment that was cut out of the pTXB1 had a length of 1186 bp. For the pHEN6a vector that was cut open, the fragment of 3208 bp is the one that is required for the cloning procedure. After digestion, the fragments were separated by agarose gel electrophoresis. This is shown in figure 34.

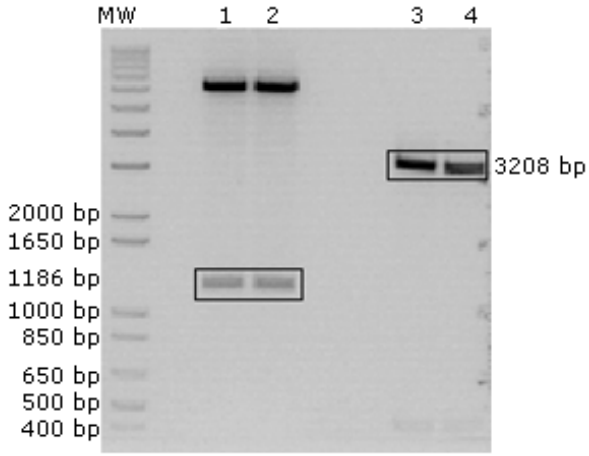

Figure 34: 0.8% agarose gel containing the BCII10-SA-intein-CBD construct cut out of the pTXB1 vector. This is shown in lane 1 and 2. In lane 3 the pHEN6a vector is shown. Out of this vector the BCII10-His_6 was removed. Digestion for both the pTXB1 and pHEN6a was done with NcoI.

The required fragments with a length of 1186 bp and 3208 bp were present. They are depicted by the black rectangles on figure 34. They were excised out of the gel and purified. The more intense fragment near the top of the gel (lane 1 and 2) is the open pTXB1 vector without the pelB-BCII10-SA-C-intein-CBD fusion protein.

The digested pHEN6a vector is shown in lane 3 and 4. The highlighted fragment is the pHEN6 vector that was cut open. It was purified out of the gel as well.

Finally the open pHEN6a vector and the BCII10-SA-intein-CBD were added together in a reaction mixture containing T4 DNA ligase. The composition of the ligation mix is mentioned in appendix 4. After ligation, the new constructs were transformed into *TOP10F'* cells for plasmid DNA amplification followed by isolation and restriction digestion analysis. To check if the cloning was correct, the samples were also sequenced. The results of the restriction digestion analysis are shown in figure 35. If cloning was successful, 2 fragments with a length of 918 bp and 3480 bp are expected after digestion with PstI.

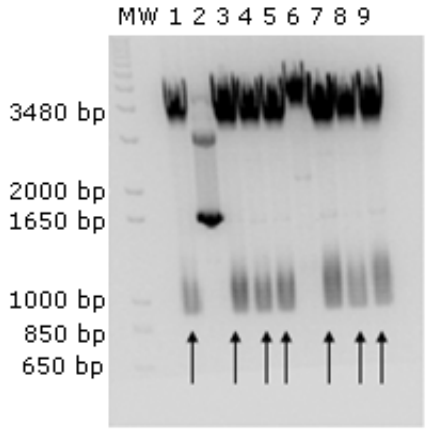

Figure 35: 1% agarose gel showing the results of the cloning of the BCII10-SA-intein-CBD construct in pHEN6a. Digestion was done with PstI. Lane 1 to 9 contain the results of 9 different, single colonies that were picked and grown overnight after which plasmid DNA was isolated and digested with PstI.

The plasmid DNA in lane 1, 3, 4, 5, 7, 8 and 9 was found to be matching expectations after digestion with PstI. The samples in lane 2 and 6 did not match the expectations and were discarded

from further cloning. A final remark that can be given about figure 35 is the smear that is present in every lane. This occurred to be typical for digestions of pHEN6a vectors and reoccurred in every pHEN6a digestion. No further investigation was performed.
Further sequencing of the good clones that were first selected by restriction digestion showed that the clones in lane 1 and lane 3 were cloned correctly. These were transformed into chemically competent *WK6 E.coli* cells. The arrows indicate the constructs that were found to be correct after digestion and were sequenced. Primers and results are shown in appendix 3 and 6. The miniprep in lane 1 and 3 was found to be correct

6.4.2 Characterisation of BCII10-SA-C-intein-CBD in pHEN6a testinductions in WK6 E.coli cell lines.

After transformation of *WK6* cells and overnight growth, colonies of each of the correct DNA samples, the ones from lane 1 and 3, were picked and inoculated in 4 ml TB medium supplemented with ampicillin. The colonies were grown for 5 hours at 37°C until OD_{600} was approximately 0.5 and protein expression was induced overnight at 28°C by addition of 1 mM IPTG. Results of the testinductions were first analyzed by SDS-PAGE and are shown below in figure 36.

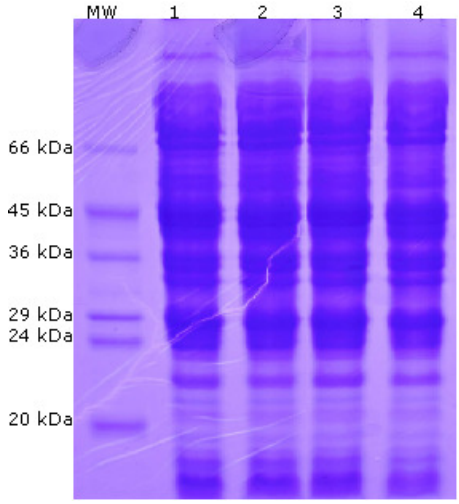

Figure 36: 12% SDS-PAGE analysis of BCII10-SA-C-intein-CBD in pHEN6a, transformed in *WK6* cells. Lane 1 and lane 3 contain the negative controls. These are cultures to which no IPTG was added. Lane 2 contains the testinduction with the DNA of clone number 1. Lane 4 contains the testinductions with the DNA of clone number 3.

As can be seen, a lot of different proteins have been expressed by the *WK6* cells. The desired protein, the BCII10-SA fusion protein should once again be visible at a height of approximately 45 kDa. As can be seen on figure 36, a thick band is indeed visible. To be sure that this is indeed the fusion protein and not another endogenous protein of the same mass, a Western blot was performed. A remarkable fact that can be observed from this SDS-PAGE is that there appears to be almost no difference between the negative controls, in lane 1 and 3, and the other samples. This is due to a leakage of the promoter region in the pHEN6a vector and will be investigated further with Western blot. Below, in figure 37, are the results of the Western blot analysis.

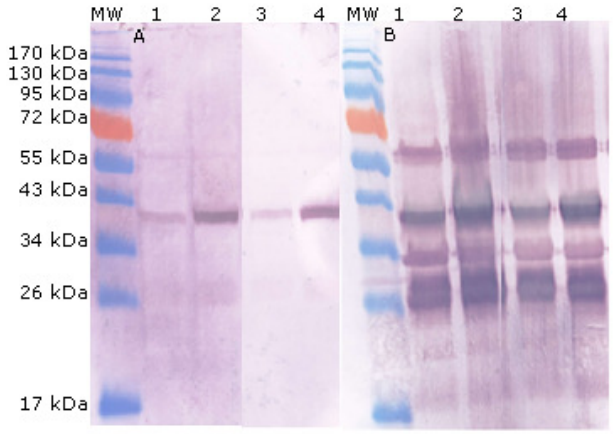

Figure 37: **A.** Western blot analysis of the SDS-PAGE gel shown in figure 36. This blot was stained using a monoclonal anti-CBD antibody as primary antibody. Lane 1 and 3 contain the negative controls. Lane 2 contains the total extract of *WK6* cells transformed with DNA from clone 1. Lane 4 contains the total extracts of *WK6* cells transformed with DNA from clone 3. **B.** Western blot analysis of the SDS-PAGE shown in figure 36. This blot was stained using a polyclonal anti-CBD antibody. Lane 1 and 3 contain the negative controls. Lane 2 contains the total extract of *WK6* cells transformed with DNA from clone 1. Lane 4 contains the total extracts of *WK6* cells transformed with DNA from clone 3 in figure 35.

First, by comparison of the blot on figure 37A, which was stained with a monoclonal antibody, to the one on figure 37B, which was stained with a polyclonal antibody, it can be seen that there is much aspecific recognition by the polyclonal antibodies. This is concluded from the high amount of bands which are visible on the polyclonal blot. Where as on the monoclonal blot, only one fragment can be distinguished. Secondly, from the Western blot analysis it can be seen that there is a clear difference between the cultures with and without IPTG as compared to on the SDS-PAGE. This difference can be especially explicit on the monoclonal blot. The difference in intensity of the fragment for the induced and non-induced cultures is clear. On the polyclonal blot, the difference between induced and non-induced cultures is less explicit. It is likely however that there is some leakage of the promoter since there are bands present in the control samples at the height of 43 kDa. This is the height where the fusion protein can be retrieved.

Figure 37 further confirms the conclusion from the SDS-PAGE in figure 36. Expression of the BCII10-SA-C-intein-CBD fusion protein was successful in *WK6* cells. The expected band at a height of 43 kDa is clearly visible. There is a very weak fragment visible as well at just about the 26 kDa fragment in the monoclonal blot. This fragment is probably a low concentration of intein-CBD which still has been cleaved off in vivo. A possible experiment which can be done to further confirm this is to do an SDS-PAGE and Western blot analysis of *WK6* cells containing no BCII10 fusion protein plasmid DNA. If these bands then show up on a Western blot with monoclonal antibodies, it is not due to in vivo cleavage but due to endogenous proteins.

For future experiments large cultures of the pHEN6a construct in *WK6* cells would have to be set up. Next the solubility will then be checked by purifying the modified nanobodies out of periplasmatic extracts. If these nanobodies are still soluble as well, C-terminal modification will be performed after which surface coupling experiments can be performed. Due to time constraints, these experiments were not conducted within this study.

7. Conclusion

During the first part of the study, a proof of concept was given by making use of MBP as a test protein. First it was proven that induction of MBP-intein-CBD fusion protein in *pLysS* cells with IPTG was possible. After inducing the expression in *E.coli* cells, the MBP-intein-CBD fusion protein was found to be soluble. MBP did not form inclusion bodies. After purification of MBP fusion proteins out of a B-PER extract, the MBP was modified C-terminally with an alkyne functionality. Both the purification and modification were successful. The final proof of concept was delivered by performing a "click" reaction in solution. As was shown by Western blot, this "click" reaction occurred successfully. After the modification, there was little to no degradation of the MBP protein.
Next the same experiments were conducted with the BCII10 nanobody. BCII10 nanobody expression in *E.coli* cells was successful. The nanobodies were targeted to the periplasmic space after expression by a pelB tag. However there were some problems for the nanobodies which did not occur with MBP. The nanobodies were not soluble after expression and there was a very high degree of in vivo cleavage. To address this problem, different constructs were cloned into different types of vectors (pTXB1, pMXB10 or pHEN6a). In these constructs the last C-terminal serine of the BCII10 nanobody was changed to (an)other amino acid(s) with PCR. The SA construct was cloned into a pTXB1 and pHEN6 vector, the SSA construct was cloned into a pTXB1 vector and the LEY construct was cloned into a pMXB10 vector. Nanobody expression with the new clones was successful. The in vivo cleavage of the nanobody fusion protein had effectively been reduced. To asses the solubility of the nanobodies, different periplasmatic extraction protocols were tested. The osmotic shock according to the protocol by Brussels university (VUB) gave the best results. However, it is very likely that a large amount of nanobodies still form inclusion bodies. The nanobody yield for clones in a pTXB1 vector after purification was low (50 µg/l culture) as compared to the yield for MBP (50 mg/l culture). This yield might be low due to the formation of inclusion bodies or because of inefficient periplasmatic trafficing. To address the solubility issue, the SA construct was cloned in a pHEN6a vector which allows for expression in *WK6 E.*coli cells. The first results here were satisfactory. However larger cultures will have to be induced and purified. These experiments were not conducted here due to time restraints.
In the future, the efficiency of the modification of MBP can be assessed by SPR, streptavidine affinity chromatography or mass spectroscopy. The efficiency of periplasmatic transfer will be assessed. Here for a pelB tag will be cloned N-terminally of a MBP fusion protein after which the experiments will be repeated. Another way to asses this problem is by preparing BCII10 nanobodies without a pelB tag and expressing them. Depending on these results other enzymatic systems for modifications will be employed or the constructs will be no longer targeted to the periplasm but purified from the cytoplasm instead.

8.References

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9. Appendices

9.1 Appendix 1: Composition of LB & TB culture media

LB medium (1 liter):	**TB Medium (1 liter):**
• 10 g Bacto-Tryptone • 5 g Bacto-yeast extract • 10 g NaCl • 1 ml 1M NaOH Add ddH_2O to 1 liter Check if the pH is approximately 7 Autoclave	Component A (1 liter): • 13,33 g Bacto-Tryptone • 26,67 g Bacto-yeast extract • 4,44 ml 100% glycerol Add ddH_2O to 1 liter Autoclave & store at room temperature Component B (100 ml): • 2,31 g KH_2PO_4 • 12,54g K_2HPO_4 Add ddH_2O to 1 liter Autoclave & store at room temperature Just before use: Mix 900 ml of component A and 100 ml of component B

9.2 Appendix 2: Buffers & solutions

TfbI Buffer (100 ml): • 30 mM KAc • 100 mM RbCl • 10 mM $CaCl_2.2H_2O$ • 50 mM $MnCl_2.4H_2O$ • 15% glycerol (v/v) Dissolve and adjust pH to 5.8 with 0,2M acetic acid Add ddH_2O to 1 liter and filter sterile with 0,22 µm syringe filters	**TfbII Buffer (100 ml):** • 10 mM Pipes (acidic) • 75 mM $CaCl_2.2H_2O$ • 10 mM RbCl • 15% glycerol (v/v) Dissolve and adjust pH to pH 6.5 with 1 M KOH Add ddH2O to 1 liter and filter sterile with 0,22 µm syringe filters
TES1 (VUB) (1 liter): • 0.5M sucrose • 0.2M Tris – HCl • 0.5 mM EDTA Dissolve and adjust to 1 liter with ddH_2O	**TES2 (Reulen et al.) (1 liter):** • 30 mM Tris • 1 mM EDTA • 20 % sucrose (w/v) Dissolve and adjust pH to 8.0 with HCl Adjust to 1 liter with ddH_2O
TES/4: • 1 volume TES • 3 volumes ddH_2O	**Column buffer (2 liter):** • 20 mM Na-HEPES • 500 mM NaCl • 1 mM EDTA Dissolve and adjust pH to 8.5 with NaOH Adjust to 1 Liter with ddH_2O
PBS (1 liter): • 137 mM NaCl • 2,7 mM KCl • 10 mM Na_2HPO_4 • 1,76 mM KH_2PO_4 Dissolve and adjust to pH 7.4 with HCl Adjust to 1 liter with ddH_2O	**SDS Sample buffer:** 10x Loading buffer: • 20g SDS (Sigma) • 20 ml 0.5M Tris-HCl pH 6.8 • 10 ml 100 mM EDTA • 5 ml 10x loading buffer • 5 ml 100% glycerol • 500 µl 0,5% Bromophenol blue in 10 mM NaOH

Coomassie staining solution Staining solution: • 400 ml methanol • 70 ml acetic acid • 2,5 g Coomassie Brilliant Blue R-250	**Coomassie destaining solution:** • 400 ml methanol • 70 ml acetic acid • 530 ml distilled water
Towbin blot buffer (1 liter): • 25 mM Tris • 192 mM glycine • 20% methanol (v/v) Dissolve and adjust to 1 liter with ddH_2O	**TBS-T (2 liter):** • 25 mM Tris • 150 mM NaCl • 0.05% Tween-20 Dissolve and adjust pH to 8.0 with HCl Adjust to 1 liter with ddH_2O
NBT/BCIP Buffer (250 ml): • 100 mM Tris • 5 mM $MgCl_2$ Dissolve and adjust pH to 9.25-9.75 with HCl Adjust volume to 250 ml with ddH_2O For 1 blot dissolve 1,5 mg BCIp and 3,0 mg NBT in 10 ml of the buffer. Use immediately	**HBS Buffer (1 liter):** • 10 mM Hepes pH7.4 • 150 mM NaCl • 3 mM EDTA • 0.005% Tween-20 Dissolve and adjust pH to 7.4 with NaOH Adjust to 1 liter with ddH_2O
6x DNA loading buffer • 30% 99% glycerol • 0.25% Bromophenol blue Add water till 10 ml	

9.3 Appendix 3: PCR primer and protocols for amplification and modification of wild type BCII10 and constructs after modification

PCR Primers:

Construct	Forward primer	Reverse primer
SSA (Bio5)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-GGC-TGA-GGA-GAC-GGT-GAC-CTG-G-3'
SA (Bio6)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-**GGC**-GGA-GAC-GGT-GAC-CTG-GGT-CC
SQ (Bio30)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-GCA-GCC-GCT-GG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-**CTG**-GGA-GAC-GGT-GAC-CTG-GGT-CC-3'
ST (Bio32)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-GCA-GCC-GCT-GG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-**GGT**-GGA-GAC-GGT-GAC-CTG-GGT-CC-3'
SG (Bio44)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-GCA-GCC-GCT-GG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-**ACC**-GGA-GAC-GGT-GAC-CTG-GGT-CC-3'
SD (Bio45)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-GCA-GCC-GCT-GG-3'	5'-GGT-GGT-TGC-TCT-TCC-GCA-**ATC**-GGA-GAC-GGT-GAC-CTG-GGT-CC-3'
LEY (Bio54)	5'-GGT-GGT-CAT-ATG-AAA-TAC-CTA-TTG-CCT-ACG-GCA-GCC-GCT-GG-3'	5'-GGT-GGT**-CTC-GAG**-TGA-GGA-GAC-GGT-GAC-CTG-GGT-3'

Sequencing primers:

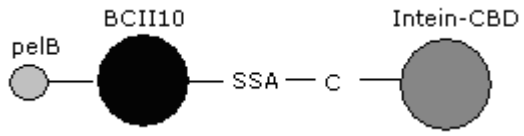
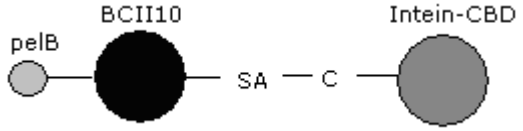
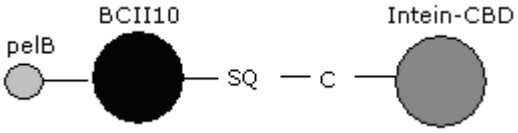
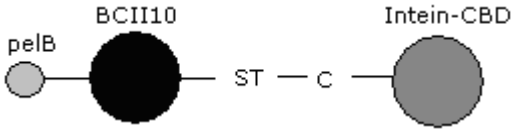
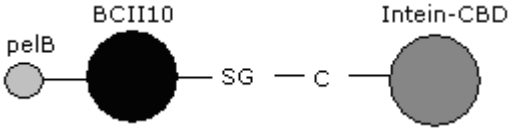
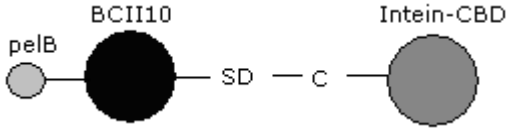
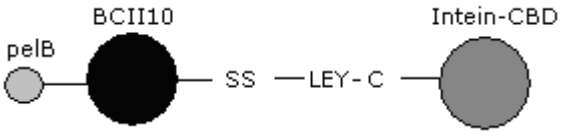
Vector	Forward primer	Reverse primer
pTXB1	TAATACGACTCACTATAGGG (T7 promoter)	GATTGCCATGCCGGTCAAGG (pTXB1R)
pMXB10	TAATACGACTCACTATAGGG (T7 promoter)	CAGGAAACAGCTATGAC (M13R-pUC)
pHEN6a	GTTTTCCCAGTCACGAC (M13F-pUC)	CAGGAAACAGCTATGAC (M13R-pUC)

DNA:

Negative control:	**BCII10-SA-intein-CBD:**
0.25 µl Taq polymerase	0.25 µl Taq polymerase
5 µl 10x Buffer	5 µl 10x Buffer
4 µl dNTP mix	4 µl dNTP mix
37.75 µl sterile water	37.75 µl sterile water
1 µl forward primer 1.1	1 µl forward primer 1.1
1 µl SA reverse primer (see appendix 1)	1 µl SA reverse primer (see appendix 1)
1 µl sterile water	1 µl pelB-BCII10-SS-RGR-His_6

BCII10-SSA-intein-CBD: 0.25 µl Taq polymerase 5 µl 10x Buffer 4 µl dNTP mix 37.75 µl sterile water 1 µl forward primer 1.1 1 µl SSA reverse primer (see appendix 1) 1 µl pelB-BCII10-SS-RGR-His_6	
Protocol 1: 5′ at 95°C 30″ at 95°C 30″ at 45°C } repeat 30 times 30″ at 72°C 5′ at 72°C Hold at 4°C	**Protocol 2: Reduction of the smear** 5′ at 95°C 30″ at 95°C 30″ at 55°C } Repeat 30 times 30″ at 72°C 5′ at 72°C Hold at 4°C

Constructs:

Bio5 	Bio6 
Bio 30 	Bio32 
Bio44 	Bio45 
Bio54 	

9.4 Appendix 4: Ligation mixtures for ligation of C-terminally modified BCII10 DNA into different vectors

Ligation of PCR products into pCR2.1 (TA-cloning) 1 µl 10x ligation buffer 5 µl sterile water 1 µl PCR product 2 µl vector pCR2.1 1 µl T4 DNA ligase → Ligate overnight at 14°C	**Negative control:** 5 µl vector (pTXB1, pMXB10 or pHEN6a) 10 µl sterile water 2 µl 10x T4 DNA ligase buffer 2 µl 50% PEG4000 1 µl T4 DNA ligase → Ligate overnight at 14°C
Ligation of BCII10-SA-intein-CBD and BCII10-SSA-intein-CBD into pTXB1 Equimolar: 5 µl BCII10-SA-intein-CBD or BCII10-SSA-intein-CBD DNA 5 µl pTXB1 vector 1 µl T4 DNA Ligase 2 µl 10X T4 Buffer 7 µl sterile water → Ligate overnight at 16°C	**Ligation of BCII10-SA-intein-CBD and BCII10-SSA-intein-CBD into pTXB1** 1:2 molar ratio: 3 µl BCII10-SA-intein-CBD or BCII10-SSA-intein-CBD DNA 7 µl pTXB1 vector 1 µl T4 DNA ligase 1 µl T4 DNA Ligase 2 µl 10x T4 Buffer → Ligate overnight at 16°C
Ligation of BCII10-SA-intein-CBD into pHEN6a 1:2 molar ratio: 5 µl pHEN6a 10 µl BCII10-SA-intein-CBD 2 µl 10x T4 DNA ligase buffer 2 µl 50% PEG4000 1 µl T4 DNA ligase	**Ligation of BCII10-SA-intein-CBD into pHEN6a** 1:6 molar ratio: 3 µl pHEN6a 19 µl BCII10-SA-intein-CBD 3 µl 10x T4 DNA ligase buffer 3 µl 50% PEG4000 2 µl T4 DNA ligase

9.5 Appendix 5: Restriction digestion mixes used for cloning procedures and to analyze if cloning was successful

Digestion of BCII10-SA-intein-CBD and BCII10-SSA-intein-CBD by EcoRI	**Digestion of BCII10-SA-intein-CBD and BCII10-SSA-intein CBD by NdeI and SapI for cloning into pTXB1**
10 µl DNA 1 µl EcoRI 2 µl 10x buffer 7 µl sterile water → Digest 1 hour at 37°C	10 µl BCII10-SA, BCII10-SSA or pTXB1 DNA 4 µl FD Buffer 1 µl NdeI 1 µl SapI 24 µl sterile water → Digest 1,5 hour at 37°C
Digestion of BCII10-SA-intein-CBD and BCII10-SSA-intein CBD by PstI to check for correct clones	**Digestion of BCII10-SA-intein-CBD and BCII10-SSA-intein CBD by BamHI and NdeI to check for correct clones**
10 µl BCII10-SA or BCII10-SSA DNA 1 µl PstI 2 µl 10x Buffer O 7 µl sterile water → Digest 1 hour at 37°C	10 µl BCII10-SA or BCII10-SSA DNA 1 µl NdeI 1 µl BamHI 2 µl 10x FD Buffer 6 µl sterile water → Digest 1 hour at 37°C
First digestion of BCII10-SA-intein-CBD by BamHI and digestion of pHEN6a VHH-His_6 for cloning First attempt: 15 µl BCII10-SA-intein-CBD DNA from clone 2 1 µl BamHI 2 µl 10x Tango Buffer 2 µl Sterile water → Digest 1 hour at 37°C 5 µl pHEN6a VHH-His_6 1,5 µl EcoRI 2 µl 10x O Buffer 11,5 µl sterile water → Digest 1 hour at 37°C	**First digestion of BCII10-SA-intein-CBD by BamHI and digestion of pHEN6a VHH-His_6 for cloning** Second attempt: 34 µl BCII10-SA-intein-CBD DNA from clone 2 4 µl 10x Tango Buffer 1,5 µl BamHI → Digest 1 hour at 37°C 6 µl pHEN6a VHH-His_6 2 µl 10x O Buffer 1,5 µl EcoRI 10,5 µl sterile water → Digest 1 hour at 37°C

Blunting of the digested pHEN6a vector and the BCII10-SA-intein-CBD DNA	**Blunting of the digested pHEN6a vector and the BCII10-SA-intein-CBD DNA**
Add to 39,5 µl of digested BCII10-SA-intein-CBD: 1 µl 10x Tango Buffer 8 µl dNTP mix 2 µl T4 DNA polymerase → Blunt 20 minutes at 11°C → Terminate the reaction by incubation for 10 minutes at 75°C	Add to 20 µl of digested pHEN6a vector: 1 µl 10x O buffer 4 µl dNTP mix 1 µl T4 DNA polymerase 4 µl sterile water → Blunt 20 minutes at 11°C → Terminate the reaction by incubation for 10 minutes at 75°C
Digestion of BCII10-SA-intein-CBD by and pHEN6a VHH-His_6 by NcoI for cloning 10 µl BCII10-SA-intein-CBD or pHEN6a 6 µl 10x Tango buffer 2 µl NcoI 2 µl sterile water → Digest 1 hour at 37°C	**Digestion of BCII10-SA-intein-CBD in pHEN6a by PstI to check for correct clones** 10 µl BCII10-SA-intein-CBD in pHEN6a DNA 1,5 µl PstI 2 µl 10x buffer O 6,5 µl sterile water → Digest 1 hour at 37°C

9.6 Appendix 6: Sequencing data

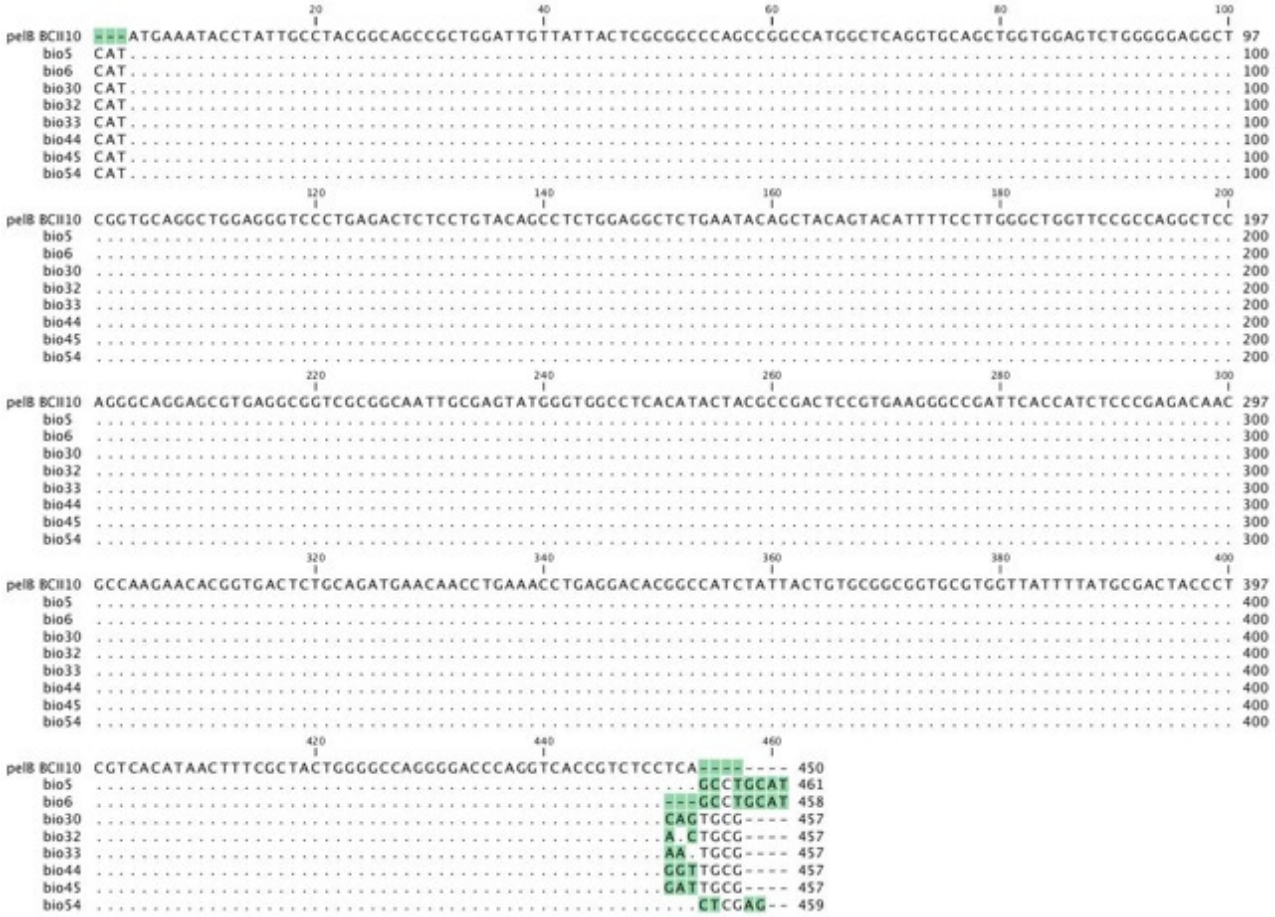


Figure S1: DNA Sequence alignment of the different constructs made and used.

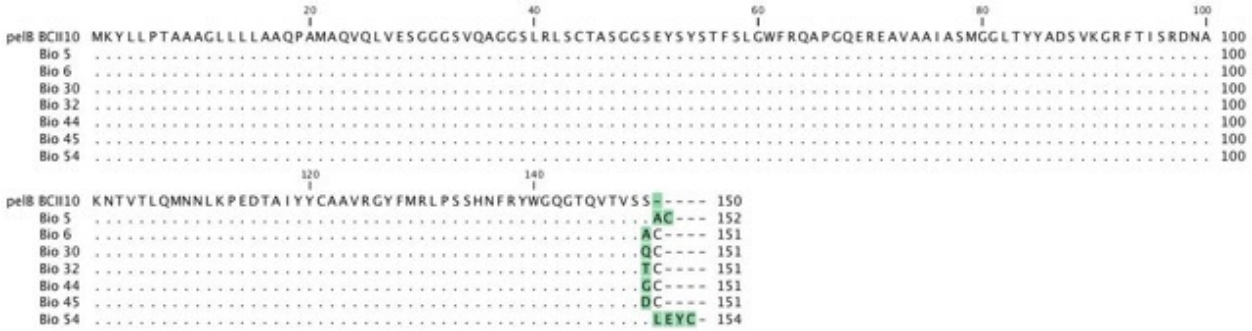


Figure S2: Protein sequence alignment of the different constructs made and used.

9.7 Appendix 7:Cleavage of proteins of the chitin beads column by MESNA and the click reaction between modified MBP and biotin-azide

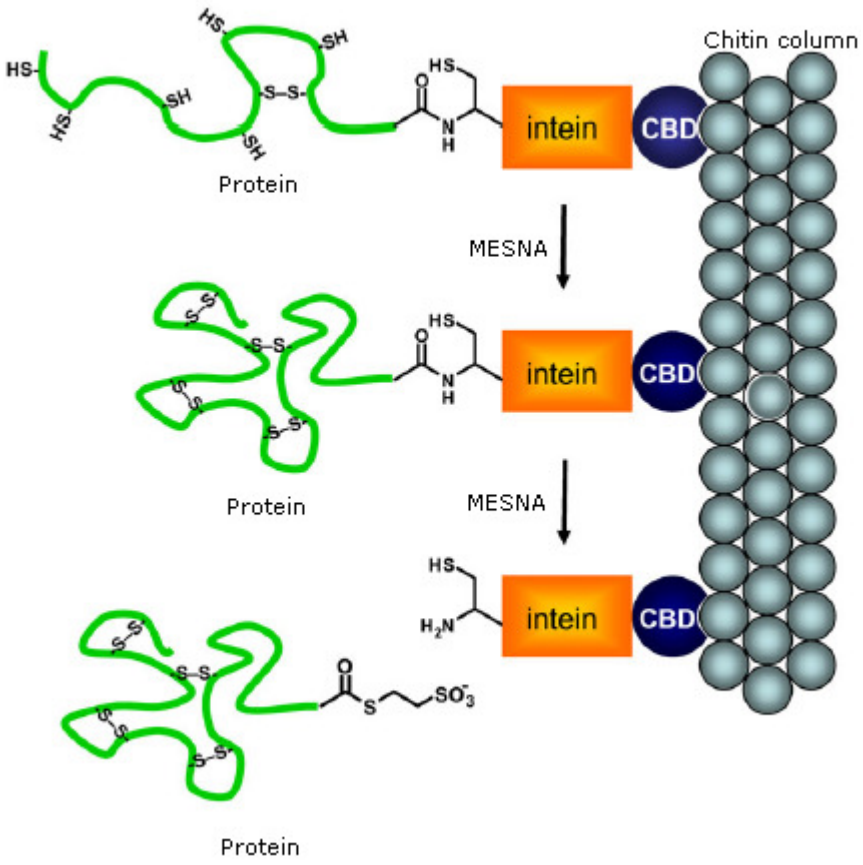


Figure S3: Cleavage of the protein from the intein-CBD domain by MESNA.

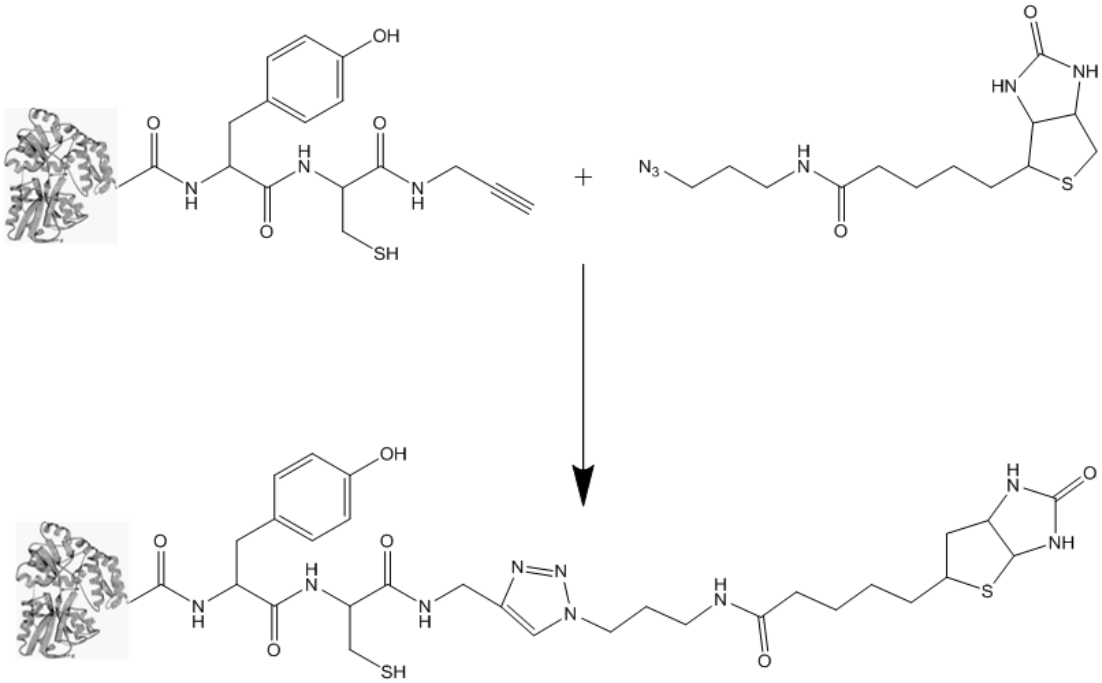


Figure S4: Click reaction between an alkyne modified MBP and an azide modified biotin.

9.8 Appendix 8: DNA & protein sequence of MBP and pelB-BCII10-intein-CBD

pelB-VHH BCII-10-Intein-CBD

cDNA sequence:

atgaaatacctattgcctacggcagccgctggattgttattactcgcggcccagccggccatggctcaggtgcagctggtggagtctgggggaggctcggtgcaggctggagggtccctgagactctcctgtacagcctctggaggctctgaatacagctacagtacattttccttgggctggttccgccaggctccagggcaggagcgtgaggcggtcgcggcaattgcgagtatgggtggcctcacatactacgccgactccgtgaagggccgattcaccatctcccgagacaacgccaagaacacggtgactctgcagatgaacaacctgaaacctgaggacacggccatctattactgtgcggcggtgcgtggttattttatgcgactaccctcgtcacataactttcgctactggggccaggggacccaggtcaccgtctcctcatgcatcacgggagatgcactagttgccctacccgagggcgagtcggtacgcatcgccgacatcgtgccgggtgcgcggcccaacagtgacaacgccatcgacctgaaagtccttgaccggcatggcaatcccgtgctcgccgaccggctgttccactccggcgagcatccggtgtacacggtgcgtacggtcgaaggtctgcgtgtgacgggcaccgcgaaccacccgttgttgtgtttggtcgacgtcgccggggtgccgaccctgctgtggaagctgatcgacgaaatcaagccgggcgattacgcggtgattcaacgcagcgcattcagcgtcgactgtgcaggttttgcccgcggaaaacccgaatttgcgcccacaacctacacagtcggcgtccctggactggtgcgtttcttggaagcacaccaccgagacccggacgcccaagctatcgccgacgagctgaccgacgggcggttctactacgcgaaagtcgccagtgtcaccgacgccggcgtgcagccggtgtatagccttcgtgtcgacacggcagaccacgcgtttatcacgaacgggttcgtcagccacgctactggcctcaccggtctgaactcaggcctcacgacaaatcctggtgtatccgcttggcaggtcaacacagcttatactgcgggacaattggtcacatataacggcaagacgtataaatgtttgcagccccacacctccttggcaggatgggaaccatccaacgttcctgccttgtggcagcttcaatga

Protein sequence:

MKYLLPTAAAGLLLLAAQPAMAQVQLVESGGGSVQAGGSLRLSCTASGGSEYSYSTFSLG
WFRQAPGQEREAVAAIASMGGLTYYADSVKGRFTISRDNAKNTVTLQMNNLKPEDTAIYY
CAAVRGYFMRLPSSHNFRYWGQGTQVTVSSCITGDALVALPEGESVRIADIVPGARPNSD
NAIDLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLL
WKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFAPTTYTVGVPGLVRFLEAHHRDPDAQ
AIADELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHAFITNGFVSHATGLTGLNSGLTT
NPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ*

The last codon of the VHH BCII-10 (tca), which codes for a serine (S), is underlined in the cDNA and protein sequence.

MBP-Intein-CBD

cDNA sequence:

atgggaagagccatggataaaatcgaagaaggtaaactggtaatctggattaacggcgataaaggctataacggtctcgctgaagtcggtaagaaattcgagaaagataccggaattaaagtcaccgttgagcatccggataaactggaagagaaattcccacaggttgcggcaactggcgatggccctgacattatcttctgggcacacgaccgctttggtggctacgctcaatctggcctgttggctgaaatcaccccggacaaagcgttccaggacaagctgtatccgtttacctgggatgccgtacgttacaacggcaagctgattgcttacccgatcgctgttgaagcgttatcgctgatttatacaaagatctgctgccgaacccgccaaaaacctgggaagagatcccggcgctggataaagaactgaaagcgaaaggtaagagcgcgctgatgttcaacctgcaagaaccgtacttcacctggccgctgattgctgctgacgggggttatgcgttcaagtatgaaaacggcaagtacgacattaaagacgtgggcgtggataacgctggcgcgaaagcgggtctgaccttcctggttgacctgattaaaaacaaacacatgaatgcagacaccgattactccatcgcagaagctgcctttaataaaggcgaaacagcgatgaccatcaacggcccgtgggcatggtccaacatcgacaccagcaaagtgaattatggtgtaacggtactgccgaccttcaagggtcaaccatccaaaccgttcgttggcgtgctgagcgcaggtattaacgccgccagtccgaacaaagagctggcaaaagagttcctcgaaaactatctgctgactgatgaaggtctggaagcggttaataaagacaaaccgctgggtgccgtagcgctgaagtcttacgaggaagagttggcgaaagatccacgtattgccgccactatggaaaacgcccagaaaggtgaaatcatgccgaacatcccgcagatgtccgctttctggtatgccgtgcgtactgcggtgatcaacgccgccagcggtcgtcagactgtcgatgaagccctgaaagacgcgcagactaattcgagctcgaagcttggcggccgcgaattcctcgagtactgcatcacgggagatgcactagttgccctacccgaggcgagtcggtacgcatcgccgacatcgtgccgggtgcgcggcccaacagtgacaacgccatcgacctgaaagtccttgaccggcatggcaatcccgtgctcgccgaccggctgttccactccggcgagcatccggtgtacacggtgcgtacggtcgaaggtctgcgtgtgacgggcaccgcgaaccacccgttgttgtgtttggtcgacgtcgccggggtgccgaccctgctgtggaagctgatcgacgaaatcaagccgggcgattacgcggtgattcaacgcagcgcattcagcgtcgactgtgcaggttttgcccgcggaaaacccgaatttgcgcccacaacctacacagtcggcgtccctggactggtgcgtttcttggaagcacaccaccgagacccggacgcccaagctatcgccgacgagctgaccgacgggcggttctactacgcgaaagtcgccagtgtcaccgacgccggcgtgcagccggtgtatagccttcgtgtcgacacggcagaccacgcgtttatcacgaacgggttcgtcagccacgctactggcctgaccggtctgaactcaggcctcacgacaaatcctggtgtatccgcttggcaggtcaacacagcttatactgcgggacaattggtcacatataacggcaagacgtataaatgtttgcagccccacacctccttggcaggatgggaaccatccaacgttcctgccttgtggcagcttcaatga

Protein sequence:

MGRAMDKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFG
GYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKS
ALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNK
GETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVN
KDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNS
SSKLGGREFLEY**CITGDALVALPEGESVRIADIVPGARPNSDNAIDLKVLDRHGNPVLADRLFHSGEHP
VYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKPEFA
PTTYTVGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHAFITN
GFVSHATGLTGLNSGL**TTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALW
QLQ*

9.9 Appendix 9: The analysis of MBP and BCII10 using Vector NTI®

Tabel S1:The analysis of the complete BCII10 fusion nanobody using Vector NTI®

Analysis	Entire Protein
Length	410 aa
Molecular Weight	43876.92
1 microgram =	22.791 pMoles
Molar Extinction coefficient	57900
1 A[280] corr. to	0.76 mg/ml
A[280] of 1 mg/ml	1.32 AU
Isoelectric Point	6.38
Charge at pH 7	-2.39

Table S2: Theanalysis of the BCII10 fusion nanobody without pelB using Vector NTI®

Analysis	Entire Protein
Length	388 aa
Molecular Weight	41666.35
1 microgram =	24.000 pMoles
Molar Extinction coefficient	56620
1 A[280] corr. to	0.74 mg/ml
A[280] of 1 mg/ml	1.36 AU
Isoelectric Point	6.21
Charge at pH 7	-3.39

Table S3:The analysis of the intein-CBD using Vector NTI®

Analysis	Entire Protein
Length	260 aa
Molecular Weight	27856.38
1 microgram =	35.898 pMoles
Molar Extinction coefficient	34760
1 A[280] corr. to	0.80 mg/ml
A[280] of 1 mg/ml	1.25 AU
Isoelectric Point	5.66
Charge at pH 7	-6.47

Table S4:The analysis of the native BCII10 nanobody using Vector NTI®

Analysis	Entire Protein
Length	128 aa
Molecular Weight	13827.99
1 microgram =	72.317 pMoles
Molar Extinction coefficient	21860
1 A[280] corr. to	0.63 mg/ml
A[280] of 1 mg/ml	1.58 AU
Isoelectric Point	9.01
Charge at pH 7	2.83

Tabel S5: The analysis of the complete MBP fusion protein using Vector NTI®

Analysis	Entire Protein
Length	646 aa
Molecular Weight	70274.20
1 microgram =	14.230 pMoles
Molar Extinction coefficient	100760
1 A[280] corr. to	0.70 mg/ml
A[280] of 1 mg/ml	1.43 AU
Isoelectric Point	5.29
Charge at pH 7	-16.15

Table S6: The analysis of the native MBP using Vector NTI®

Analysis	Entire Protein
Length	386 aa
Molecular Weight	42435.84
1 microgram =	23.565 pMoles
Molar Extinction coefficient	66000
1 A[280] corr. to	0.64 mg/ml
A[280] of 1 mg/ml	1.56 AU
Isoelectric Point	5.10
Charge at pH 7	-9.92

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Covalent and site-specific coupling of nanobodies onto solid substrates for biosensor applications

Richting: **master in de biomedische wetenschappen-bio-elektronica en nanotechnologie**
Jaar: **2011**

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Datum: **14/06/2011**